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Hypothalamic Vitamin D Improves Glucose Homeostasis and Reduces Weight

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Despite clear associations between vitamin D deficiency and obesity and/or type 2 diabetes, a causal relationship is not established. Vitamin D receptors (VDRs) are found within multiple tissues, including the brain. Given the importance of the brain in controlling both glucose levels and body weight, we hypothesized that activation of central VDR links vitamin D to the regulation of glucose and energy homeostasis. Indeed, we found that small doses of active vitamin D, 1 α ,25-dihydroxyvitamin D₃ (1,25D₃) (calcitriol), into the third ventricle of the brain improved glucose tolerance and markedly increased hepatic insulin sensitivity, an effect that is dependent upon VDR within the paraventricular nucleus of the hypothalamus. In addition, chronic central administration of 1,25D₃ dramatically decreased body weight by lowering food intake in obese rodents. Our data indicate that 1,25D₃-mediated changes in food intake occur through action within the arcuate nucleus. We found that VDR colocalized with and activated key appetite-regulating neurons in the arcuate, namely proopiomelanocortin neurons. Together, these findings define a novel pathway for vitamin D regulation of metabolism with unique and divergent roles for central nervous system VDR signaling. Specifically, our data suggest that vitamin D regulates glucose homeostasis via the paraventricular nuclei and energy homeostasis via the arcuate nuclei.

Vitamin D is a fat-soluble vitamin available in some foods but also produced from sunlight. Whatever the source,

vitamin D requires hydroxylation in the liver and kidney to produce the active form, 1 α ,25-dihydroxyvitamin D₃ (1,25D₃), also called calcitriol. 1,25D₃ binds to the vitamin D receptor (VDR), which forms a heterodimer with the retinoid X receptor and modulates gene expression. Although vitamin D has important roles in calcium/phosphorus regulation and bone health, it also has important actions in immunity, inflammation, and differentiation (1).

Interestingly, low vitamin D status is associated with obesity and impaired glucose tolerance (2,3). Whether this is a causal relationship is unclear. When combined with a low-energy diet, vitamin D supplementation results in greater decreases in body weight and fat mass in humans (4–6) and prevents dietary-induced weight gain in rodents (7). Additionally, vitamin D intake at breakfast can increase thermogenesis and fat oxidation rates in subsequent meals, suggesting direct metabolic action (8). Preclinical studies also demonstrate a clear pathway by which vitamin D affects glucose homeostasis. VDR is present in pancreatic β -cells (9), the VDR-null mouse has impaired glucose tolerance with decreased insulin levels (10), and supplementation with 1,25D₃ improves fasting glucose levels in mice (11). Given the strong association between vitamin D status and the metabolic syndrome, the inconsistency of vitamin D supplementation in improving weight or abnormal glucose tolerance is perplexing (4,12–17).

Understanding how vitamin D might regulate glucose and body weight is paramount to creating effective

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strategies for using vitamin D in the management of obesity and diabetes. Previous research has focused on the peripheral actions of vitamin D. However, the brain, specifically the hypothalamus, is well-known to control both body weight and glucose (18). Interestingly, the brain has VDR in key hypothalamic nuclei including the arcuate (ARC) and paraventricular (PVN) nuclei, which regulate both body weight and glucose metabolism (19,20). The purpose of this manuscript is to explore the novel hypothesis that vitamin D regulates energy and glucose homeostasis via direct actions in the brain.

RESEARCH DESIGN AND METHODS

Animals

Animals were singly housed at the University of Cincinnati Laboratory Animal Medical Services Facility or at Baylor College of Medicine (BCM) on a 12-h light/dark cycle with ad libitum access to water and food. Studies used adult male Long-Evans rats (Harlan, Indianapolis, IN), mice bearing a floxed VDR allele (VDR^{fl/fl} [21]), or C57Bl/6 mice from an internal colony at BCM. Animal numbers are stated in the figure legends. All studies were approved by the University of Cincinnati Institutional Animal Care and Use Committee (IACUC) or the BCM IACUC as applicable.

Diet

Rats were fed a 40% high-fat butter diet (Research Diets, New Brunswick, NJ) for at least 13 weeks prior to all experiments except in the lentiviral studies, which were performed after 6 weeks of an HFD. Mice were fed a Western diet (45% fat, cat. no. D12451; Research Diets).

Surgeries

Intracerebroventricular Cannulas

Cannulas were surgically implanted into the third cerebral ventricle (i3vt) or PVN as previously described (22,23); coordinates were as follows: i3vt 2.2A/P, 7.8D/V, PVN 1.35A/P, 7.6D/V, and 0.2M/L as determined by the atlas of Paxinos and Watson (24,25).

Viral Injections

Rats received 2 μ L of replication-defective VDR knockdown lentivirus or its control (SPWGM-V463 [73e6 transducing units/ μ L] or SPWGM-NC; Viral Vector Core, University of South Carolina School of Medicine, Columbia, SC) unilaterally into the PVN (coordinates as above). Mice received 20 nL replication-defective adenovirus-associated virus (AAV) containing Cre-recombinase or its control (AAV9.CMV.H1eGFP-Cre.WPRE.SV40 9.82e12 genome copies/mL or AAV-CMV-GFP-9 4.0e12 gc/mL; University of Pennsylvania Vector Core, Chapel Hill, NC) injected bilaterally into the PVN (coordinates 0.94A/P, 4.75D/V, and 0.20M/L). We chose bilateral injection in the mice in order to assess physiologic effects of vitamin D action in the PVN. At sacrifice, PVN were dissected microscopically and RT-PCR was performed for quantification of VDR. Quantification of VDR knockdown only occurred in AAV-treated mice, as technical difficulties prevented quantification in lentiviral vector-treated rats.

