

Activation of *N*-Methyl-D-aspartate (NMDA) Receptors in the Dorsal Vagal Complex Lowers Glucose Production^{*[S]}

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Diabetes is characterized by hyperglycemia due partly to increased hepatic glucose production. The hypothalamus regulates hepatic glucose production in rodents. However, it is currently unknown whether other regions of the brain are sufficient in glucose production regulation. The *N*-methyl-D-aspartate (NMDA) receptor is composed of NR1 and NR2 subunits, which are activated by co-agonist glycine and glutamate or aspartate, respectively. Here we report that direct administration of either co-agonist glycine or NMDA into the dorsal vagal complex (DVC), targeting the nucleus of the solitary tract, lowered glucose production *in vivo*. Direct infusion of the NMDA receptor blocker MK-801 into the DVC negated the metabolic effect of glycine. To evaluate whether NR1 subunit of the NMDA receptor mediates the effect of glycine, NR1 in the DVC was inhibited by DVC NR1 antagonist 7-chlorokynurenic acid or DVC shRNA-NR1. Pharmacological and molecular inhibition of DVC NR1 negated the metabolic effect of glycine. To evaluate whether the NMDA receptors mediate the effects of NR2 agonist NMDA, DVC NMDA receptors were inhibited by antagonist D-2-amino-5-phosphonovaleric acid (D-APV). DVC D-APV fully negated the ability of DVC NMDA to lower glucose production. Finally, hepatic vagotomy negated the DVC glycine ability to lower glucose production. These findings demonstrate that activation of NR1 and NR2 subunits of the NMDA receptors in the DVC is sufficient to trigger a brain-liver axis to lower glucose production, and suggest that DVC NMDA receptors serve as a therapeutic target for diabetes and obesity.

Diabetes and obesity are partly characterized by a disruption in glucose homeostasis. An elevation of glucose production

and/or a decrease in glucose uptake lead to a rise in plasma glucose levels and the breakdown of gluco-regulatory homeostatic mechanisms. In fact, an increased production of glucose by the liver is determined to be the major contributing factor to fasting hyperglycemia in type 2 diabetes (1).

To date, the mechanisms underlying the regulation of hepatic glucose production and homeostasis in healthy and obese/diabetic conditions remain to be elucidated. In this regard, the hypothalamus detects a rise in nutrients and hormones to regulate peripheral glucose homeostasis and specifically lower glucose production (2–14). However, the neuronal network that controls glucose homeostasis remains unclear.

The *N*-methyl-D-aspartate (NMDA)⁵ receptor is composed of NR1 and NR2 subunits, which are activated by co-agonist glycine and glutamate or aspartate, respectively (15). NMDA is a selective agonist of NMDA receptors and NMDA receptors are fundamental to excitatory neurotransmission (Fig. 1*a*). In the CNS, the NMDA receptors have important roles in synaptic plasticity and neuronal development (16). In addition, direct administration of NMDA receptor blocker into the DVC negates the ability of lipid/cholecystokinin-sensing mechanisms in the gut to regulate glucose production (17, 18). However, it remains unknown whether direct activation of NMDA receptors in the DVC control glucose homeostasis. Here we tested the hypothesis that activation of NR1 and NR2 subunits of the NMDA receptors in the DVC triggers the hepatic vagus to lower glucose production and regulate glucose homeostasis *in vivo*.

MATERIALS AND METHODS

Ethics Statement—All study protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the University Health Network.

Animal Preparation—Eight-week-old male Sprague-Dawley (SD) rats weighing between 260 and 280 g (Charles River Laboratories, Montreal, QC) were used for our studies. Rats were housed in individual cages and maintained on a standard light-dark cycle with access to standard rat chow and water *ad libitum*. Rats were anesthetized by intraperitoneal (intraperitoneal) injection of ketamine (60 mg/kg) and xylazine (8 mg/kg). A 26-gauge bilateral guide cannula made of stainless steel (Plas-

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⁵ The abbreviations used are: NMDA, *N*-methyl-D-aspartate; DVC, dorsal vagal complex; D-APV, D-2-amino-5-phosphonovaleric acid; HVAG, hepatic vagotomy; i.v., intravenous; NTS, nucleus of solitary tract; MBH, mediobasal hypothalamus; MOPS, 4-morpholinepropanesulfonic acid; pfu, plaque-forming units.

tic Ones Inc., Roanoke, VA) was stereotactically implanted (David Kopt Instruments, Tujunga, CA), as previously described (18), into the dorsal vagal complex (DVC) targeting the nucleus of solitary tract (NTS, 0.0 mm on occipital crest, 0.4 mm lateral to midline, 7.9 mm below skull surface). The guide cannula was secured in place with mounting screws, cyanoacrylate gel, and the dental cement. To create a closed system, the guide cannula was kept free of obstruction by inserting a dummy cannula, which was then covered with a dust cap (Plastics One Inc.).

After 1 week of recovery, the rats underwent intravenous (i.v.) and intra-arterial catheterization, where the internal jugular vein and the carotid artery were cannulated, respectively, for infusion and sampling during *in vivo* clamp studies (described below). The catheter for vascular cannulation was made of PE50 tubing (Clay Adams, 0.023" ID \times 0.038" OD) attached to a silastic tubing (Corning, 0.020" ID \times 0.037" OD) at the tip. Recovery from surgery was monitored by measuring daily food intake and body weight, ensuring that only animals that have at least attained within 10% of starting body weight were used for infusion studies.

DVC Administration in Clamp Procedure—Four days post-i.v. catheterization, animals underwent the clamp studies. Rats were restricted to 20 g of food the night before the experiment to ensure the same nutritional status during the clamp procedure, which lasted a total of 210 min. At $t = 0$ min, central infusion of various treatments into DVC via indwelling cannulae were initiated and maintained throughout the entire duration of the clamp at a constant infusion rate of 0.33 μ l/h (all central infusion performed with CMA/400 syringe microdialysis infusion pumps). Briefly, the DVC treatment groups were: [1] saline, [2] (+)-MK-801 (0.06 ng/min, dissolved in saline), [3] glycine (10 μ M, dissolved in saline), [4] glycine (10 μ M) + (+)-MK-801 (0.06 ng/min), [5] 7 chlorokynurenic acid (10 μ M), [6] glycine (10 μ M) + 7 chlorokynurenic acid (10 μ M), [7] glycine (10 μ M) + Strychnine (0.6 μ M), [8] NMDA (100 μ M), [9] NMDA (100 μ M) + D-APV (300 μ M) and [10] D-APV (300 μ M).

