



# Thioredoxin-1 Overexpression in the Ventromedial Nucleus of the Hypothalamus Preserves the Counterregulatory Response to Hypoglycemia During Type 1 Diabetes in Male Rats

Chunxue Zhou and Vanessa H. Routh

*Diabetes* 2018;67:120–130 | <https://doi.org/10.2337/db17-0930>

We previously showed that the glutathione precursor, *N*-acetylcysteine (NAC), prevented hypoglycemia-associated autonomic failure (HAAF) and impaired activation of ventromedial hypothalamus (VMH) glucose-inhibited (GI) neurons by low glucose after recurrent hypoglycemia (RH) in nondiabetic rats. However, NAC does not normalize glucose sensing by VMH GI neurons when RH occurs during diabetes. We hypothesized that recruiting the thioredoxin (Trx) antioxidant defense system would prevent HAAF and normalize glucose sensing after RH in diabetes. To test this hypothesis, we overexpressed Trx-1 (cytosolic form of Trx) in the VMH of rats with streptozotocin (STZ)-induced type 1 diabetes. The counterregulatory response (CRR) to hypoglycemia in vivo and the activation of VMH GI neurons in low glucose using membrane potential sensitive dye in vitro was measured before and after RH. VMH Trx-1 overexpression normalized both the CRR and glucose sensing by VMH GI neurons in STZ rats. VMH Trx-1 overexpression also lowered the insulin requirement to prevent severe hyperglycemia in STZ rats. However, like NAC, VMH Trx-1 overexpression did not prevent HAAF or normalize activation of VMH GI neurons by low glucose in STZ rats after RH. We conclude that preventing HAAF in type 1 diabetes may require the recruitment of both antioxidant systems.

Hypoglycemia is a severe side effect of intensive insulin therapy (1). Recurrent hypoglycemia (RH) impairs the brain's ability to sense subsequent hypoglycemia and generate the autonomic and neuroendocrine counterregulatory responses (CRRs) that restore euglycemia (1). This condition, known as hypoglycemia-associated autonomic failure

(HAAF), is the major limiting factor in treating diabetes with insulin.

The ventromedial hypothalamus (VMH) plays a key role in hypoglycemia detection and CRR initiation (2,3). VMH glucose-sensing neurons are likely candidates for detecting hypoglycemia. The glucose sensitivity of VMH glucose-inhibited (GI) neurons, in particular, parallels CRR magnitude (4); for example, RH blunts activation of VMH GI neurons by decreased glucose (5–7). Manipulations that increase activation of VMH GI neurons in vitro also enhance the CRR (6–9). Nitric oxide (NO) production by neuronal NO synthase and activation of the NO receptor soluble guanylyl cyclase (sGC) are critical for activation of VMH GI neurons in low glucose and for the CRR (7,10). Elevated reactive oxygen species (ROS) level causes sGC s-nitrosation, a posttranslational modification that desensitizes sGC to NO (11). Hypoglycemia increases VMH ROS (6). Thus, ROS production during hypoglycemia may impair NO signaling in VMH GI neurons and blunt their activation by decreased glucose. In support of this hypothesis, we found that enhancing the glutathione antioxidant defense system with *N*-acetylcysteine (NAC) in nondiabetic rats reduced VMH s-nitrosation, restored normal glucose sensing by VMH GI neurons after RH, and completely prevented HAAF (6).

Although these results in nondiabetic rats are promising, for clinical relevance, the effect of NAC must persist in the presence of diabetic hyperglycemia, which exacerbates brain oxidative stress by triggering mitochondrial ROS production (12). VMH GI neurons from rats with diabetes induced by streptozotocin (STZ) show a severely blunted activation in low glucose. High glucose incubation also prevents subsequent

Department of Pharmacology, Physiology and Neurosciences, New Jersey Medical School, Rutgers University, Newark, NJ

Corresponding author: Vanessa H. Routh, [routhvh@njms.rutgers.edu](mailto:routhvh@njms.rutgers.edu).

Received 9 August 2017 and accepted 23 October 2017.

This article contains Supplementary Data online at <http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db17-0930/-/DC1>.

© 2017 by the American Diabetes Association. Readers may use this article as long as the work is properly cited, the use is educational and not for profit, and the work is not altered. More information is available at <http://www.diabetesjournals.org/content/license>.

activation of VMH GI neurons by low glucose *in vitro* (13). These data are consistent with the impaired CRR in diabetes (14). Therefore, diabetic hyperglycemia *per se* may also impair VMH GI glucose sensing and the CRR by increasing oxidative stress. However, NAC treatment did not restore activation of VMH GI neurons in low glucose after RH in STZ rats (Supplementary Figs. 1 and 2). This suggests that the combined oxidative stress resulting from RH during diabetic hyperglycemia exceeded the capacity of the glutathione system or that recruitment of a different antioxidant defense system is necessary.

The thioredoxin (Trx) system, composed of NADPH, Trx, and Trx reductase (TrxR), facilitates enzymatic protein reduction and denitrosation (15). Trx is the primary regulator of cellular nitrosative status (16). Hyperglycemia reduces Trx activity in the aorta of diabetic rats (17) and blunts exercise-induced upregulation of Trx activity in rat brain (18). Conversely, Trx overexpression in pancreatic  $\beta$ -cells slowed the progression of STZ-induced type 1 diabetes (19) and prevented the formation of type 2 diabetes in mice (20). Moreover, overexpression of Trx-1, the cytosolic isoform of Trx, attenuated oxidative stress-induced osteopenia in STZ mice (21). Therefore, this study tests the hypothesis that VMH Trx-1 overexpression preserves glucose sensing and the CRR in rats with STZ-induced type 1 diabetes.

## RESEARCH DESIGN AND METHODS

All procedures were approved by the Rutgers University Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (3 or 5 weeks old) were purchased from Charles River Laboratories. Animals were housed and maintained on a 12-h light-dark cycle at 22–23°C with *ad libitum* access to food and water.

### VMH Trx-1 Overexpression

Purified and packaged adeno-associated virus (AAV)/DJ9-hybrid cytomegalovirus/chicken  $\beta$ -actin promoter (CAG)-green fluorescent protein (GFP)-2A-Trx-1 and AAV/DJ9-CAG-GFP were purchased from Vector BioLabs. Three-week-old rats were stereotaxically injected bilaterally in the VMH over 10 min with 1  $\mu$ L PBS containing  $2 \times 10^9$  genomic particles of AAV/DJ9 per side expressing Trx-1 + EGFP or EGFP. The coordinates were 2.3 mm caudal to the bregma, 0.58 mm lateral to the midline, and 8.80 mm down from the dural surface.

### Type 1 Diabetes

Two weeks after the AAV injections, rats received one intraperitoneal injection of STZ (65 mg/kg in 0.1 mol/L citrate buffer; pH 4.5). Controls received vehicle. Rats with glycemia below 150 mg/dL 24 h after the STZ injection received a second dose of STZ. Glycemia exceeding 300 mg/dL by the third day after the STZ injection was considered type 1 diabetes. This was defined as day 1 of diabetes. For the following 2 weeks, diabetic rats received one daily injection of long-acting insulin, ProZinc insulin (PZI, Boehringer Ingelheim), to prevent illness caused by severe hyperglycemia. The glycemic response to PZI was

monitored for 3 h postinjection. Daily doses were adjusted to each individual's response. Rats that experienced inadvertent hypoglycemia were excluded from the study. Non-diabetic rats received saline.

### RH

Two hours after PZI or saline treatment, rats were fasted for one hour and intraperitoneally injected with 6, 5, and 4 units/kg regular human insulin (Humulin R, Lilly) respectively, for the final 3 days of the study. Less insulin was administered on subsequent days to decrease the chance of severe hypoglycemia. Controls received saline. Tail blood was taken to measure glycemia every 30 min to verify 2 h of hypoglycemia ( $\sim$ 30 to 60 mg/dL).