Carotid/Jugular Catheters

Carotid and jugular catheters were implanted as previously described with the exception that a mouse antenna for sampling access was not used (26). Surgery occurred after 13 weeks on HFD.

Drugs

Hydroxypropyl- β -cyclodextrin (THPB-EC; CTD, Inc., Alachua, FL) was dissolved in saline to a concentration of 0.5 g/mL and used as the vehicle. 1,25D₃ (cat. no. 17936, 1 mg; Sigma-Aldrich, St. Louis, MO) was dissolved in 0.5 g/mL hydroxypropyl- β -cyclodextrin. ZK159222, a VDR antagonist (27,28), was a generous gift from Bayer Pharma AG (Berlin, Germany) and was dissolved in saline.

Glucose Tolerance Test

Glucose tolerance tests (GTTs) were performed as previously described (23) with the following modifications: animals fed the HFD for 18 weeks (Fig. 1A) were fasted for 4 h; intraperitoneal GTT (i.p.GTT) used 1.5 g/kg dextrose; 1 h prior to dextrose administration, body weight-randomized rats were injected i3vt with 0.1 μ g/2 μ L 1,25D₃ or 2 μ L vehicle; and an additional glucose measurement was performed prior to the i3vt injection (−60 min). This dose was chosen based on a dose response curve and was the lowest effective dose (data not shown). For lentiviral studies (Fig. 2D–F), 1 g/kg dextrose was used and the GTT was performed 4 weeks after lentiviral injection. Insulin levels during intravenous GTT (i.v.GTT) were determined with a rat insulin ELISA (Crystal Chem, Downers Grove, IL). Mouse GTTs were performed after a 4-h fast with 1.5 g/kg dextrose i.p. No vitamin D was administered. Insulin levels were not measured during i.p.GTTs.

Hyperinsulinemic-Euglycemic Clamp

Rats, which had previously undergone an i.v.GTT the week before, underwent a hyperinsulinemic-euglycemic clamp as previously described (23,29) with the following modifications: animals were fasted for 4 h, and at 120 and 180 min, 0.1 μ g/1 μ L 1,25D₃ or 1 μ L vehicle was infused i3vt. Only animals that were in a steady state at the end of the clamp were included in the analyses.

Tracer Calculations

Plasma tracer concentrations were determined through a modification of the Somogyi procedure as previously published (30,31). Glucose R_a , endogenous glucose production, and glucose utilization were calculated according to previous methods (31,32).

Acute Food Intake Studies

Food intake studies were performed as previously described (22) with the modification that rats were injected i3vt with 0.1 μ g/2 μ L 1,25D₃ or 2 μ L vehicle 60 min prior to dark cycle onset. This dose was used based on the dose response curve of the GTT.

Chronic Studies

After 20 weeks of HFD, rats underwent i3vt cannula placement with verification as previously described (22).

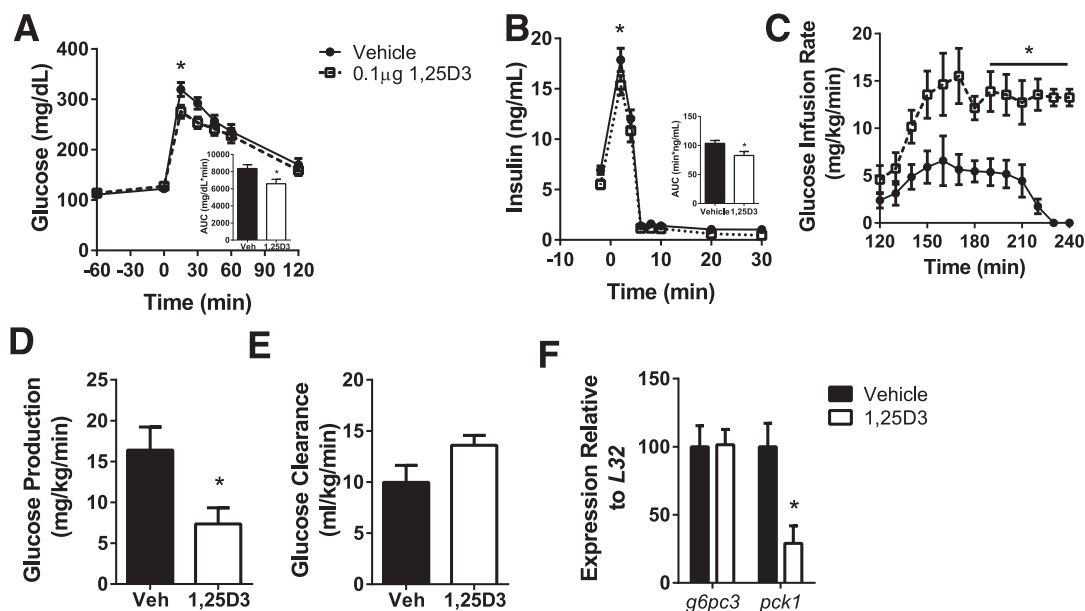


Figure 1—Hypothalamic 1,25D₃ lowers plasma glucose by inhibition of hepatic glucose production. **A:** i3vt 1,25D₃ improves glucose excursion after an intraperitoneal bolus of dextrose in DIO rats ($n = 12$ vs. 13). **B:** Insulin levels were lower in rats treated with i3vt 1,25D₃ after a bolus of 0.5 g/kg i.v. dextrose ($n = 7$ –8/group). **C–E:** HFD-fed rats treated with 0.1 μ g i3vt 1,25D₃ at 120 and 180 min during a physiologic hyperinsulinemic-euglycemic clamp had increased glucose infusion rate (**C**) and during the last 30 min had decreased glucose production (**D**) with no change in glucose clearance compared with controls (**E**) ($n = 4$ –5/group). **F:** At sacrifice, liver samples from rats treated with 1,25D₃ during the clamp had no change in mRNA expression of *g6pc3* but decreased *pck1* compared with controls. * $P < 0.05$. AUC, area under the curve; Veh, vehicle.