A primed-continuous intravenous infusion of [3-³H]glucose (Perkin Elmer; 40 μ Ci bolus; 0.4 μ Ci/min; all infusion performed with Harvard Apparatus PHD 2000 infusion pumps) was also initiated at the onset of the procedure ($t = 0$ min) and maintained for the entire duration of the clamp to assess glucose kinetics based on the tracer-dilution methodology. A pancreatic (basal insulin)-euglycemic clamp was started at $t = 90$ min until 210 min to evaluate the effect of DVC treatments on glucose metabolism independent of differences among groups in plasma insulin and glucose. To achieve this, somatostatin (3 μ g/kg/min) was continuously infused to suppress endogenous insulin and glucagon secretions. Insulin (0.8 mU/kg/min) was replaced back to basal level (see supplemental Tables S1-S2). During this period, a variable infusion of 25% glucose solution was started and periodically adjusted to maintain the plasma glucose concentration near the basal state (see supplemental Tables S1-S2 for basal and clamp glucose levels).

Samples for the determination of [3-³H]glucose specific activity were obtained at 10-min intervals. Plasma samples were also collected at regular intervals for determination of plasma insulin levels. At the end of the experiment, rats were anesthe-

tized and tissue wedges (*i.e.* DVC and mediobasal hypothalamus (MBH)) were freeze-clamped *in situ* and stored at -80°C for later analysis. To verify the anatomical placement of the DVC bilateral cannulae (targeting the NTS), we infused radioactive tracer [3-³H]glucose via the DVC cannulae at the same infusion rate (0.33 μ l/hr) and duration (210 min) as in the pancreatic-euglycemic clamp procedure. By the end of the 210-min infusion, DVC, MBH and cortical tissue wedges were obtained and were homogenized for counting. We discovered that DVC infusion selectively increased radioactivity (dpm/mg tissue) in the DVC but not in the MBH or the cortex (supplemental Fig. S3).

Construct and Virus Propagation—A small interfering RNA template of NRI 5'-GAATGTCCATCTACTCTGATTCAAGAGATCAGAGTAGATGGACATTC-3' or the mismatch 5'-GTATGACCATGTACTCTCATTCAAGAGATGAGAGTACATGGTCATAC-3' (28) was inserted into a shuttle vector provided by the pSilencer adeno 1.0-CMV system (Invitrogen). The shuttle vector was then used to generate the recombinant adenovirus and packaged in HEK293 cells according to the manufacturer's instructions. A viral titer in plaque forming unit (pfu)/ml was determined by end point dilution assay (adenovirus containing small hairpin RNA-NRI: 4.0×10^{11} pfu/ml; MM, 4.0×10^{11} pfu/ml).

Western Blot Analysis—MBH and DVC/NTS tissue samples (~ 8 mg) were sonicated in homogenization buffer [in mM: 20 MOPS, 1 EDTA disodium dihydrate, 1 tablet of Roche EDTA-free minipill]. 60 μ g of total protein was subjected to 8% SDS-PAGE and transferred to polyvinylidene difluoride membrane. The membranes were probed with primary antibody mouse anti-NMDAR1 (BD Pharmingen) at 1:1000 overnight at 4°C and then with secondary anti-mouse horseradish peroxidase (Santa Cruz) at 1:5000 for 1 hour at room temperature. The horseradish peroxidase signal was detected with an enhanced chemiluminescence substrate solution. Rabbit anti- β -tubulin (Cell Signaling) was used as loading control.

Immunofluorescence Staining Method—Male Sprague-Dawley rats were anesthetized and sacrificed after 11 days of viral brain injection, followed by transcardial perfusion of saline (40 ml) and 4% paraformaldehyde (35 ml). Brains were removed, and 4 mm coronal sections containing the medulla (for DVC staining) were embedded, frozen, and stored in -80°C in Optimal Cutting Temperature (O.C.T.) compound (Tissue-Tek). 10 μ m coronal tissue sections mounted on glass slides were obtained via cryostat sectioning of the frozen brain sample. For NR1 of NMDA receptor immunostaining, tissue were blocked with 10% normal goat serum with 0.2% Triton X-100 for 1 h and incubated overnight with mouse anti-NMDAR1 antibody (1:250, BD Pharmingen). After washing and incubation with goat anti-mouse immunoglobulin antibody (1:1000, Alexa-Fluor 488, Molecular Probes), the slides were viewed under fluorescence microscope.

DVC Adenoviral Infection in Clamp Procedure—Immediately post-stereotaxic surgery, rats were administered 3 μ l of adenovirus per side of cannulae over a 30-s injection with microsyringes. Microsyringes were left in cannulae for 20 min before removal to prevent backflow of volume. The adenovirus infected-groups were: [1] DVC MM, [2] DVC shRNA-NR1. To