### CRR

One week after diabetes induction, rats were anesthetized with ketamine/xylazine (80/10 mg/kg *i.p.*) and surgically implanted with vascular catheters in the right jugular vein. For RH, the animals were allowed 3 or 4 days to recover from surgery before the first episode of hypoglycemia. The CRR was examined 5–7 days after cannulation of the jugular vein or the day after RH. Thus, the range for the study was from day 13 to 16 of diabetes or the corresponding days for nondiabetic controls. On the day of the CRR evaluation, rats were fasted for 1 h starting 2 h after the PZI or saline treatment, and then Humulin R (1.3–1.5 units/kg) was administered via jugular catheter. Blood glucose was monitored every 15 min from 0 to 120 min after the insulin infusion. Blood samples withdrawn from the jugular catheter were collected at 0, 15, 30, 60, and 90 min in chilled tubes containing EGTA (1.6 mg/mL, Sigma-Aldrich) and aprotinin (250 kallikrein inhibitor units, Sigma-Aldrich) for measurement of plasma glucagon (6). For catecholamines, blood was collected in chilled tubes containing reduced glutathione (1.2 mg/mL, Sigma-Aldrich) and EDTA (1.8 mg/mL, Sigma-Aldrich) at 0, 60, and 120 min (6). Plasma samples were shipped to the Yale Diabetes Center for analysis. Erythrocytes were resuspended in isovolumetric sterile artificial plasma and infused back via the jugular catheter to prevent volume depletion and anemia (22). The animals were sacrificed at the end of study, and the brain was harvested. Slices containing the VMH were made to verify the position of the stereotaxic injections.

### Auranofin Study

Five-week-old rats were anesthetized with ketamine/xylazine (80/10 mg/kg, *i.p.*) and then underwent catheter placement in the jugular vein and stereotaxic surgery to implant cannula bilaterally in the VMH according to the following coordinates: 2.4 mm caudal to the bregma, 0.60 mm lateral to the midline, and 9.0 mm down from the dural surface. The CRR was examined 5–7 days later. On the day of the experiment, 1  $\mu$ L of 1  $\mu$ mol/L auranofin in 0.1% DMSO in artificial cerebrospinal fluid or same amount of vehicle was infused into the VMH bilaterally via the brain cannula over 15 min. Humulin R (3 units/kg) was subcutaneously administered 1 h after the auranofin infusion. Blood glucose was

monitored every 15 min from 0 to 120 min after the insulin injection.

### Measurement of Membrane Potential Changes Using Fluorescence Imaging Plate Reader Membrane Potential Dye

VMH neurons from tissue punches that excluded the arcuate nucleus were dissociated and placed in 2.5 mmol/L glucose Neurobasal-A Media (Life Technologies) with B27, as previously described (10), and used within 6 h. Neurons were visualized on an Olympus BX81 WI microscope with a 10 $\times$  objective equipped with a red filter for visualization of fluorescence imaging plate reader membrane potential dye (FLIPR-MPD; excitation, 548 nm; emission, 610–675 nm). Perfusion of VMH neurons in extracellular solution (in mmol/L: 25 HEPES, 121 NaCl, 4.7 KCl, 1.2 MgSO<sub>4</sub>, 5 NaHCO<sub>3</sub>, 2 CaCl<sub>2</sub>, 0.23 KH<sub>2</sub>PO<sub>4</sub>, 0.97 K<sub>2</sub>HPO<sub>4</sub>; pH 7.4;  $\sim$ 300  $\mu$ Osm) containing 0.5% MPD (FLIPR MPD Blue, Molecular Devices) at 34 $^{\circ}$ C began 30 min before and continued throughout the duration of all experiments. Glucose concentrations are indicated in the figures. Fluorescence images were recorded to evaluate changes in membrane potential as glucose level changes. Increases in fluorescence indicate depolarization. Cells that have a reversible increase in fluorescence  $\geq$ 12% compared with that observed in 2.5 mmol/L glucose are considered to be GI neurons. This threshold was defined as exceeding twice the SD of fluorescence variation observed in response to a solution change in which the glucose concentration was held constant (i.e., noise). The amplitude of fluorescence intensity change was not correlated with the change in the glucose concentration. However, the number of neurons that depolarized as glucose decreased (GI neurons) was correlated with the glucose concentration, with an IC<sub>50</sub> of  $\sim$ 0.7 mmol/L, as we have shown previously (23). Hence, data are expressed as the percentage of GI neurons detected per dish.

### Real-time Quantitative PCR

Total RNA was extracted from  $\sim$ 2–4 mg VMH punches using TRIzol reagent (Invitrogen) and then transcribed to cDNA using SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. cDNA quantization was performed using the TaqMan assay by the LightCycler 480 system (Roche) for 45 PCR cycles. Results were calculated from the standard curves relative to cyclophilin A mRNA levels in the same samples. Tumor necrosis factor- $\alpha$  mRNA was measured and found to be below detection, indicating a minimal inflammation response caused by the AAV injections.

### Trx Activity

VMH punches were sonicated in Tris-EDTA buffer (pH 7.5). Trx activity was measured using the Trx activity fluorescent assay kit (Cayman Chemical) following the manufacturer's manual and normalized to the amount of protein.

### Data Analysis

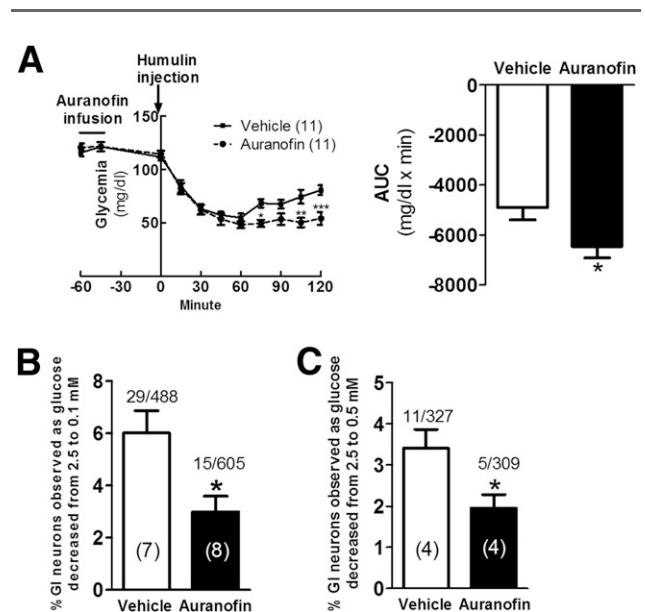
All data are presented as mean  $\pm$  SEM. Statistical analysis was performed using GraphPad Prism 5.0 software by

two-way ANOVA, followed by the Bonferroni post hoc test, one-way ANOVA, followed by the Tukey post hoc test, or the unpaired Student *t* test, as indicated in the figures. *P* < 0.05 indicates statistical significance.