Four weeks later, rats were anesthetized and had osmotic pump placement (cat. no. 1004; Alzet Osmotic Pumps, Cupertino, CA) under isoflurane with subcutaneous tubing connecting the pump to the cannula. Pumps delivered 0.264 μ g/day 1,25D₃ or 0.11 μ L/h vehicle for 28 days. Food and body weights were measured at 0, 1, 2, 3, 4, 5, 6, 7, 14, 21, and 28 days relative to pump placement. Body composition was measured with QMR (EchoMRI, Houston, TX) on days 0 and 29. A second cohort of animals was placed in a continuous monitoring system (Physioscan Metabolic Monitoring system, Accuscan Instruments, Columbus, OH) for 96 h to determine energy expenditure 3 days after surgery. We chose this time point as one where the rats were starting to recover from surgery but still had similar body weights between the groups. The first 24 h were considered adaptation, and the data from the next 72 h were analyzed. Data for indirect calorimetry analysis were sampled every 10 min.

Immunohistochemistry

Animals

For *c-fos* studies, ad libitum-fed rats had i3vt cannulas placed, were allowed to recover for 11 weeks, and were injected with 2 μ L saline or antagonist ZK159222 followed 30 min later by 0.1 μ g/1 μ L i3vt 1,25D₃ or 1 μ L vehicle. They were sacrificed 1 h after injections. VDR location studies were performed on ad libitum-fed rats without cannulas. Brain sections were taken from C57Bl/6, neuropeptide Y (NPY)-GFP, or proopiomelanocortin (POMC)-GFP mice (both GFP-expressing mice were a generous gift from Yong Xu,

BCM). Animals were deeply anesthetized with ketamine (70 mg/kg) and xylene (6.2 mg/kg) and perfused transcardially with 0.9% saline followed by 4% formalin. Brains were stored overnight in 20% sucrose/PBS plus 0.01% sodium azide at 4°C. Serial coronal sections were collected at 25 μ m and stored in cryopreservative at –20°C.

Immunohistochemistry

Rinses with 0.1 mol/L PBS occurred between each incubation step. Incubations were as follows: 0.3% H₂O₂/PBS for 30 min, 0.1 mol/L PBS/3% normal donkey serum/0.25% Triton-X-100/0.01% sodium azide for 2 h, 1:2,500 rabbit anti-*c-fos* (sc52; Santa Cruz Biotechnology, Santa Cruz, CA) or 1:100 rabbit anti-VDR (N20) (sc-1009; Santa Cruz Biotechnology) overnight, 1:1,000 biotinylated donkey anti-rabbit IgG or 1:200 donkey anti-rabbit IgG Alexa Fluor 594 conjugate (A-21207; Thermo Fisher Scientific) in PBS/3% normal donkey serum/0.25% Triton-X-100 for 2 h, 1:500 avidin biotin complex/PBS solution (PK6100; Vector Laboratories, Burlingame, CA) for 1 h (*c-fos* only), 0.04% DAB (D5905; Sigma-Aldrich)/0.01% H₂O₂/PBS for 10 min (*c-fos* only), and PBS plus 0.01% sodium azide; mounted on slides; air dried overnight; and coverslipped (vectashield hard-set mounting medium with DAPI used for slides [101098-050; VWR]). Quantification of *c-fos* was performed as previously described (22). An individual blind to the experimental treatment groups scored the sections. For determination of the degree of overlap between cells with GFP expression by POMC and VDR