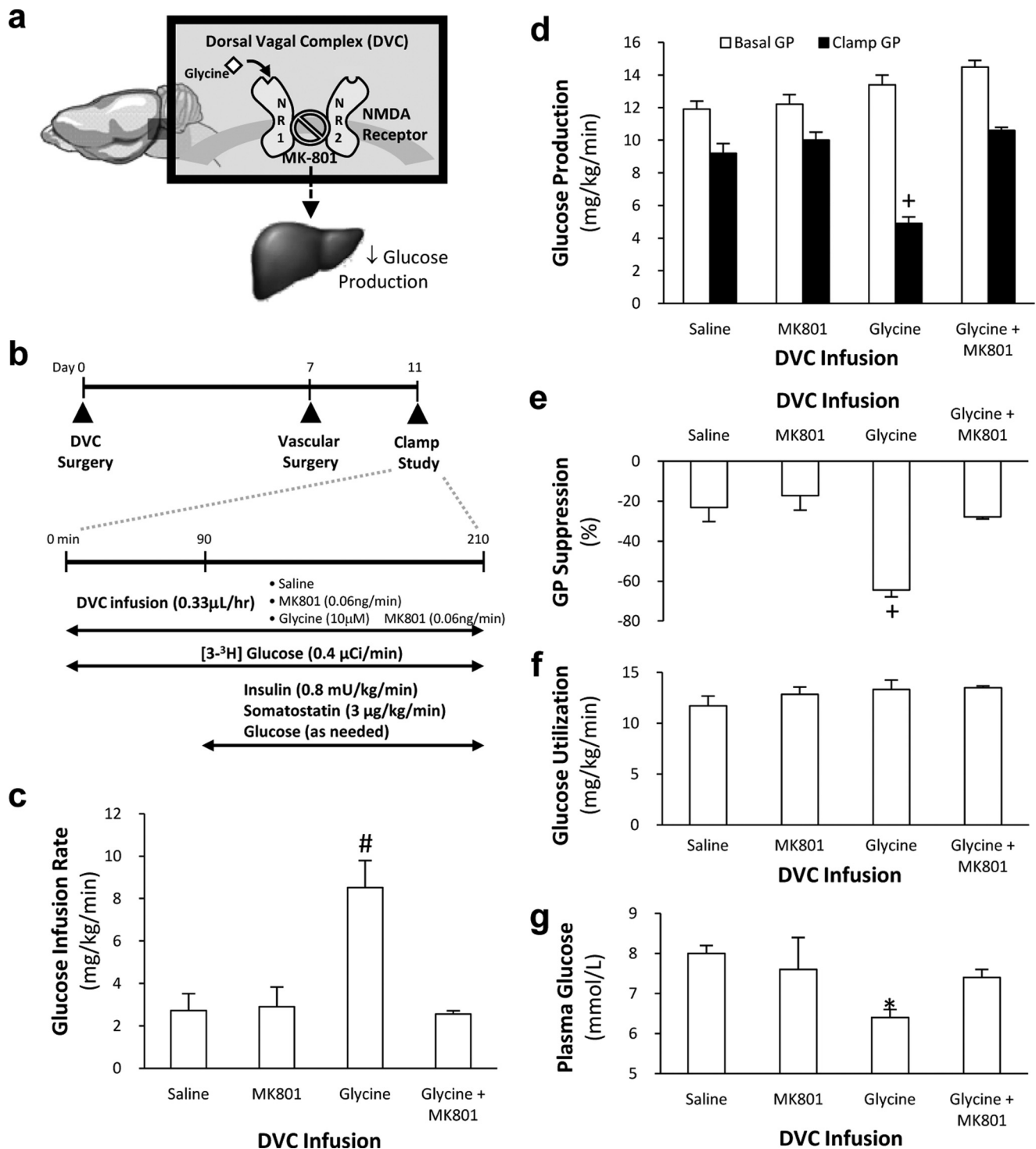


FIGURE 1. DVC administration of glycine lowers glucose production, an effect that is negated by NMDA receptor antagonist MK-801. *a*, schematic representation of working hypothesis: DVC co-agonist glycine binds to the NR1 subunit to potentiate NMDA receptor activation, leading to a decrease in hepatic glucose production. NMDA receptor antagonist MK-801 binds to the NMDA receptor, preventing ion flow through these channels and blocks the glucose production-lowering effect of glycine. *b*, schematic representation of experimental design: DVC (targeting the NTS) stereotaxic surgery was performed on male Sprague-Dawley rats (~260–280 g). After 1 week of recovery (day 7), vascular surgery (*i.e.* intravenous catheterization) was performed. Rats were given 5 recovery days until clamp studies (day 11). During the pancreatic-euglycemic clamp procedure, DVC administration of glycine increased (c) glucose infusion rate and lowered (d) glucose production, effects that were fully negated by co-administration with MK-801 (c and d). *e*, suppression of glucose production during the clamp period was expressed as percent decrease from basal glucose production. *f*, glucose utilization was comparable in all groups. Under non-clamped settings, DVC glycine infusion lowered (g) plasma glucose levels, an effect that was fully negated by co-administration with MK-801. DVC saline (*n* = 6), DVC MK-801 (*n* = 5), DVC glycine (*n* = 6), DVC glycine + MK-801 (*n* = 5). *, *p* < 0.05; #, *p* < 0.01; +, *p* < 0.001.