## RESULTS

### TrxR Inhibition Impairs the CRR and Activation of VMH GI Neurons in Low Glucose

Bilateral VMH infusion of the TrxR inhibitor auranofin 1 h before insulin-induced hypoglycemia administration compared with vehicle-treated controls (Fig. 1A) showed the absolute value for the area under the curve (AUC) for glucose after the insulin injection was increased 22% (*P* < 0.05) by VMH auranofin ( $-6,264 \pm 523$  mg/dL  $\times$  min, *n* = 11), compared with the vehicle-treated controls ( $-4,907 \pm 492$  mg/dL  $\times$  min, *n* = 11). This indicates a slower recovery to euglycemia after insulin-induced hypoglycemia. Incubation of isolated VMH neurons in 1  $\mu$ mol/L auranofin for 1 h decreased the percentage of detectable VMH GI neurons observed in response to both 0.1 and 0.5 mmol/L glucose by 50% (*P* < 0.05) and 43% (*P* < 0.05), respectively (Fig. 1B and C). Together, these data support the hypothesis that the Trx antioxidant defense



**Figure 1**—TrxR inhibition impairs the glycemia recovery after insulin-induced hypoglycemia and the activation of VMH GI neurons by low glucose. **A:** The time course of glycemia from 1 h before to 2 h after insulin injection (left panel). Repeated two-way ANOVA showed a main effect of auranofin ( $F_{1,200} = 7.390$ , *P* < 0.05). The AUC of the glycemia was calculated relative to the basal glycemia (at the time of insulin injection) (right panel). VMH GI neurons that depolarized in response to a 2.5 to 0.1 mmol/L (**B**) or 0.5 mmol/L (**C**) glucose decrease were identified using FLIPR MPD imaging. Data are expressed as the percentage of GI neurons per dish. \**P* < 0.05, \*\**P* < 0.01, and \*\*\**P* < 0.001 according to the Student *t* test or Tukey post hoc tests. The (*n*) refers to the number rats in **A** and the number of dishes of neurons prepared from four to six rats of each group in **B** and **C**. The digits on each bar in **B** and **C** reflect the number of GI neurons/number of total neurons.

plays a role in both the CRR and glucose sensing by VMH GI neurons.

### Trx-1 Overexpression Restores the CRR in Type 1 Diabetes

Trx activity was significantly lower in STZ-induced diabetic rats compared with nondiabetic controls (Fig. 2A). Therefore, we determined whether VMH Trx-1 overexpression preserved hypoglycemia counterregulation and glucose sensing by VMH GI neurons in these animals. To do this, we injected AAV/DJ9-CAG-EGFP-2A-Trx-1 (Trx-1 and EGFP expression) or AAV/DJ9-CAG-EGFP (EGFP expression alone; control) into the VMH bilaterally. Figure 2B shows that EGFP expression was largely constrained to the VMH. Trx-1 AAV injection increased VMH Trx-1 mRNA expression by fivefold and doubled Trx activity 2 weeks after the injection (Fig. 2C and D). Trx-1 overexpression was maintained in control and STZ rats for the duration of the study (Fig. 2E).

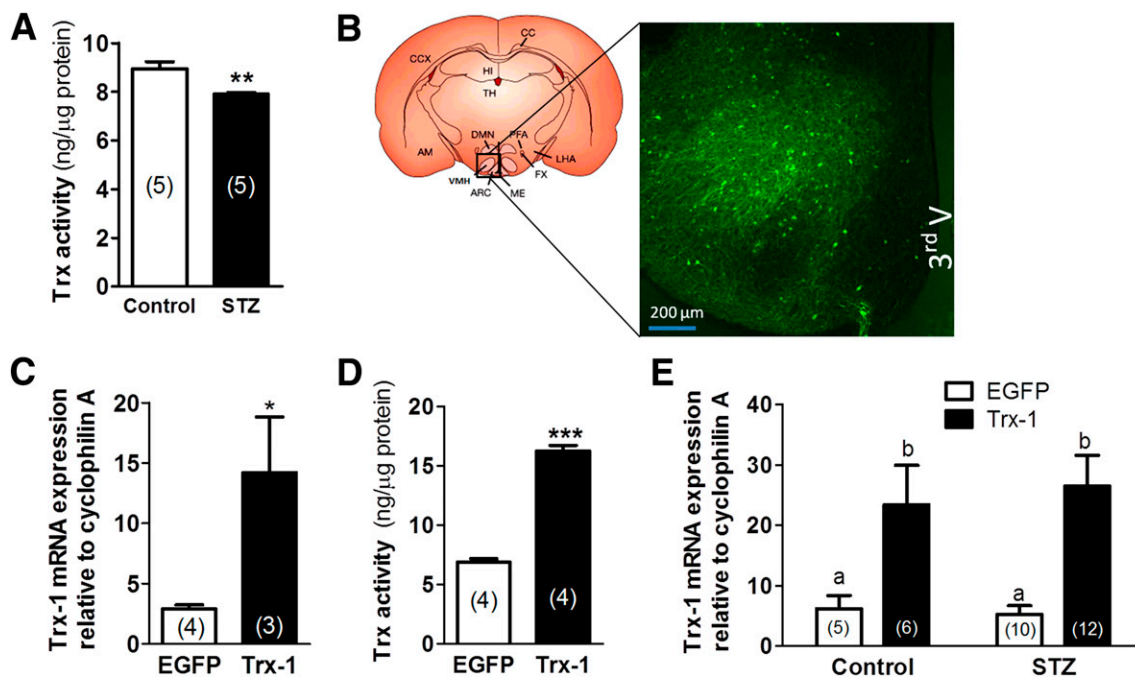
Interestingly, the proportion of rats with bilateral VMH Trx-1 AAV injections that failed to develop diabetes in response to a single STZ injection (24 of 90) was significantly greater than that of rats that received the control EGFP AAV injection (2 of 86;  $P < 0.0001$  two-sample  $z$  test (Table 1). Moreover, glycemia was lower on the day after injection in the Trx-1-overexpressing rats that developed diabetes after the first STZ injection compared

**Table 1—Rate of failure to induce diabetes after one and two STZ injections**

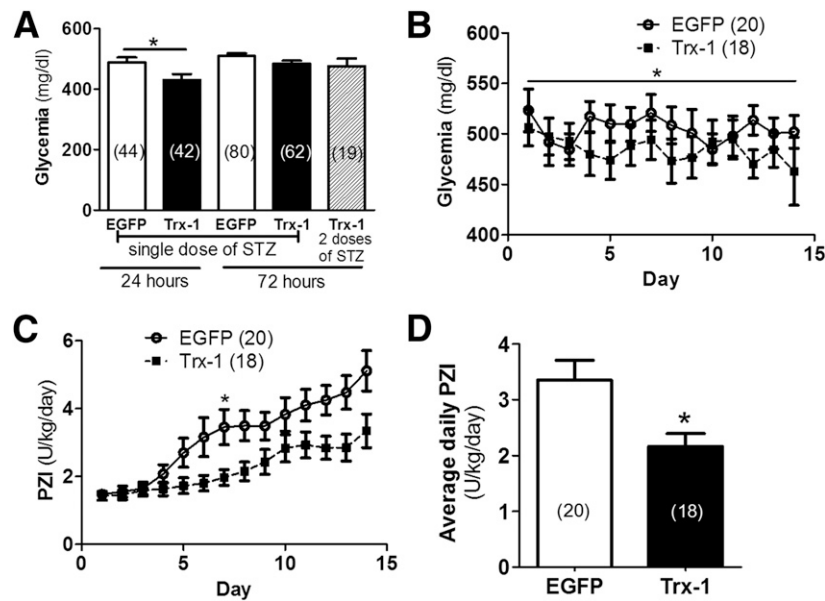
	Failure rate	
	After the first dose	After the second dose
EGFP	2.3 (2/86)	0 (0/86)
Trx-1	26.7 (24/90)***	4.4 (4/90)*

Glycemia was measured 24 h after STZ injection and a second dose of STZ was administered if the glycemia was  $<150$  mg/dL. Diabetes was defined as glycemia  $>300$  mg/dL. Data are expressed as rate of failure (%) to induce diabetes (number of nondiabetic rats/total number of rats injected with STZ). \*\*\* $P < 0.0001$  and \* $P < 0.05$ , two-sample  $z$  test.