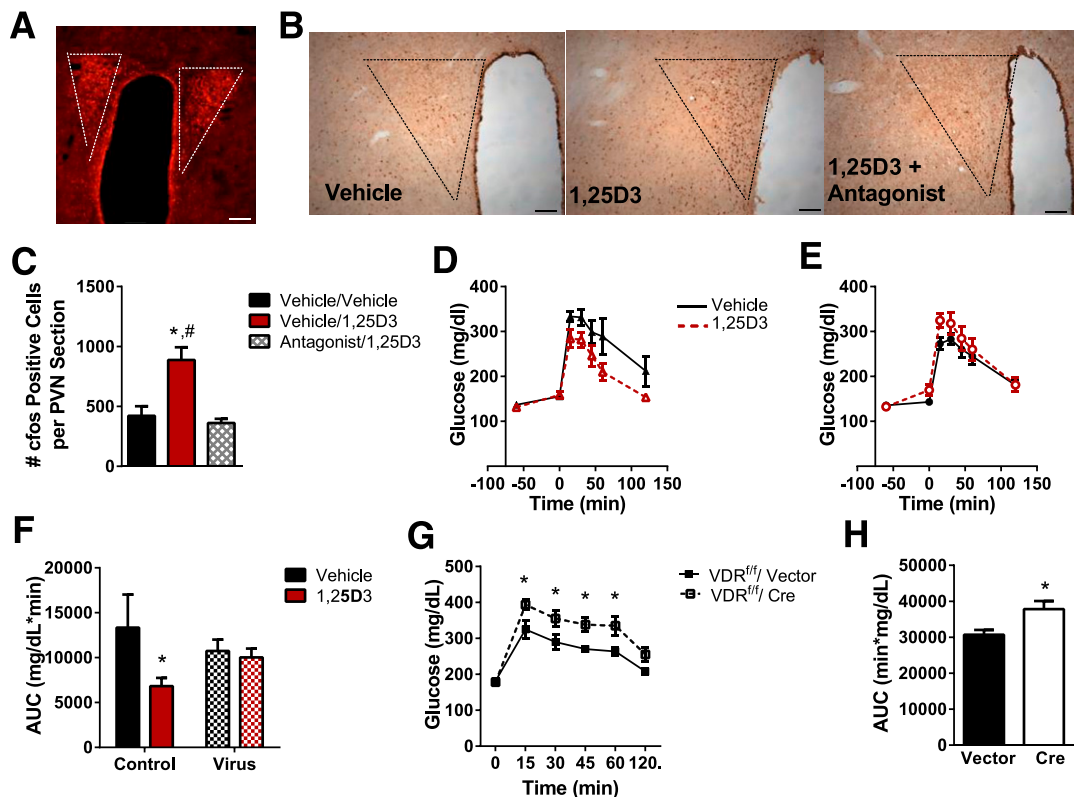


Figure 2—Vitamin D receptors in PVN are important in regulating glucose. **A:** The VDR is present in the PVN of HFD-fed rats ($\times 10$ magnification). **B and C:** i3vt 1,25D₃ increases c-fos in the PVN of DIO rats. This effect is diminished with pretreatment of a VDR antagonist, ZK159222 ($\times 10$ magnification). **D and E:** Intra-PVN 1,25D₃ (0.1 μ g) 60 min prior to glucose injection tends to improve glucose excursions in control virus-treated animals (**D**) but not in animals with VDR shRNA (**E**) ($n = 4$ –6/group). **F:** PVN 1,25D₃ causes a decrease in the area under the curve (AUC) in **D** but not in **E**. **G and H:** VDR^{fl/fl} mice with bilateral PVN knockdown of VDR mediated through AAV-Cre delivery (VDR^{fl/fl}/Cre) demonstrate increased glucose excursions after a bolus of 1.5 g/kg i.p. dextrose 16 weeks after AAV-Cre injection from 15–60 min (**G**) and in the area under the curve (**H**) ($n = 7$ –8/group). Dashed lines outline the PVN. Scale bars = 100 μ m. * $P < 0.05$ vs. vehicle or control; # $P < 0.05$ vs. antagonist/1,25D₃.

immunoreactivity, three different sections of the ARC were examined in each of two separate animals. Mean percent \pm SE of POMC-GFP-expressing cells coexpressing VDR was determined by averaging the results obtained for each of the three sections of ARC in the two mice mentioned above. A similar analysis was done with NPY-GFP-expressing cells. Display images were adjusted for brightness and contrast. N20 was chosen as our VDR antibody because it produced nuclear staining, as opposed to D6 (Santa Cruz Biotechnology), which shows cytoplasmic staining in the brain (data not shown and ref. 33). N20 has been shown to be specific for the VDR, producing a clear band on Western blots with absence of staining in a VDR-null animal (34).

Electrophysiology

POMC-CreER/Rosa26-tdTOMATO mice (at 6–10 weeks of age) were used for electrophysiological recordings as previously described (35) with the following modifications. Patch pipettes were filled with intracellular solution (adjusted to pH 7.3) containing (in mmol/L) 128 K gluconate, 10 KCl, 10 HEPES, 0.1 EGTA, 2 MgCl₂, 0.3 Na-GTP, and 3 Mg-ATP. Current clamp was engaged to test neural firing

frequency at baseline and after puff of 1 μ mol/L 1,25D₃ for 1 s. The values for firing frequency were averaged within a 2-min bin at baseline or after 1,25D₃ treatment.

Cell Culture

Cells from a mouse neuroblastoma cell line, Neuro-2A (American Type Culture Collection, Manassas, VA) were routinely cultured at 37°C in normoxia conditions (5% CO₂, 95% air) in DMEM (Life Technologies) supplemented with 10% FBS (Life Technologies) and 1% penicillin-streptomycin. Cells were plated at 6×10^5 cells/cm². At 85–90% confluence, cells were incubated for 24 h with 1,25D₃ (10^{-7} mol/L) or vehicle. A separate group also received a VDR antagonist, ZK159222 (10^{-5} mol/L).

RT-PCR

Quantification of mRNA expression was performed as previously described (36). TaqMan primers (Thermo Fischer Scientific) are listed in Supplementary Table 1.

Statistical Analysis

Results are presented as mean \pm SE. Results are analyzed by a one-way or two-way ANOVA as appropriate with

Tukey post hoc analysis where appropriate. The level of significance was set as $P < 0.05$. Data were analyzed with GraphPad Prism, version 6.