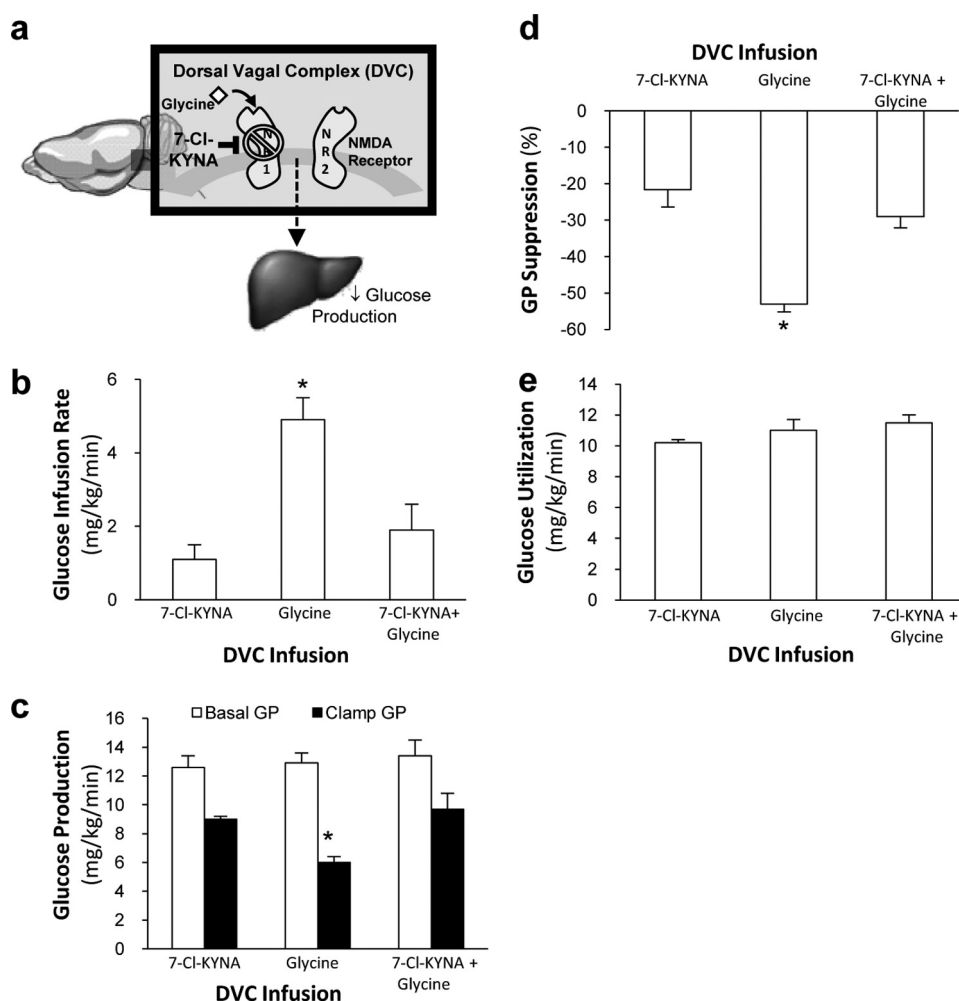


FIGURE 2. DVC administration of 7-chlorokynurenic acid (7-Cl-KYNA), an NR1 antagonist, abolishes the suppressive effect of glycine on glucose production. *a*, schematic representation of working hypothesis: Infusion of 7-Cl-KYNA disables the NMDA receptors, leading to the negation of the glucose production-lowering effect of DVC glycine. DVC administration of 7-Cl-KYNA fully obliterated the glycine-induced increase in (*b*) glucose infusion rate and decrease in (*c*) glucose production. *d*, suppression of glucose production during the clamp period was expressed as percent decrease from basal glucose production. *e*, glucose utilization was comparable in all groups. DVC 7-Cl-KYNA ($n = 4$), DVC glycine ($n = 7$), DVC glycine + 7-Cl-KYNA ($n = 6$). *, $p < 0.05$.

verify the presence of the adenovirus, Western blot analysis (NR1 expression in MM *versus* shRNA-NR1 animals) were performed in the appropriate tissue (*i.e.* DVC) taken right after the clamp procedure on Day 11 using the method detailed above. Adenovirus-injected animals used for clamp procedures underwent intravenous catheterization as discussed. Four days post-intravenous catheterization, animals whose daily food intake and body weight had recovered back to within 10% of baseline underwent the clamp studies in the aforementioned manner. DVC MM and DVC hRNA-NR1 rats were centrally infused with either saline or glycine (10 μ M).

Hepatic Branch Vagotomy—A group of rats, in addition to vascular cannulation, underwent a sectioning of the hepatic branch of the anterior abdominal vagal trunk as described (18). A laparotomy incision was made on the ventral midline just caudal to the xiphoid process, and the abdominal muscle wall was opened with a second incision. The stomach and the lower esophagus were exposed and gently retracted from the abdominal cavity by severing the gastrohepatic ligament using fine forceps, revealing the descending ventral esophagus

and the ventral subdiaphragmatic vagal trunk. The hepatic branch of the ventral subdiaphragmatic vagal trunk was transected and the hepatoesophageal omentum was severed so that no tissue connections between the esophagus and the liver remained anywhere along the length of the subdiaphragmatic esophagus. The transection of the hepatic vagus nerve interrupts neural communication between the brain and the liver, and to a much lesser degree, slightly decreases the innervations to the intestine. Sham-operated rats underwent all procedures except transection of the neural tissue. The abdominal cavity was bathed with saline during the procedure to prevent drying of the viscera.

Biochemical Analysis—Plasma glucose concentrations were measured by the glucose oxidase method (Glucose Analyzer GM9, Lunenbertg, MA). Plasma insulin levels were determined by radioimmunoassay (RIA) (Linco Research, St. Charles, MO).

Statistical Analysis—The statistical differences between groups were determined by two-way analysis of variance followed by the Tukey post hoc test with a probability of $p < 0.05$ taken as significant. Data are presented as means \pm S.E.

RESULTS

DVC Glycine Activates NMDA Receptors to Lower Glucose Production—An elevation of glycine concentration enhances NMDA receptor function in oocytes or hippocampal pyramidal neurons *in vitro* (19, 20) and potentiates the NMDA-mediated neuronal firing rate of the dorsal horn *in vivo* (21). Here we first examined whether direct activation of the NMDA receptor in the DVC targeting the NTS via DVC glycine administration regulates glucose homeostasis *in vivo* (Fig. 1*a*). We first infused glycine at 10 μ M, \sim 1.5-fold higher than the CSF [glycine] in both humans and rodents (22, 23), directly into the DVC of conscious unrestrained rats, and evaluated the gluco-regulatory effects of DVC glycine with the pancreatic clamp technique (Fig. 1*b*). The use of the pancreatic-euglycemic clamp technique and tracer-dilution methodology allows for the assessment of the effects that treatments have on the rate of hepatic glucose production and peripheral glucose uptake independent of differences among groups in peripheral circulating insulin and glucose levels (7, 18, 24). During the pancreatic clamp, when circulating insulin and glucose were maintained at near basal levels (supplemental Table S1), DVC glycine administration mark-