with the analogous controls (Fig. 3A). Although nearly all of the rats in both groups developed diabetes after a second STZ injection, the proportion of rats that did not develop diabetes was still larger for those with VMH Trx-1 overexpression ( $P < 0.05$ ) (Table 1). Glycemia in both diabetic groups reached the same level 3 days after the last STZ injection (Fig. 3A). Furthermore, Trx-1 AAV-injected rats had lower blood glucose levels during 2 weeks of diabetic hyperglycemia than did rats expressing the control AAV (Fig. 3B). This was associated with a decreased amount of PZI required to lower morning glycemia from  $>300$  to 150–200 mg/dL (Fig. 3C and D). Although STZ rats weighed



**Figure 2—Verification of VMH Trx-1 overexpression.** A: VMH Trx activity was reduced in STZ rats. B: A representative image illustrates the site of AAV injection in a coronal section of a brain slice. The EGFP signal is mainly confined to the VMH 2 weeks after the AAV injection (adapted with permission from Schwartz MW, Woods SC, Porte D Jr., Seeley RJ, Baskin DG. Central nervous system control of food intake. *Nature* 2000;404:661–671). C: VMH Trx-1 mRNA expression 2 weeks after the AAV injection. D: VMH Trx activity 2 weeks after the AAV injection. E: VMH Trx-1 mRNA expression at the end of the study in STZ and control rats (2 weeks after the induction of diabetes). Two-way ANOVA showed a main effect of the Trx-1 AAV injections ( $F_{1,29} = 15.71$ ,  $P < 0.001$ ). \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  according to the Student  $t$  test. Different letters indicate statistical significance among the four groups determined by two-way ANOVA, followed by Tukey post hoc tests. The number of animals is given within the data bars ( $n$ ). AM, amygdala; ARC, arcuate nucleus; CC, corpus callosum; CCX, cerebral cortex; DMN, dorsomedial nucleus; FX, fornix; HI, hippocampus; LHA, lateral hypothalamic area; ME, median eminence; PFA, perifornical area; TH, thalamus; 3<sup>rd</sup>V, third ventricle.



**Figure 3**—VMH Trx-1 overexpression improves glycemia management in STZ rats. *A*: Glycemia of rats that developed diabetes after one or two doses of STZ. Glycemia readings that indicated values above detectable limits were quantified as 600 mg/dL, because this value was the upper limit of detection for the glucose meter. *B*: Morning glycemia over 2 weeks in STZ rats. The overall glycemia was lower in diabetic rats with VMH Trx-1 overexpression vs. EGFP controls (repeated measures two-way ANOVA,  $F_{1,465} = 7.545$ ,  $P < 0.01$ ). *C*: The amount of PZI required to reduce basal hyperglycemia ( $>300$  mg/dL) to 150–200 mg/dL was decreased in diabetic rats with Trx-1 overexpression (repeated measures two-way ANOVA,  $F_{1,453} = 47.94$ ,  $P < 0.0001$ ). *D*: The average amount of PZI required for diabetic rats.  $*P < 0.05$  by the Student *t* test or repeated-measures two-way ANOVA, followed by Tukey post hoc tests. The number of animals is given within the data bars (*n*).

less than the nondiabetic rats, VMH Trx-1 overexpression had no effect on body weight (Supplementary Fig. 3).

In response to insulin-induced hypoglycemia, STZ rats with the control EGFP AAV injection did not recover to euglycemia during the 2 h monitoring period, and the absolute value of their glucose AUC was significantly greater than nondiabetic rats (Fig. 4A). In contrast, glucose levels and the glucose AUC in diabetic rats with VMH Trx-1 overexpression were not significantly different from the nondiabetic animals. A similar effect was observed for the glucagon response (Fig. 4B). Interestingly, epinephrine levels at 120 min were higher in both groups of diabetic rats compared with the nondiabetic rats (Fig. 4C). Two-way ANOVA indicated that the absolute value of the AUC for epinephrine was greater in diabetic than in nondiabetic rats; however, there was no interaction between Trx-1 overexpression and diabetes, and post hoc analysis showed no differences between individual groups. The norepinephrine level at each time point was comparable among all the groups, whereas the AUC for norepinephrine was similar to that of epinephrine (Fig. 4D).

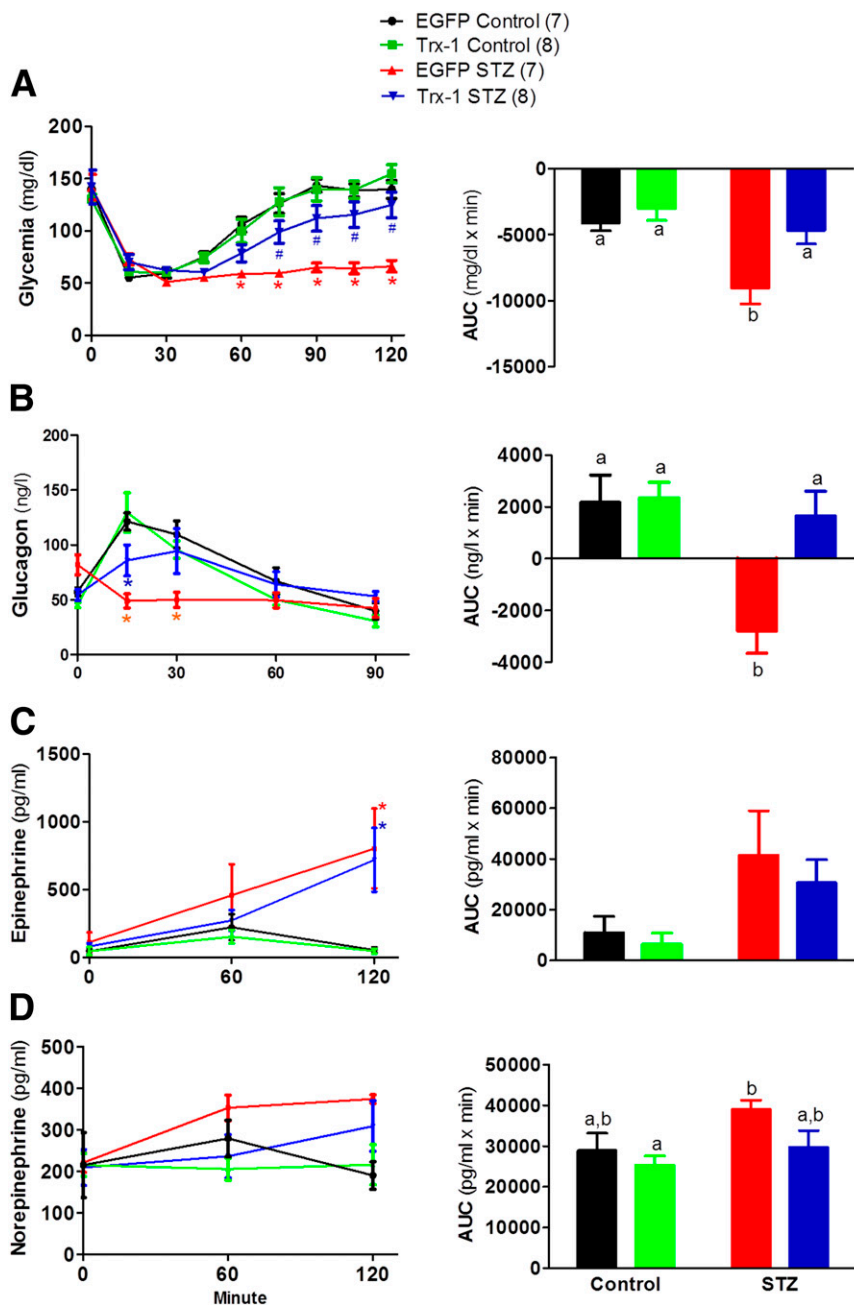
#### Trx-1 Overexpression Prevents Impaired Activation of VMH GI Neurons by Low Glucose in Type 1 Diabetes

Neurons that were transfected with the Trx-1 EGFP or control EGFP AAV were analyzed separately from neurons in the same animals that were not transfected (e.g., did not show EGFP fluorescence). Compared with VMH neurons isolated from nondiabetic controls, the percentage of control EGFP+ VMH GI neurons that were detected in response to

0.1 and 0.5 mmol/L glucose was reduced by 84% and 85%, respectively, in rats with STZ-induced diabetes. Trx-1 overexpression completely normalized activation of VMH GI neurons in both 0.1 and 0.5 mmol/L (Fig. 5A and B, left panels). Activation by low glucose of nontransfected GI neurons from control EGFP AAV-STZ rats but not Trx-1 AAV-STZ rats was also significantly lower than in nondiabetic rats (Fig. 5A and B, right panels).