RESULTS

Vitamin D and Glucose Regulation in the Brain

For determination of the effect of acute central 1,25D₃ administration on glucose regulation, GTTs and hyperinsulinemic-euglycemic clamps were performed on HFD rats after 1,25D₃ was provided into the third ventricle (i3vt). In HFD-fed obese rats, i3vt administration of 1,25D₃ lowered glucose levels during an i.p.GTT at both the 15-min time point and as measured by the incremental area under the curve (iAUC) (Fig. 1A and inset). This improvement was not observed when the same dose of 1,25D₃ was given intraperitoneally, indicating a central effect of 1,25D₃ (Supplementary Fig. 1A). Interestingly, we found that diet had a profound effect on the glucose-regulating effects of 1,25D₃, since there was no effect of i3vt 1,25D₃ in chow-fed animals (Supplementary Fig. 1B). As designed, i3vt 1,25D₃ treatment did not alter blood glucose levels during an i.v.GTT (Supplementary Fig. 1C). However, the insulin response was lower 2 min after glucose injection and as measured by the iAUC (Fig. 1B and inset). Together, these data indicate that improvements in glucose levels after 1,25D₃ administration were not due to enhanced insulin secretion. Since glucose clearance during an i.v.GTT is determined primarily by mass action and therefore does not assess an impact of central nervous system (CNS) 1,25D₃ on insulin sensitivity, we next used a hyperinsulinemic-euglycemic clamp to further test whether i3vt 1,25D₃ improves insulin sensitivity. i3vt 1,25D₃-treated animals required a markedly higher glucose infusion rate throughout the clamp (Fig. 1C) to maintain glucose levels matched to those of vehicle-treated animals (Supplementary Fig. 1D). During the last 30 min of the clamp, 1,25D₃-treated animals required an almost sevenfold increase in glucose infusion rate, which corresponded to a significant decrease in glucose production (Fig. 1D) without differences in glucose clearance (Fig. 1E). Since the liver is a major insulin-responsive source of glucose, we evaluated hepatic expression levels of key genes involved in gluconeogenesis. Phosphoenolpyruvate carboxykinase 1 (*pck1*), the rate-limiting enzyme in gluconeogenesis, was decreased in 1,25D₃-treated animals, but there was no difference in the expression of glucose-6-phosphatase 3 (*g6pc3*) (Fig. 1F).

We then considered what population of VDR regulates this potent CNS effect of vitamin D. Consistent with previous research showing strong expression of VDR in the human PVN (37), we observed abundant VDR staining within the rodent PVN (Fig. 2A). i3vt 1,25D₃ increased *c-fos* expression in the PVN compared with vehicle but had no effect when animals were pretreated with a VDR antagonist (Fig. 2B and C). While we also saw VDR staining in the ventromedial nucleus of the hypothalamus, another area known to have glucoregulatory functions,

there was only *c-fos* activation in the PVN. Thus, to determine whether 1,25D₃ could act in the PVN to regulate glucose, we administered 1,25D₃ directly into the PVN (representative injection location in Supplementary Fig. 2A) and found it decreased glucose excursion during an i.p.GTT (Fig. 2D), evidenced by a decrease in the iAUC analysis (Fig. 2F). In contrast, 1,25D₃ did not decrease glucose excursion when administered to rats that previously had a PVN injection of lentiviral short hairpin (sh) RNA targeting the VDR (Fig. 2E and F). These effects were independent of body weight, since there was no body weight phenotype in the viral-treated rats (Supplementary Fig. 2B). While we were unable to quantify the VDR knockdown in our lentiviral-treated rats, the attenuation of the 1,25D₃-mediated glucose phenotype suggests a role for the VDR in the PVN in glucose regulation. Thus, we hypothesized that VDR in the PVN has a physiological role in glucose homeostasis. We tested this using a mouse model with a floxed VDR (21) and site-specific knockdown of the VDR through an AAV-Cre (VDR^{fl/fl}/Cre) compared with a control virus (VDR^{fl/fl}/vector) bilaterally in the PVN. AAV-Cre injection decreased VDR expression by ~50% in the PVN (Supplementary Fig. 2C). Interestingly, VDR^{fl/fl}/Cre mice had an increased glucose excursion during an i.p.GTT on HFD (Fig. 2G and H) but not on standard chow (Supplementary Fig. 2D). This effect was not secondary to weight differences (Supplementary Fig. 2E) or an effect of the virus itself, as there was no difference in glucose tolerance in C57Bl/6 mice after AAV-Cre PVN injection (Supplementary Fig. 2F). It is possible that the effect of the diet on glucose tolerance may have been secondary to the aging of the animals, since the GTTs were performed in the same animals at different time points. However, i3vt 1,25D₃ improved glucose tolerance in HFD-fed rats but not chow-fed rats of equivalent ages (HFD 14 weeks [Fig. 1A] and chow 16 weeks [Supplementary Fig. 1B]), which supports a differential effect of diet in the effects of vitamin D in the brain. Together, these results identify a novel pathway by which 1,25D₃ and VDR may act to affect glucose regulation in the brain and support the PVN as an integral location in these actions.

Vitamin D and Weight Regulation

Given the association of vitamin D deficiency and obesity, we sought to determine whether vitamin D had central effects on body weight or food intake. i3vt 1,25D₃ did not acutely alter food intake (Fig. 3A) but, when given chronically to HFD-fed rats, caused a marked decrease in body weight (Fig. 3B) and food intake (Fig. 3C). 1,25D₃-treatment specifically decreased fat mass without altering lean mass (Fig. 3D). There were no differences in energy expenditure between the groups (Fig. 3E). In addition, i3vt 1,25D₃ did not cause a conditioned aversion to saccharin (Fig. 3F). Smaller doses (0.132 and 0.066 µg/day) of chronic i3vt 1,25D₃ resulted in similar weight loss (Supplementary Fig. 3A) and increases in peripheral calcium