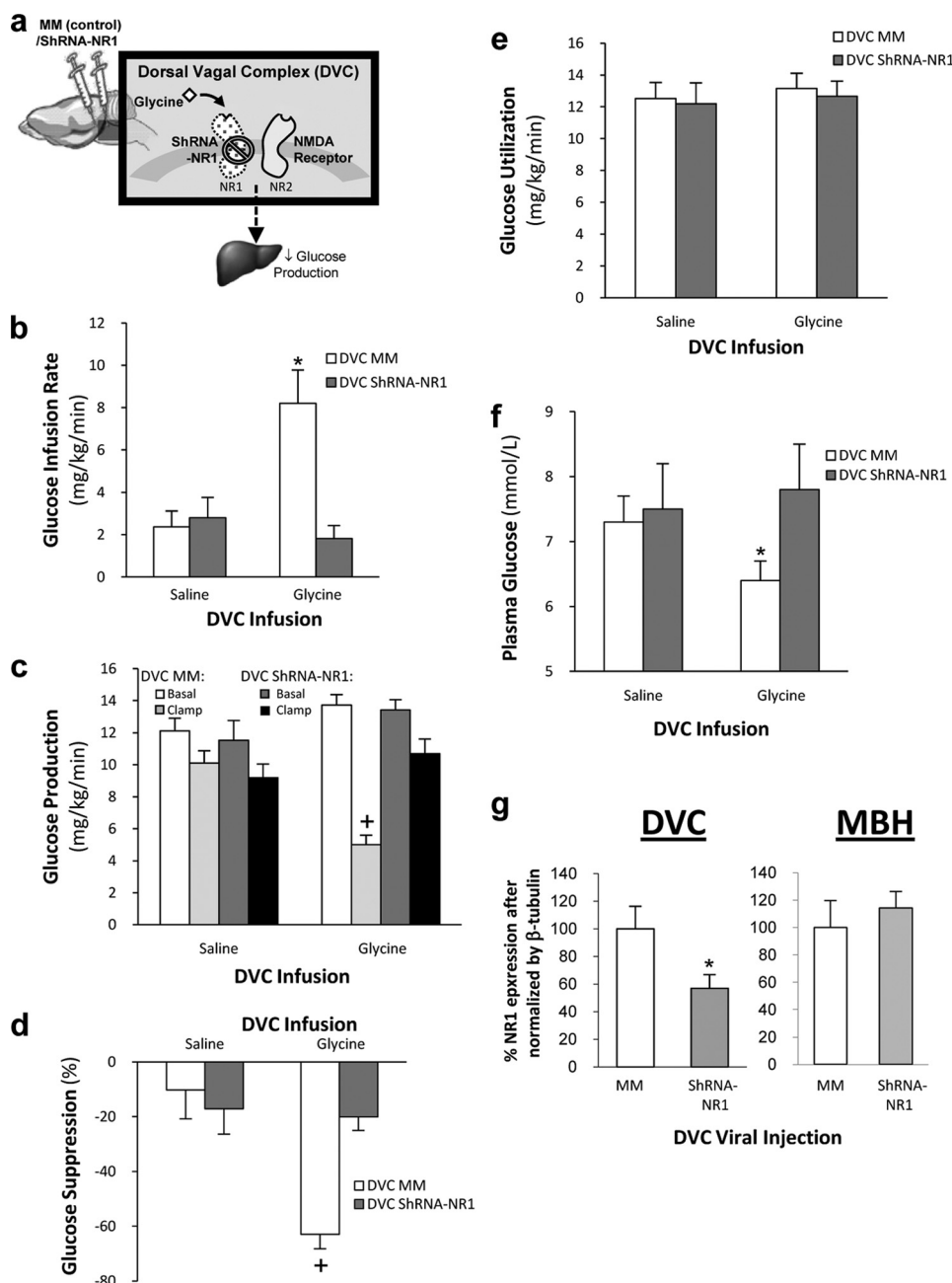


FIGURE 3. Molecular knockdown of DVC NR1 negates the suppressive effect of glycine on glucose production. *a*, schematic representation of working hypothesis: Injection of the adenovirus containing shRNA-NR1 in the DVC disables the NMDA receptors, leading to negation of the glucose production-lowering effect of DVC glycine. DVC administration of glycine in rats injected with shRNA-NR1-expressing adenovirus failed to increase (*b*) glucose infusion rate and lower (*c*) glucose production. *d*, suppression of glucose production during the clamp period was expressed as percent decrease from basal glucose production. *e*, glucose utilization was comparable in all groups. Molecular knockdown of DVC NR1 negated the glycine infusion-induced drop in (*f*) plasma glucose levels. *g*, representative Western blot analysis of NR1 expression in the DVC ($n = 4$) and MBH ($n = 4$) tissue of adenovirus-infected clamp animals. *In vivo* infection of adenovirus expressing shRNA-NR1 in the DVC led to a decrease in NR1 expression in the DVC but not in the MBH (11 days post-infection). Infection of control adenovirus expressing mismatch (MM) in the DVC did not alter NR1 expression in either tissue. DVC MM + saline ($n = 5$), DVC shRNA-NR1 + saline ($n = 5$), DVC MM + glycine ($n = 5$), DVC shRNA-NR1 + glycine ($n = 7$). *, $p < 0.05$; #, $p < 0.01$; +, $p < 0.001$.

edly increased the rate of exogenous systemic glucose infusion required to maintain euglycemia compared with DVC saline (Fig. 1c). Based on the tracer dilution methodology, it was determined that the increase in glucose infusion rate induced by DVC glycine was accounted for entirely by an inhibition of

glucose production (Fig. 1, *d* and *e*), rather than an increase in glucose uptake (Fig. 1*f*).

We also administered DVC glycine at $1 \mu\text{M}$ ($n = 4$) or $1000 \mu\text{M}$ ($n = 4$) and glucose production during the clamp was $10.9 \pm 1.5 \text{ mg/kg/min}$ or $2.0 \pm 0.3 \text{ mg/kg/min}$, respectively. These data indicate that DVC glycine dose-dependently lowers glucose production, and the metabolic effects of DVC glycine administered at $10 \mu\text{M}$ is not maximized. Under non-clamped settings where plasma gluco-regulatory hormones were allowed to change at will, DVC glycine ($10 \mu\text{M}$) administration for 90 min was still sufficient to lower plasma glucose levels by $\sim 1\text{--}1.5 \text{ mM}$ (Fig. 1*g*). Together, these data demonstrate that direct DVC glycine administration lowers glucose production and plasma glucose levels.

To verify whether DVC glycine administration lowers glucose production and plasma glucose levels through the activation of DVC NMDA receptors, we co-infused NMDA receptor ion channel blocker MK-801 with glycine directly into the DVC (Fig. 1*a*). MK-801 is a high affinity blocker of the NMDA receptor (25). During the clamps, it was first noted that DVC MK-801 alone did not affect any of the glucose kinetics parameters compared with DVC saline (Fig. 1, *c–g*). However, co-administration of DVC MK-801 with glycine was sufficient to fully negate the increase in glucose infusion rate (Fig. 1*c*) and the decrease in glucose production (Fig. 1, *d* and *e*) induced by DVC glycine independent of differences in circulating insulin levels among groups (supplemental Table S1). In addition, DVC MK-801 fully reversed the plasma glucose-lowering effect induced by glycine under the unclamped conditions (Fig. 1*g*). Thus, glycine enhances NMDA receptor activation in the

DVC to lower glucose production and plasma glucose levels.