#### VMH Trx-1 Overexpression Did Not Preserve the CRR in Type 1 Diabetes After RH

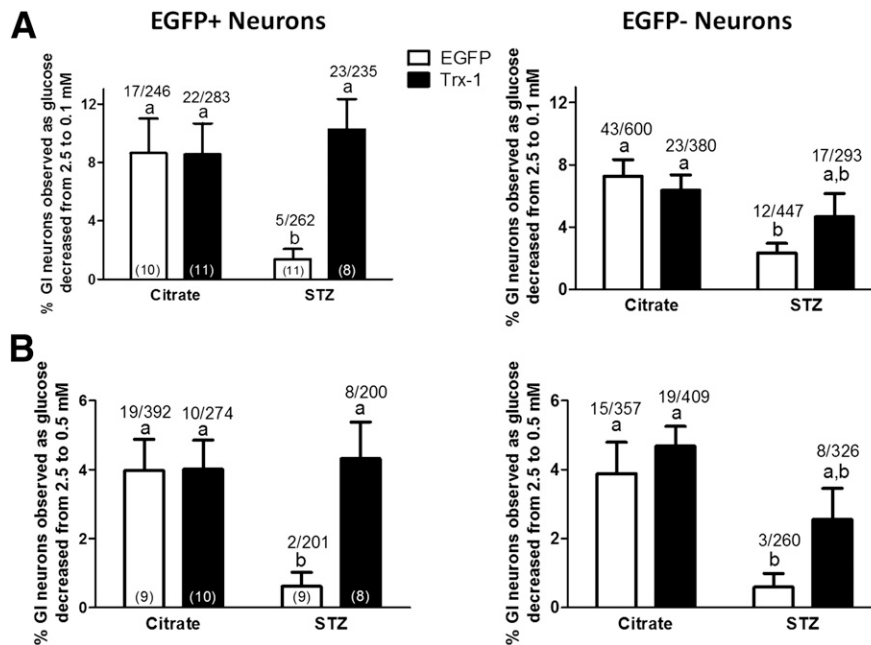
We next examined the CRR in STZ rats that experienced one daily 2-h episode of hypoglycemia for the final 3 days of the study. After RH, VMH Trx-1 overexpression did not restore the glycemic recovery after insulin-induced hypoglycemia in diabetic rats. However, the AUC for glycemia in the Trx-1-overexpressing diabetic rats showed a slight improvement, but this was not significantly different from the nondiabetic controls or the EGFP-expressing diabetic controls ( $P = 0.052$  [Trx-1 vs. EGFP] two-way ANOVA). In contrast to our previous results with NAC, VMH Trx-1 overexpression did not enhance glycemic recovery in nondiabetic animals after RH (Fig. 6A). VMH Trx-1 overexpression also failed to increase glucagon secretion in nondiabetic or diabetic rats after RH (Fig. 6B). Interestingly, although epinephrine secretion in control EGFP-injected diabetic rats exposed to RH was similar to that of nondiabetic controls at all time points, VMH Trx-1 overexpression tended to increase epinephrine levels at 60 and 120 min. A similar



**Figure 4**—VMH Trx-1 overexpression restores the CRR to hypoglycemia in STZ rats. The left panels show the time course for glycemia (A), plasma glucagon (B), epinephrine (C), and norepinephrine (D) in response to the insulin injection (at time 0). The right panels show the corresponding AUC for each parameter relative to the initial value for that parameter. **A:** Repeated two-way ANOVA showed a significant effect for STZ and Trx-1 ( $F_{3,208} = 11.82, P < 0.0001$ ) (left panel). Two-way ANOVA showed main effects of STZ ( $F_{1,26} = 25.33, P < 0.001$ ) and Trx-1 ( $F_{1,26} = 5.639, P < 0.05$ ) as well as interaction ( $F_{1,26} = 5.612, P < 0.05$ ) (right panel). **B:** Repeated two-way ANOVA showed a main effect of time ( $F_{4,104} = 24.00, P < 0.0001$ ) and its interaction with the treatments ( $F_{12,104} = 5.509, P < 0.0001$ ) (left panel). Two-way ANOVA showed the main effects of STZ ( $F_{1,26} = 10.30, P < 0.001$ ) and Trx-1 ( $F_{1,26} = 6.803, P < 0.01$ ) and the interaction between them ( $F_{1,26} = 5.827, P < 0.05$ ) (right panel). **C:** Repeated two-way ANOVA showed a significant effect of STZ and Trx-1 ( $F_{3,52} = 3.178, P < 0.05$ ) (left panel). Two-way ANOVA showed a main effect of STZ ( $F_{1,26} = 7.202, P < 0.05$ ) (right panel). **D:** Repeated two-way ANOVA showed no significant differences (left panel). Two-way ANOVA showed a main effect of STZ ( $F_{1,26} = 4.590, P < 0.05$ ) (right panel). \* $P < 0.05$  between any group vs. EGFP control and # $P < 0.05$  between Trx-1 STZ vs. EGFP STZ, determined by Tukey post hoc tests after repeated two-way ANOVA. Different letters indicate statistical significance determined by two-way ANOVA, followed by Tukey post hoc tests. The number of rats in each group is given as (n) in the group legend.

trend was observed for the epinephrine AUC (Fig. 6C). Norepinephrine secretion after insulin-induced hypoglycemia was significantly higher in the Trx-1-overexpressing STZ

rats compared with the other groups at 120 min, and the norepinephrine AUC was greater in these rats compared with STZ rats expressing EGFP alone (Fig. 6D).



**Figure 5**—Trx-1 overexpression prevents impaired activation of VMH GI neurons by low glucose in STZ rats. Transfected (left panels) and nontransfected (right panels) VMH GI neurons that depolarized in response to a 2.5 to 0.1 mmol/L (A) or 2.5 to 0.5 mmol/L (B) glucose decrease were identified using FLIPR MPD imaging. A: Two-way ANOVA showed a main effect of Trx-1 ( $F_{1,36} = 5.290$ ,  $P < 0.05$ ) and interaction ( $F_{1,36} = 5.526$ ,  $P < 0.05$ ) (left panel). Two-way ANOVA showed the main effect of STZ ( $F_{1,36} = 10.28$ ,  $P < 0.01$ ) (right panel). B: Two-way ANOVA showed a main effect of Trx-1 ( $F_{1,32} = 5.042$ ,  $P < 0.05$ ) and interaction ( $F_{1,32} = 4.862$ ,  $P < 0.05$ ) (left panel). Two-way ANOVA showed a main effect of STZ ( $F_{1,32} = 14.49$ ,  $P < 0.001$ ) (right panel). Data are expressed as the percentage of GI neurons per dish. Different letters indicate statistical significance determined by two-way ANOVA, followed by Tukey post hoc tests. The number of dishes of neurons from 7–11 rats in each group is given as (n). The digits on each bar mean the number of GI neurons/number of total neurons.