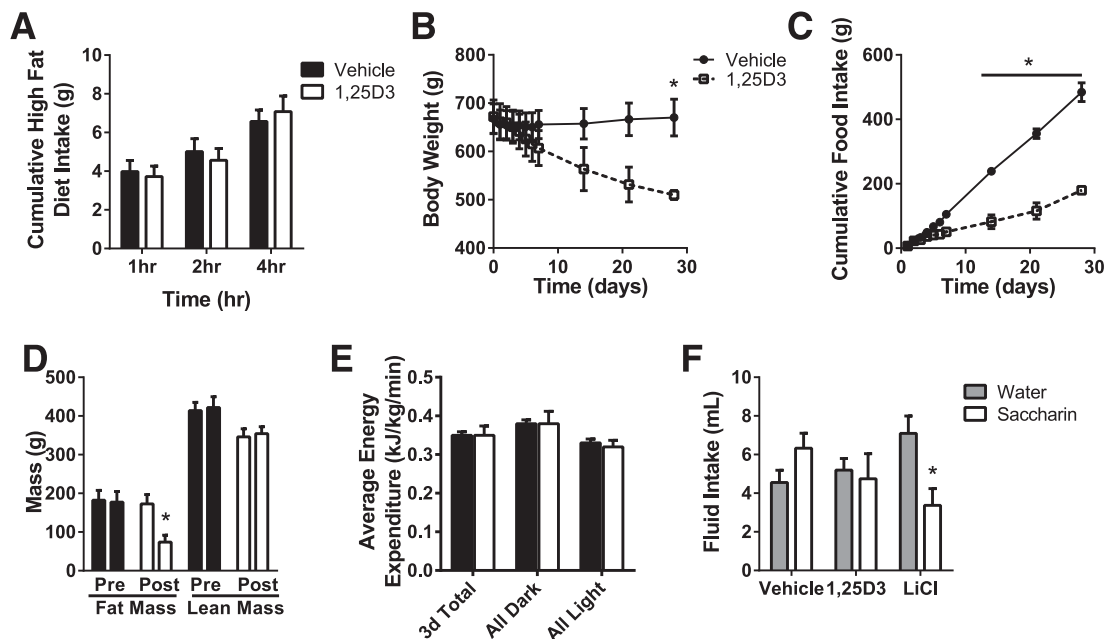


Figure 3—Chronic hypothalamic 1,25D₃ decreases body weight by decreasing food intake. **A:** Acute delivery of i3vt 1,25D₃ (0.1 μg) did not alter food intake in HFD-fed rats ($n = 10$ –11/group). **B** and **C:** Chronic delivery of i3vt 1,25D₃ (0.01 μg/h, 0.26 μg/day) decreased body weight (**B**) and food intake (**C**) over 28 days ($n = 3$ –4/group). **D:** After 28 days of chronic 1,25D₃, fat mass, but not lean mass, was decreased. **E:** After 3 days (3d) of chronic 1,25D₃ delivery, when the animals were still body weight matched, average energy expenditure was not different between the groups. **F:** No conditioned taste aversion occurred after 1,25D₃ (0.1 μg) treatment. The positive control, lithium chloride (LiCl), produced a positive conditioned taste aversion. hr, hour. * $P < 0.05$ vs. vehicle treatment (**B**–**D**) or water (**F**).

levels (Supplementary Fig. 3B). However, chronic subcutaneous delivery of 1,25D₃ did not cause weight loss (Supplementary Fig. 3C) despite a comparable increase in peripheral calcium concentration (Supplementary Fig. 3D). These results suggest that 1,25D₃ can decrease body weight through changes in food intake without changes in energy expenditure and that these effects are independent of aversive or hypercalcemic effects of 1,25D₃.

Given the potent effects of chronic 1,25D₃ on food intake, we hypothesized that 1,25D₃ might regulate key appetite-regulating neurons. We confirmed that VDRs were present in the ARC (Fig. 4A) and discovered VDR colocalized with $80.5 \pm 2.04\%$ POMC-GFP-expressing (Fig. 4B) and $86.9 \pm 5.0\%$ NPY-GFP-expressing (Supplementary Fig. 4A) neurons. 1,25D₃ had direct transcriptional effects in a hypothalamic cell line, Neuro2A, increasing mRNA expression of *cyp24a1* (Fig. 4C), a direct target of the VDR (38). Pretreatment with a VDR genomic antagonist attenuated the effects of 1,25D₃ on *cyp24a1*, indicating that 1,25D₃ acted via VDR. More importantly, we found that 1,25D₃ directly activated POMC neurons. In electrophysiology experiments with synaptic inputs blocked, 1,25D₃ activated 7/18 POMC neurons (representative tracing, Fig. 4D), causing a mean membrane potential change of 4.4 ± 1.0 mV compared with 0.17 ± 0.28 mV by vehicle treatment (Fig. 4E). The remaining neurons had no response. Interestingly, without blockade

of synaptic inputs, 4/17 POMC cells were inhibited (Supplementary Fig. 4C), indicating an additional population of POMC neurons inhibited by 1,25D₃.

DISCUSSION

The current results provide clear evidence that vitamin D has potent CNS effects to improve glucose tolerance and hepatic insulin sensitivity that do not occur when the same small dose is administered peripherally. Previous literature has shown vitamin D is important for insulin secretion, specifically, that vitamin D increases insulin secretion (10,39,40). Conversely, we found that central administration of 1,25D₃ caused a small reduction in peripheral insulin levels. It is unlikely that we missed the effect of 1,25D₃ on the insulin peak secondary to the frequent sampling that occurred during the i.v.GTT. Therefore, our data suggest that in addition to VDR action in the β -cell, 1,25D₃ may also improve glucose tolerance by insulin secretory-independent mechanisms. In fact, our data strongly suggest that CNS 1,25D₃ improves glucose tolerance by improving hepatic insulin sensitivity in HFD-fed rats.