NR1 Subunit of the DVC NMDA Receptors Is Required for Glycine to Lower Glucose Production—NMDA-induced increases in neuronal intracellular calcium and membrane currents is abol-

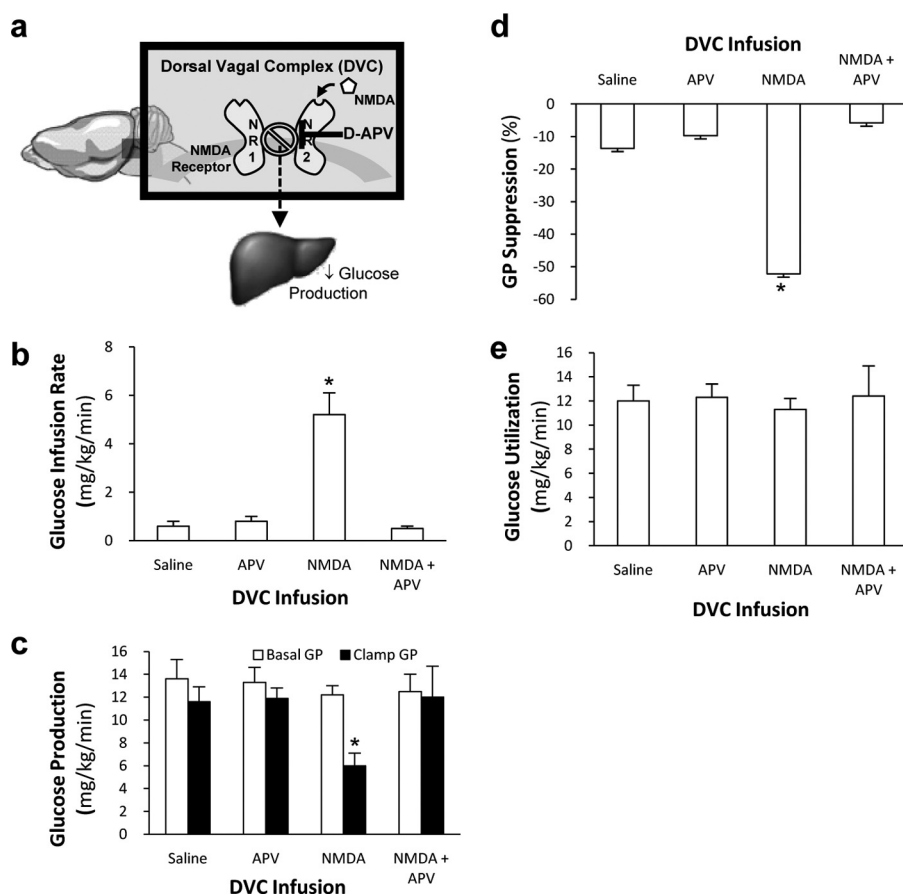


FIGURE 4. DVC administration of D-2-amino-5-phosphonovaleric acid (D-APV), a NMDA receptor antagonist, negates the suppressive effect of NMDA on glucose production. a, schematic representation of working hypothesis: Infusion of D-APV disables the NMDA receptors, leading to the negation of the glucose production-lowering effect of DVC NR2 agonist NMDA. DVC administration of D-APV fully reversed the NMDA-induced increase in (b) glucose infusion rate and decrease in (c) glucose production. d, suppression of glucose production during the clamp period was expressed as percent decrease from basal glucose production. e, glucose utilization was comparable in all groups. DVC saline ($n = 4$), DVC D-APV ($n = 4$), DVC NMDA ($n = 6$), DVC NMDA + D-APV ($n = 5$). *, $p < 0.01$.

ished in NR1-null mice (26). In parallel, selective knockdown of the glycine-binding NR1 subunit of NMDA receptor in the spinal cord dorsal horn was sufficient to reduce NMDA-induced neuronal currents (27). These data indicate that a selective inhibition or knockdown of NR1 subunit of the NMDA receptor is sufficient to negate glycine-induced NMDA neurotransmission. To evaluate whether DVC glycine activates NR1 subunit of the NMDA receptors to potentiate NMDA transmission to lower glucose production, we inhibited NR1 in the DVC with pharmacological and molecular approaches. We first co-infused NR1 competitive antagonist 7-chlorokynurenic acid with glycine directly into the DVC (Fig. 2a). DVC 7-chlorokynurenic acid administered at $10 \mu\text{M}$ alone did not affect glucose metabolism (Fig. 2, b–d) but was sufficient to fully negate the ability of DVC glycine ($10 \mu\text{M}$) to increase glucose infusion rate (Fig. 2b) and to decrease glucose production (Fig. 2, c and d) during the clamps. Glucose uptake was comparable in all groups (Fig. 2e). We have also co-administered DVC 7-chlorokynurenic acid at 0.5, 1, 5, 20, or $30 \mu\text{M}$ ($n = 3$ per group) with glycine ($10 \mu\text{M}$). Glucose production during the clamps was 5.1 ± 1.0 mg/kg/min, 5.6 ± 0.9 , 7.7 ± 0.8 , 10.0 ± 1.7 or 12.9 ± 1.3 ,

respectively. These findings together indicate that DVC NR1 antagonist 7-chlorokynurenic acid negates the ability of glycine to lower glucose production in a dose-dependent manner.

To alternatively evaluate whether glycine in the DVC regulates glucose homeostasis through the activation of NR1 subunit, we developed a molecular approach to knockdown the NR1 subunit of the NMDA receptor in the DVC *in vivo* (supplemental Fig. S1a). We first constructed an adenoviral vector expressing the shRNA of NR1 or mismatch sequence control under the cytomegalovirus (CMV) promoter as previously described with modifications (28) (supplemental Fig. S1a). Direct injection of NR1 antisense into the spinal cord reduces NR1 by 30% but is sufficient to attenuate nociception in rodents (29). In addition, an injection of adeno-associated virus expressing the shRNA-NR1 into the spinal cord dorsal horn decreases NR1 protein levels by ~60% but fully reversed the injury-induced pain in rodents (28). We here injected rats with adenoviral vectors expressing shRNA-NR1 or mismatch control directly into the DVC at the time of the stereotaxic surgery on day 1.

These injected rats fully recovered

by day 7 and were then subjected to vascular surgeries (supplemental Fig. S1b). On day 11, the same rats underwent the pancreatic-euglycemic clamp studies for evaluation of the effect of DVC adenovirus injections on glucose metabolism (supplemental Fig. S1b). We directly infused glycine or saline into the DVC of adenoviral injected rats (shRNA NR1 or mismatch control) on day 11 (Fig. 3a). We first confirmed that similar to normal rats, DVC glycine administration increased glucose infusion rate (Fig. 3b) and lowered glucose production (Fig. 3, c and d) during the pancreatic-euglycemic clamps in rats injected with DVC mismatch control. In contrast, in the presence of similar basal plasma insulin and glucose levels (supplemental Table S2), DVC glycine administration failed to increase glucose infusion rate (Fig. 3b) and lower glucose production (Fig. 3, c and d) in rats injected with DVC shRNA-NR1. DVC injections of adenovirus encoding the shRNA-NR1 or mismatch control alone did not affect glucose kinetics (Fig. 3, b–d), and glucose uptake was comparable between all groups (Fig. 3e).