### Trx-1 Overexpression Did Not Normalize Glucose Sensing by VMH GI Neurons in Type 1 Diabetes After RH

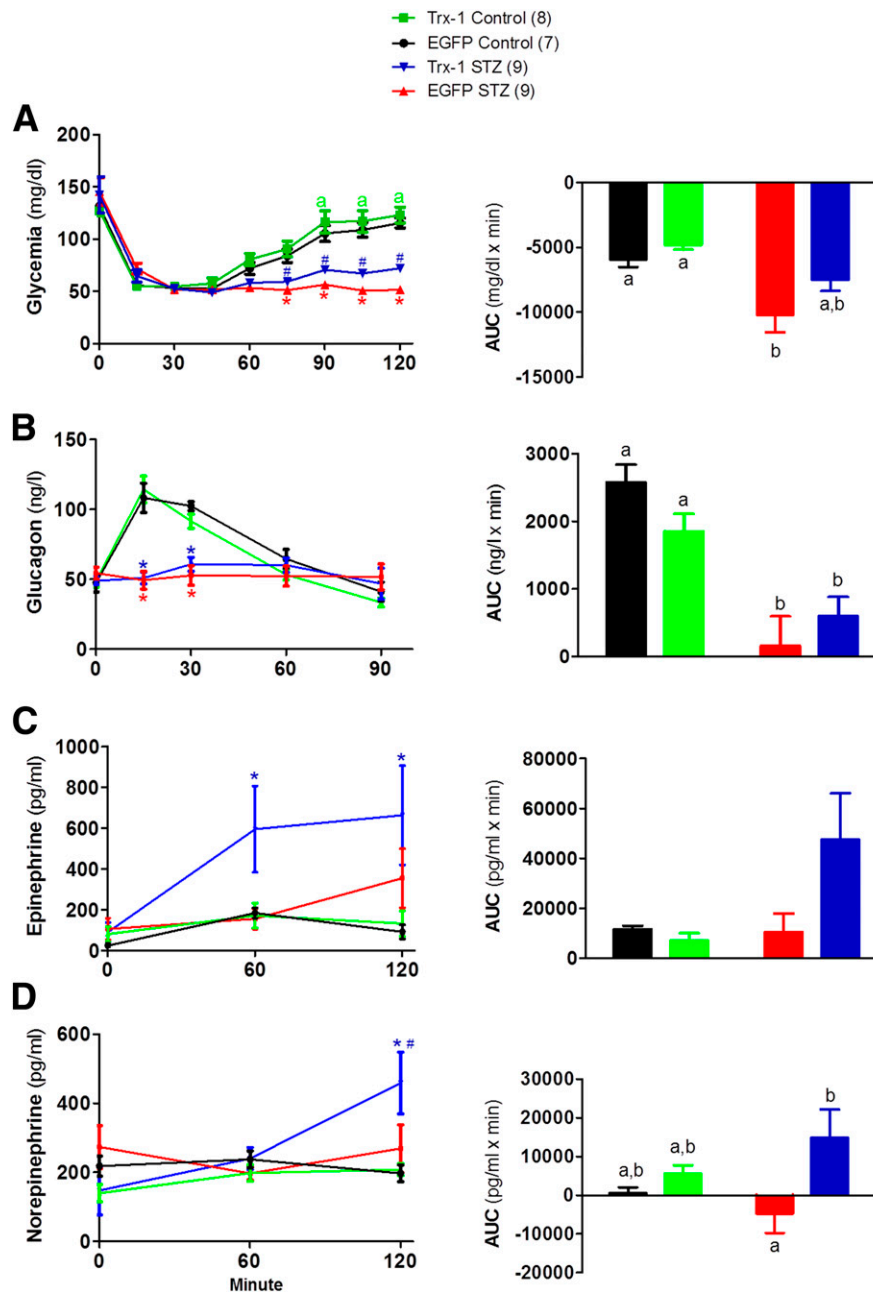
Trx-1 overexpression increased the number of VMH GI neurons from STZ rats after RH that were detected in response to a maximal glucose decrease (0.1 mmol/L) to that of RH-exposed nondiabetic controls that received the Trx-1 EGFP or control EGFP vectors. However, unlike our previous results with NAC, Trx-1 overexpression did not increase the number of detectable VMH GI neurons in the nondiabetic controls after RH. Moreover, VMH Trx-1 overexpression had no effect on the response of VMH GI neurons isolated from diabetic or nondiabetic rats to a more physiological glucose decrease (0.5 mmol/L) after RH (Fig. 7A and B, left panels). Detection of VMH GI neurons from the Trx-1-overexpressing diabetic and nondiabetic rats that were not transfected was not different from the EGFP controls in response to either glucose decrease (Fig. 7A and B, right panels). In addition, RH reduced the percentage of KCl-responsive neurons regardless of treatment group, suggesting that RH, but not hyperglycemia, slightly reduced neuronal viability ( $P < 0.05$  [RH vs. saline] two-way ANOVA) (Fig. 7C).

## DISCUSSION

Pretreatment with the glutathione precursor NAC decreased VMH ROS and sGC s-nitrosation after RH (6). NAC pretreatment also preserved normal activation of

VMH GI neurons in low glucose and the CRR after RH (6). These observations suggest that increasing glutathione may be a viable treatment for HAAF. However, NAC did not preserve activation of VMH GI neurons in low glucose when RH occurred in rats with type 1 diabetes (Supplementary Fig. 2). This may be because the NAC-driven increase in glutathione is not able to compensate for the combined oxidative stress resulting from RH and hyperglycemia. The Trx system compensates for oxidative stress in diabetes (18,24). Trx-1 and Trx-2 are two isoforms of Trx. Trx-1 is considered the major protein denitrosylase in mammalian cells (25). Trx-1 overexpression alleviated oxidative stress-induced osteopenia in STZ mice (21). We thus hypothesized that VMH Trx-1 overexpression would similarly preserve activation of VMH GI neurons by low glucose and the CRR in type 1 diabetes.

In support of our hypothesis, VMH Trx activity decreased in rats with type 1 diabetes. Moreover, TrxR inhibition by auranofin reduced glycemia recovery after insulin-induced hypoglycemia and the activation of VMH GI neurons in low glucose. These data suggest that the Trx antioxidant system is necessary for glucose sensing by VMH GI neurons and the full CRR. Consistent with this concept, VMH Trx-1 overexpression completely restored glycemic recovery and glucagon release after insulin-induced hypoglycemia in STZ rats to that observed in nondiabetic rats. VMH Trx-1 overexpression also completely normalized the