Like humans (37), we found that rodents also have strong VDR signaling in the PVN. In our animals, unilateral direct injection of 1,25D₃ into the PVN decreased glucose excursions but had no effect in animals treated with lentiviral VDR shRNA. This supports a direct action of 1,25D₃ via the VDR in the PVN to control glucose

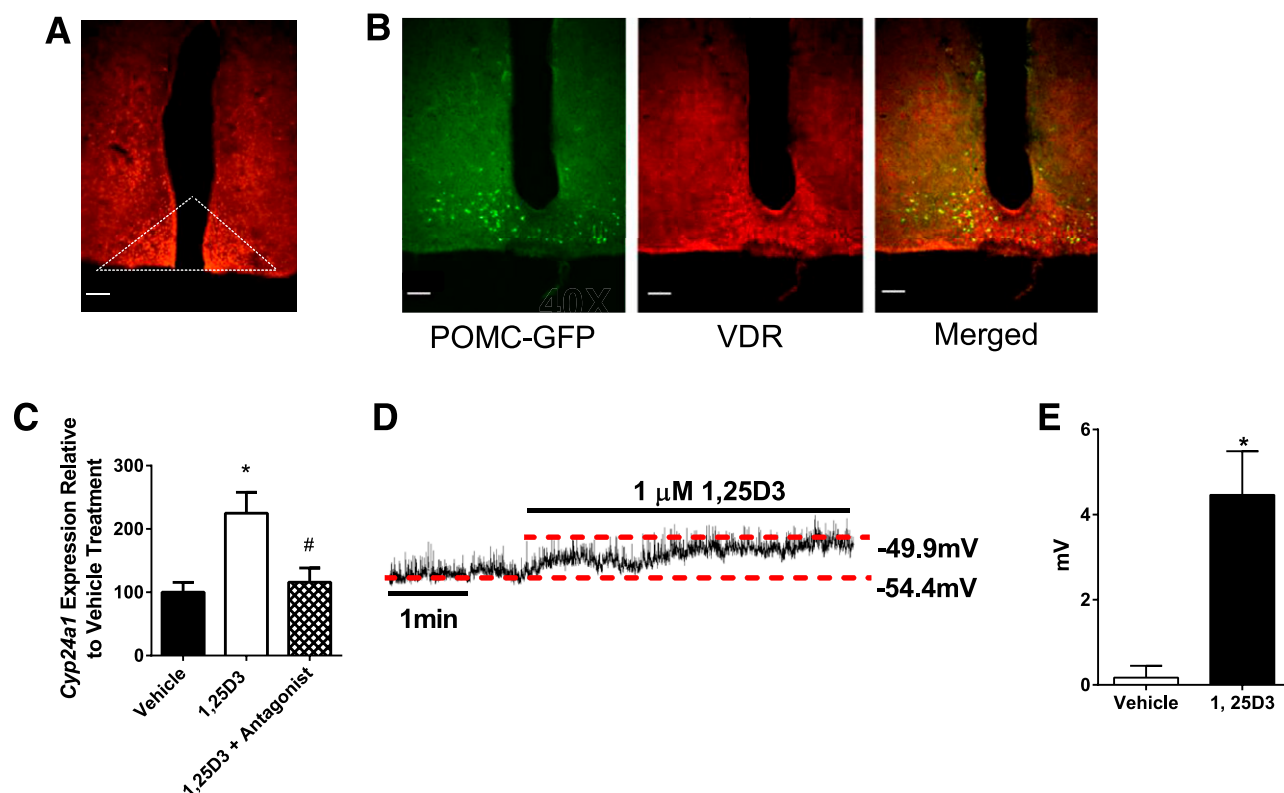


Figure 4—1,25D₃ depolarizes POMC neurons. **A:** VDRs are present in the ARC ($\times 10$ magnification). **B:** POMC (green; first panel) and VDR (red; middle panel) neurons colocalize (yellow; third panel). **C:** 1,25D₃ increases *cyp24a1* levels in Neuro2A cells. The VDR antagonist ZK159222 attenuated the 1,25D₃-induced expression of *cyp24a1*. **D:** Representative tracing of depolarization of a POMC neuron by 1,25D₃. **E:** Change in membrane potential by 1,25D₃ ($n = 7$) vs. vehicle ($n = 11$). Dashed lines outline the ARC. Scale bars = 100 μ m. * $P < 0.05$ vs. vehicle; # $P < 0.05$ vs. ZK159222.

levels, which is novel and consistent with research demonstrating the importance of the PVN in glucose control (18,41,42).

The effect of diet on the action of vitamin D in the brain is important for both exogenous and endogenous vitamin D action. First, in HFD but not chow conditions, i3vt 1,25D₃ improved glucose excursions. The lack of an effect of 1,25D₃ in a chow state is not surprising, since it is difficult to improve upon an already normal glucose tolerance. However, knockdown of the VDR in the PVN resulted in a worsened GTT only in HFD-fed mice. Thus, PVN VDRs are not necessary for normal glucose tolerance in a chow-fed state, but in a state of excess nutrients act to improve glucose tolerance. This may explain the association between low vitamin D levels and impaired glucose tolerance in obese patients, since a lower vitamin D level would theoretically lead to decreased VDR action in the PVN.

With regard to energy homeostasis, our data show that 1,25D₃ has actions within the brain to decrease food intake, thereby decreasing body weight and fat mass. One previous report of intracerebral 1,25D₃ also showed significant weight loss over 6 days of treatment at a comparable dose ($\sim 0.24 \mu$ g/day) (43). This anorectic effect of i3vt 1,25D₃ is not secondary to visceral illness, since

1,25D₃ did not cause a conditioned taste aversion. We ruled out hypercalcemia (a known cause of anorexia) as a possible mechanism of weight loss, since peripherally administered 1,25D₃ increased calcium but did not change body weight. Additionally, since only central 1,25D₃, not peripheral 1,25D₃, caused weight loss, this ruled out a peripheral mechanism of action. Thus, i3vt 1,25D₃-mediated weight loss is not likely to be due to hypercalcemia or peripheral action of 1,25D₃.