Under non-clamped settings, DVC glycine administration for 90 min lowered plasma glucose levels in DVC mismatch control and this lowering effect was nullified in DVC

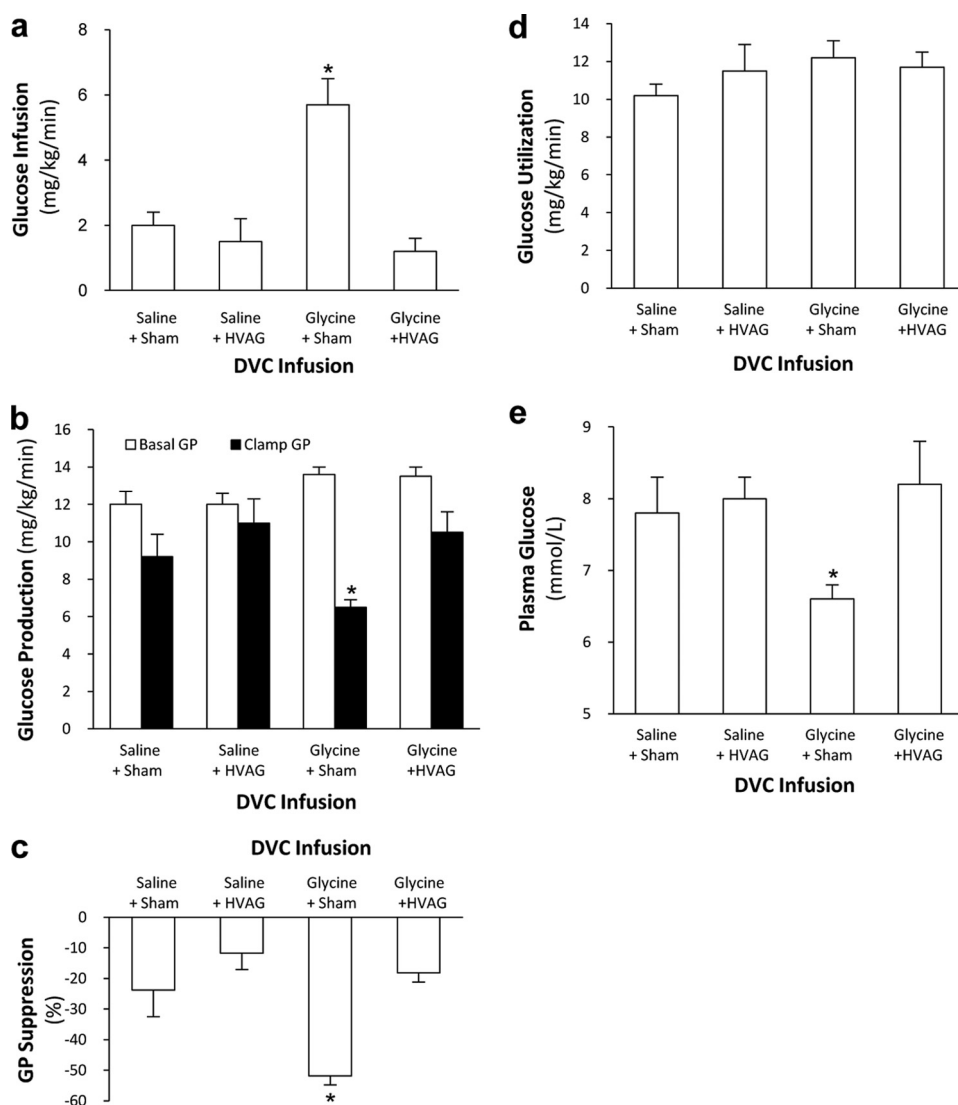


FIGURE 5. Hepatic vagotomy (HVAG) negates the suppressive effect of glycine on glucose production. During the pancreatic-euglycemic clamp procedure, DVC administration of glycine in sham-operated (SHAM) rats increased (a) glucose infusion rate and lowered (b) glucose production. These effects were fully negated by hepatic vagotomy (a and b). c, suppression of glucose production during the clamp period was expressed as percent decrease from basal glucose production. d, glucose utilization was comparable in all groups. Under non-clamped settings, DVC glycine infusion lowered (e) plasma glucose levels in sham-operated rats, an effect that was fully negated by hepatic vagotomy. DVC saline + Sham ($n = 5$), DVC saline + HVAG ($n = 4$), DVC glycine + Sham ($n = 6$), DVC glycine + HVAG ($n = 5$). *, $p < 0.05$.

shRNA-NR1 rats (Fig. 3f). Brain wedges were harvested right after the clamp studies for NR1 protein determination. Direct DVC injection of the adenovirus encoding the shRNA of NR1 (comparing to the mismatch control) decreased NR1 protein levels in the DVC/NTS-containing homogenate (Fig. 3g and supplemental Fig. S1c). The protein levels of NR1 in the hypothalamus were unaffected by DVC injection of adenovirus-shRNA-NR1 (Fig. 3g and supplemental Fig. S1c). In addition, immunofluorescence images show that NR1 expression within the DVC was greatly reduced in rats injected with DVC shRNA-NR1 versus control (supplemental Fig. S2). Together with the pharmacological loss-of-function studies (via DVC delivery of 7 chlorokynurenic acid), these molecular loss-of-function studies on DVC NR1 suggest that the activation of the NR1 subunit of the NMDA receptors is required for gly-

cine to lower glucose production and plasma glucose levels *in vivo*.

DVC NMDA Receptors Are Required for NR2 Agonist NMDA to Lower Glucose Production—To alternatively evaluate whether activation of DVC NMDA receptors lowers glucose production, we directly infused NR2 agonist NMDA into the DVC and evaluated glucose kinetics with the clamp technique (Fig. 4a). DVC NMDA markedly increased glucose infusion rate required to maintain euglycemia during the pancreatic basal insulin clamps (Fig. 4b). The increased in glucose infusion rate induced by DVC NMDA was accounted for by an inhibition of glucose production (Fig. 4, c and d), rather than a change in glucose uptake (Fig. 4e). To assess whether the activation of NMDA receptors is required for the metabolic effect of DVC NMDA, NMDA receptors were inhibited via the administration of DVC NMDA receptor antagonist D-APV. DVC D-APV at 300 μ M alone did not affect glucose kinetics (Fig. 4, b–e) but fully negated the ability of DVC NMDA to increase glucose infusion rate and lower glucose production during the clamps (Fig. 4, b–d). Thus, activation of DVC NMDA receptors via NR2 agonist NMDA lowers glucose production.