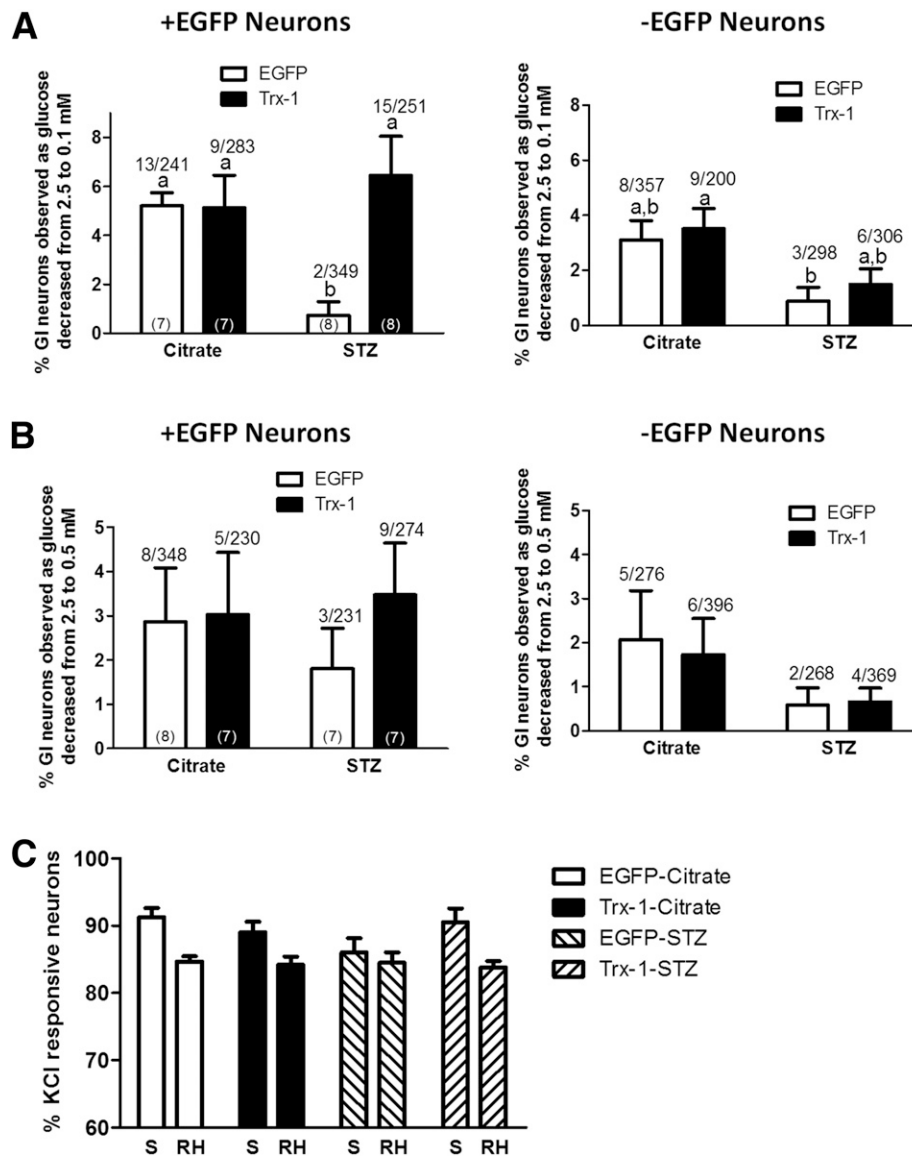


**Figure 6**—VMH Trx-1 overexpression fails to restore the CRR to hypoglycemia in STZ rats after RH. Left panels show the time course for glycemia (A), plasma glucagon (B), epinephrine (C), and norepinephrine (D) in response to the insulin injection (at time 0). Right panels show the corresponding AUC for each parameter relative to the initial value for that parameter. A: Significant main effects of STZ and Trx-1 ( $F_{3,261} = 38.45$ ,  $P < 0.0001$ ) were determined by repeated two-way ANOVA (left panel). Significant main effects of STZ ( $F_{1,28} = 13.54$ ,  $P = 0.001$ ) and Trx-1 ( $F_{1,28} = 4.116$ ,  $P = 0.052$ ) were determined by two-way ANOVA (right panel). B: A significant main effect of STZ ( $F_{1,28} = 32.38$ ,  $P < 0.0001$ ) was determined by two-way ANOVA (right panel). C: Significant main effects of time ( $F_{2,58} = 9.435$ ,  $P < 0.001$ ) and the interaction between time and treatments ( $F_{6,58} = 3.010$ ,  $P < 0.05$ ) were determined by repeated two-way ANOVA (left panel). The AUC analysis did not show any effect of and interaction between STZ and Trx-1 (right panel). D: Significant main effects of time ( $F_{2,87} = 3.264$ ,  $P < 0.05$ ) and the interaction between time and treatments ( $F_{6,87} = 2.814$ ,  $P < 0.05$ ) were determined by repeated two-way ANOVA (left panel). A significant main effect of STZ ( $F_{1,29} = 5.938$ ,  $P < 0.05$ ) was determined by two-way ANOVA (right panel). \* $P < 0.05$  between any group vs. EGFP control and # $P < 0.05$  between Trx-1 STZ vs. EGFP STZ determined by Tukey post hoc tests after repeated two-way ANOVA. Different letters indicate statistical significance determined by two-way ANOVA, followed by Tukey post hoc tests. The number of rats in each group is given as (*n*) in the group legend.

activation of VMH GI neurons in low glucose. Although epinephrine and norepinephrine secretion was elevated in diabetic compared with nondiabetic rats, levels were the

same in diabetic rats injected with VMH Trx-1 or EGFP control AAV. Therefore, the restoration of glycemia recovery is likely due to the normalized glucagon response.





**Figure 7**—VMH Trx-1 overexpression did not enhance activation of VMH GI neurons in low glucose after RH. Transfected (EGFP-expressing) (left panels) and untransfected (non-EGFP-expressing) (right panels) VMH GI neurons from rats injected with Trx-1 overexpression or control AAV that depolarized in response to a 2.5 to 0.1 (A) or 0.5 mmol/L (B) glucose decrease were identified using FLIPR-MPD imaging. Data are expressed as the percentage of GI neurons per dish. A: Two-way ANOVA showed a main effect of Trx-1 ( $F_{1,26} = 6.564$ ,  $P < 0.05$ ) and an interaction between STZ and Trx-1 ( $F_{1,26} = 6.244$ ,  $P < 0.05$ ) (left panel). Two-way ANOVA showed a main effect of STZ ( $F_{1,26} = 11.36$ ,  $P < 0.01$ ) (right panel). B: Two-way ANOVA showed no main effects or interaction between STZ and Trx-1 in both transfected and untransfected neurons. C: Percentage of KCl-responsive neurons analyzed for glucose sensing in Fig. 5A and band in Fig. 7A and B with 7–10 dishes per group. Two-way ANOVA showed a main effect of RH ( $F_{1,57} = 11.94$ ,  $P = 0.001$ ). Different letters indicate statistical significance determined by Tukey post hoc tests after two-way ANOVA followed. The number of dishes of neurons from 10 rats in each group is given as (n). The digits on each bar mean the number of GI neurons/number of total neurons. S, saline.

This is very important, because glucagon secretion is impaired early in the development of type 1 diabetes (26). Moreover, hypothalamic glucose-sensing appears to be normalized only when glycemia is normalized, strengthening the causal relationship between glucose sensing and CRR failure.

The mechanisms underlying the impaired glucagon response in diabetes are not clear. Campanucci et al. (27) and Taborsky and colleagues (28) suggest that reduced sympathetic activation of the islet  $\alpha$ -cells plays an important role. Alternatively, inhibiting intrapancreatic zinc

(cosecreted with insulin) infusion restored the glucagon response to hypoglycemia in STZ rats, suggesting that the loss of  $\beta$ -cell inhibition may also play a role (29,30). Although these mechanisms could result from local changes caused by autoimmune destruction of the  $\beta$ -cell, the important role VMH plays in the impaired glucagon response in diabetes is becoming increasingly clear. For example, increased VMH  $\gamma$ -aminobutyric acid-ergic (GABAergic) output contributes to defective glucagon secretion in response to insulin-induced hypoglycemia in STZ rats (31). Conversely,

inhibiting VMH GABA transmission restored glucagon secretion (31). These observations suggest that despite of  $\beta$ -cell loss, reduced VMH GABAergic transmission can restore the glucagon response to insulin-induced hypoglycemia. Similarly, VMH AMPK activation increased glucagon secretion in response to hypoglycemia in rats with type 1 diabetes (32). AMPK activation is crucial for activation of VMH GI neurons by low glucose (10). Interestingly, Trx-1 is a cofactor for AMPK that maintains this enzyme in its activated state (33). Together, our data suggest that VMH Trx-1 overexpression preserved glucagon secretion and led to normalization of glycemic recovery in STZ rats after insulin-induced hypoglycemia by normalizing VMH hypoglycemia detection. Finally, the observation that epinephrine secretion in diabetic rats 2 h after insulin injection was more than twice that of the nondiabetic rats is consistent with one study that evaluated patients with type 1 diabetes and also STZ rats (34).