Although we demonstrated VDR within both the PVN and ARC, there does not seem to be a role for the PVN in weight regulation directly. There was no weight phenotype in the mice with bilateral PVN VDR knockdown. Although it is possible that the importance of the PVN in weight regulation was not seen secondary to only achieving a $\sim 50\%$ knockdown, it is more likely that the anorectic action of 1,25D₃ lies within the ARC.

Our study is the first to show colocalization of VDR with POMC and NPY neurons, which supports the hypothesis that action of 1,25D₃ in the brain to cause weight loss may occur through VDR in the ARC. Using a neuronal cell line, we demonstrated that 1,25D₃ has VDR-dependent transcriptional activity in neurons, since 1,25D₃ treatment increased expression of a known VDR target gene, *cyp24a1*, which was blocked with antagonism

of the VDR. However, VDR also acts through nongenomic/rapid-response pathways involving calcium channels (44). The rapid depolarization of POMC cells by $1,25D_3$ indicates a rapid-response action of $1,25D_3$. One possible mechanism by which $1,25D_3$ may depolarize POMC cells is through activation of transient receptor potential channels (TRPCs). Activation of TRPC5 cells can excite POMC neurons (45). Although there are no published studies on the effects of $1,25D_3$ on TRPC5 channels, there are published reports of $1,25D_3$ activating TRPC3 channels (46). Thus, future studies will examine the molecular mechanism of action of $1,25D_3$ on POMC neurons. It is unclear whether the food intake effects of $1,25D_3$ in the brain are through rapid or genomic effects, since we saw food intake reduction in chronic, but not acutely, treated animals. Although we used well-handled animals, it is possible that the acute effects of vitamin D on food intake are masked by the unavoidable stress occurring in animals after handling. Additionally, it is also possible that the genomic and rapid effects are dependent upon one another in vivo, as has been suggested by data on the rapid effects of $1,25D_3$ on insulin secretion (44).

Our data provide elegant explanations for the conflicting research surrounding vitamin D and obesity/diabetes. First, our data clearly show the importance of central action of vitamin D to affect glucose homeostasis or body weight. Clinical trials have used a vitamin D precursor, ergocalciferol (vitamin D2) or cholecalciferol (vitamin D3), and not the active compound, $1,25D_3$. Since $1,25D_3$ concentrations are tightly regulated in the body, supplementation of precursors may not lead to increases in $1,25D_3$ levels, particularly in the CNS. Previous research demonstrates that both cholecalciferol and $1,25D_3$ have limited uptake into the brain (20,47). Our own data show no effect of peripheral $1,25D_3$ on weight loss or glucose but clearly demonstrate that $1,25D_3$ can act within the brain to effect change in glucose and weight regulation. Thus, peripheral supplementation of a vitamin D derivative may have limited effects due to its limited access to the brain or the lack of change in the concentration of central $1,25D_3$, despite increases in peripheral levels of 25-hydroxyvitamin D. Secondly, our data clearly indicate the necessity of an obese state to illuminate the effects of $1,25D_3$ /VDR in the brain. Paradoxically, the VDR-null animal has a lean phenotype and on an HFD has decreased fat mass thought to occur through an upregulation of uncoupling proteins (48,49). The relevance of this effect is unclear in humans, since vitamin D supplementation does not cause increases in fat mass or decreases in energy expenditure. Additionally, it is unknown what developmental compensation occurs, especially in the brain, in a VDR-null animal. Our study highlights the role of the VDR in adulthood without any confounding developmental compensation, which is more relevant to the clinical model of vitamin D deficiency associated with obesity. Vitamin D supplementation, though, has not improved glucose, insulin resistance, or adiposity measures in overweight people in multiple studies (12–15).

Interestingly, trials showing beneficial effects of vitamin D supplementation occurred in conjunction with diet modifications and/or changes in BMI (4,16,17). Thus, it is possible that peripheral administration of vitamin D has impaired transport into the brain in an obese setting, which can be improved with diet modification or weight loss. Overall, the contrast between the clinical studies and our data underscores the necessity of understanding the mechanisms underlying the association of low vitamin D levels and obesity/diabetes in order to better inform clinical trial design.

In conclusion, our studies are the first to show an effect of $1,25D_3$ in the brain on both glucose and weight regulation. We have demonstrated that these effects are dependent upon action of vitamin D in distinct hypothalamic nuclei. Additionally, we have demonstrated a physiological and pharmacologic effect of vitamin D in the PVN on glucose homeostasis. Our data support the ARC as a possible site of action for $1,25D_3$ actions on weight regulation. Further elucidation of the signaling mechanisms involved in central VDR signaling are important for understanding the clinical association of vitamin D deficiency and obesity/diabetes but also for developing vitamin D derivatives that accentuate the pharmacological effects on weight loss and improving glucose homeostasis.

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Author Contributions. S.R.S. designed and performed experiments, analyzed data, and wrote the manuscript. D.M.A. contributed to discussion and performed data collection. A.P.C., R.G.-A., and Y.H. performed data collection. Y.X. contributed to discussion and provided materials. D.G. provided materials and advice. D.D.M. contributed to the discussion and helped with experimental design. R.J.S. and D.A.S. contributed to discussion, helped with experimental design, and reviewed and edited the manuscript. S.R.S. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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