Hepatic Vagal Innervation Is Required for DVC NMDA Receptor Activation to Lower Glucose Production—Finally, to delineate the downstream neuronal circuit that mediates the ability of DVC NMDA receptor activation to lower glucose

production and plasma glucose levels, we repeated the DVC glycine and clamp experiments in rats underwent hepatic branch vagotomy as well as the sham-operated procedure. DVC glycine increased glucose infusion rate (Fig. 5a) and decreased glucose production (Fig. 5, b and c) in sham-operated rats. In contrast, hepatic vagotomy fully negated the ability of DVC glycine to increase glucose infusion rate and to lower glucose production (Fig. 5, a–c). Glucose uptake was comparable in all groups (Fig. 5d). Under non-clamped conditions, DVC glycine was sufficient to lower plasma glucose levels in sham-operated rats and this glucose-lowering effect was fully reversed by hepatic vagotomy (Fig. 5e). Thus, hepatic vagal innervation is required for DVC NMDA receptor activation to lower glucose production and plasma glucose levels.

DISCUSSION

The DVC is a critical region of the hindbrain to receive peripheral nutritional and hormonal signals to regulate food intake and maintain energy balance (30–33). We here demonstrate with pharmacological, molecular and surgical approaches that direct activation of DVC NMDA receptors triggers the hepatic vagus to lower glucose production *in vivo*.

The current study expands the role of DVC in the control of homeostasis and suggests that DVC is a crucial region of the brain, which is sufficient for the regulation of glucose homeostasis. More importantly, selective activation of NMDA receptors in the DVC via co-agonist glycine or NMDA lowers glucose production via the binding of NR1 and NR2 subunits, respectively.

In addition to the established role of glycine binding on the NR1 subunit of NMDA receptor to potentiate NMDA transmission (19–21, 34), glycine could also facilitate glutamate release via the binding to its strychnine-sensitive glycine receptors on presynaptic components that can indirectly activate NMDA receptors (35). We co-infused DVC glycine (10 μ M) with strychnine (0.6 μ M) during the clamp experiments ($n = 4$) in order to inhibit strychnine-sensitive glycine receptors in the presence of glycine administration. DVC glycine with strychnine was equally potent to lower glucose production (4.8 ± 0.5 mg/kg/min) as compared with when DVC glycine was administered alone (Fig. 1), indicating at this preliminary stage that strychnine-sensitive glycine receptor is not required for DVC glycine to lower glucose production. Together with the potent metabolic effect of DVC NMDA on glucose production via the binding of NR2 subunit of NMDA receptors (Fig. 4), these data strengthened the claim that direct activation of NMDA receptors by the co-agonist glycine or NMDA in the DVC regulates glucose homeostasis.

Molecular and pharmacological inhibition of DVC NR1 subunit of the NMDA receptors in our short-term settings *per se* did not alter glucose production. One possible explanation for a lack of ability of DVC NR1 inhibition alone to increase glucose production and induce hyperglycemia is that the glycine binding site is unsaturated at basal conditions (34). This is supported by the fact that glycine concentration at the DVC glutamatergic synapses is lower than CSF glycine concentration because of the presence of local high-capacity glycine transporters (34). Thus, DVC NMDA receptors in resting basal conditions are not sufficiently activated to lower glucose production. In contrast, when exogenous glycine is applied to the DVC (Figs. 1–3), NMDA receptor is potentially activated to lower glucose production via the NR1 binding subunit. In parallel, since our DVC NR1 subunit inhibition occurs in a short-term setting, studies also await to determine whether chronic knock-down of DVC NMDA receptors disrupts glucose homeostasis, especially in light of Claude Bernard's observation in 1855 reporting that lesioning the floor of the 4th ventricle (containing DVC) results in hyperglycemia (36).

Our present findings indicate that DVC NMDA receptors are required for NR2 agonist NMDA to lower glucose production. However, because the use of NMDA and D-APV (Fig. 4) do not distinguish which of the four different NR2 subunits

(NR2A, NR2B, NR2C, and NR2D) (37, 38) are involved, future studies are required to selectively assess the individual or combinational involvement of the various NR2 subunit(s) in the DVC control of glucose metabolism. In addition to the NR1 and NR2 subunit-containing NMDA receptors, there are also the non-classical NMDA receptors that contain NR1 and NR3 subunits, which are selectively activated by glycine (39). However, NR1/NR3-containing NMDA receptors are resistant to the inhibitory effect of NMDA receptor blocker MK-801 (39). In light of the fact that DVC MK-801 fully negated the ability of glycine to lower glucose production (Fig. 1), our current findings argue against a potential role of NR3 subunit in the control of glucose production by DVC glycine. Future studies designed to selectively inhibit NR3 subunit in the DVC are required to fully address this issue.

The mechanisms underlying the CNS control of glucose homeostasis remain undefined. Although the hypothalamus regulates glucose homeostasis (2, 14), it is currently unknown whether the hypothalamus relays neuronal signal(s) to other regions of the brain to regulate glucose homeostasis. The hypothalamic-medullary modulatory pathways, in particular the neuronal cross-talk among brain regions such as the arcuate nucleus, paraventricular nucleus, DVC, have been implicated in various homeostatic control such as food intake regulation (40) and osmoregulation (41). Together with current findings, a potential neuronal circuit linking the hypothalamus and DVC in glucose regulation is warranted for future investigations. Furthermore, because our DVC delivery approach targets the NTS, it is equally important to begin identifying the specific subnucleus within the NTS (*i.e.* medial/dorsomedial subnucleus) that could potentially control glucose homeostasis.

In summary, selective activation of the NR1/NR2-containing NMDA receptors in the DVC lowers glucose production. In light of the fact that direct inhibition of DVC NMDA receptors negates the ability of lipid-sensing mechanisms in the gut to lower glucose production (17, 18), we propose that NMDA receptors in the DVC integrate nutrient-sensing related signals in the body and are sufficient to regulate glucose homeostasis.

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