VMH Trx-1 overexpression did not restore the glucagon response or glycemic recovery to subsequent insulin-induced hypoglycemia after RH in STZ rats or normalize activation of VMH GI neurons in response to a physiological glucose decrease. As we hypothesized for NAC, one antioxidant system alone may not be able to compensate for the combined oxidative stress of RH and diabetic hyperglycemia; that is, although glutathione and Trx overlap to some extent, they also have unique functions. On the one hand, glutathione can, for example, regulate cellular processes through protein glutathionylation (35). On the other hand, Trx but not glutathione is a cofactor for AMPK that maintains normal activation (33). Thus, glutathione and Trx in combination may possibly be more effective in preventing the deleterious effects of hypoglycemia and hyperglycemia. This suggests that a treatment that enhanced both antioxidant systems may be needed to prevent the impaired CRR and reduced activation of VMH GI neurons in low glucose when RH occurs during diabetes. However, this hypothesis requires further study.

A slight increase in the percentage of detectable VMH GI neurons in response to low glucose was also observed in nontransfected GI neurons from diabetic rats with VMH Trx-1 overexpression. It is possible that these neurons were actually transfected with virus. However, they could have been mistakenly counted as nontransfected neurons because of photobleaching or weak transfection in which EGFP expression was below the detection threshold. Alternatively, NO diffusion from adjacent VMH GI neurons that were transfected may have activated nontransfected GI neurons. Also important to consider is that Trx-1 overexpression was unlikely to be limited to GI neurons and probably occurred in GE neurons as well as in nonglucose-sensing neurons and glial cells.

An unexpected finding with potential high impact was the effect of VMH Trx-1 overexpression on peripheral glucose homeostasis in STZ rats. Although almost all EGFP control rats became diabetic after one STZ injection, a statistically larger proportion (~27%) of those with VMH Trx-1 overexpression required a second injection. VMH lesions change the expression of genes related to differentiation, proliferation,

apoptosis, metabolism, and immune signaling pathways in pancreatic  $\beta$ -cells (36–38). VMH lesions also increase mortality after STZ administration (39). These data suggest that the VMH modulates islet  $\beta$ -cell vulnerability to STZ. VMH Trx-1 overexpression also lowered the insulin requirement in STZ rats without affecting body weight. This is consistent with a recent report showing that VMH activation increases peripheral insulin sensitivity (40).

In summary, NAC pretreatment preserved the activation of VMH GI neurons by low glucose in RH-exposed rats but did not preserve normal glucose sensing when RH occurred during diabetes. VMH Trx-1 overexpression preserved the CRR and activation of VMH GI neurons by low glucose in rats with type 1 diabetes before but not after RH. Our data suggest that increasing both glutathione and Trx antioxidant defenses in the VMH may restore the activation of VMH GI neurons and prevent HAAF during diabetes.

---

**Acknowledgments.** The authors are deeply grateful to Dr. Owen Chan, University of Utah School of Medicine, Salt Lake City, UT, and Dr. Xavier Fioramonti, INRA, Université de Bordeaux, Bordeaux, France, for advice in evaluating the CRR in diabetic rats and also thank Dr. Chan for advice in the care and prevention of severe hyperglycemia in diabetic rats. Dr. Zhenyu Sheng, Rutgers New Jersey Medical School, Newark, NJ, provided assistance with care of the diabetic rats.

**Funding.** This work was partly supported by JDRF Award 2-SRA-2014-269-M-R and National Institutes of Health grant R01-DK-081538-01A1.

**Duality of Interest.** No potential conflicts of interest relevant to this article were reported.

**Author Contributions.** C.Z. collected the data. C.Z. and V.H.R. designed the study and wrote the manuscript. V.H.R. edited the manuscript. V.H.R. 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.

**Prior Presentation.** Parts of this study were presented in abstract form at the 77th Scientific Sessions of the American Diabetes Association, San Diego, CA, 9–13 June 2017, and at the American Society for Nutrition Scientific Sessions and Annual Meeting at Experimental Biology, Chicago, IL, 22–26 April 2017.

## References

1. Cryer PE. Mechanisms of hypoglycemia-associated autonomic failure and its component syndromes in diabetes. *Diabetes* 2005;54:3592–3601
2. Borg WP, Sherwin RS, During MJ, Borg MA, Shulman GI. Local ventromedial hypothalamus glucopenia triggers counterregulatory hormone release. *Diabetes* 1995;44:180–184
3. Borg WP, During MJ, Sherwin RS, Borg MA, Brines ML, Shulman GI. Ventromedial hypothalamic lesions in rats suppress counterregulatory responses to hypoglycemia. *J Clin Invest* 1994;93:1677–1682
4. Routh VH, Donovan CM, Ritter S. Hypoglycemia detection. *Transl Endocrinol Metab* 2012;3:47–87
5. Song Z, Routh VH. Recurrent hypoglycemia reduces the glucose sensitivity of glucose-inhibited neurons in the ventromedial hypothalamus nucleus. *Am J Physiol Regul Integr Comp Physiol* 2006;291:R1283–R1287
6. Fioramonti X, Deak A, Deshpande S, et al. Hypothalamic S-nitrosylation contributes to the counter-regulatory response impairment following recurrent hypoglycemia. *PLoS One* 2013;8:e68709
7. Fioramonti X, Marsollier N, Song Z, et al. Ventromedial hypothalamic nitric oxide production is necessary for hypoglycemia detection and counterregulation. *Diabetes* 2010;59:519–528
8. Song Z, Routh VH. Differential effects of glucose and lactate on glucosensing neurons in the ventromedial hypothalamic nucleus. *Diabetes* 2005;54:15–22

9. McCrimmon RJ, Fan X, Cheng H, et al. Activation of AMP-activated protein kinase within the ventromedial hypothalamus amplifies counterregulatory hormone responses in rats with defective counterregulation. *Diabetes* 2006;55:1755–1760
10. Murphy BA, Fakira KA, Song Z, Beuve A, Routh VH. AMP-activated protein kinase and nitric oxide regulate the glucose sensitivity of ventromedial hypothalamic glucose-inhibited neurons. *Am J Physiol Cell Physiol* 2009;297:C750–C758
11. Sayed N, Baskaran P, Ma X, van den Akker F, Beuve A. Desensitization of soluble guanylyl cyclase, the NO receptor, by S-nitrosylation. *Proc Natl Acad Sci U S A* 2007;104:12312–12317
12. Mastrocola R, Restivo F, Vercellinato I, et al. Oxidative and nitrosative stress in brain mitochondria of diabetic rats. *J Endocrinol* 2005;187:37–44
13. Canabal DD, Potian JG, Duran RG, McArdle JJ, Routh VH. Hyperglycemia impairs glucose and insulin regulation of nitric oxide production in glucose-inhibited neurons in the ventromedial hypothalamus. *Am J Physiol Regul Integr Comp Physiol* 2007;293:R592–R600
14. Kleinbaum J, Shamoon H. Impaired counterregulation of hypoglycemia in insulin-dependent diabetes mellitus. *Diabetes* 1983;32:493–498
15. Lu J, Holmgren A. The thioredoxin antioxidant system. *Free Radic Biol Med* 2014;66:75–87
16. Sengupta R, Holmgren A. The role of thioredoxin in the regulation of cellular processes by S-nitrosylation. *Biochim Biophys Acta* 2012;1820:689–700
17. Schulze PC, Yoshioka J, Takahashi T, He Z, King GL, Lee RT. Hyperglycemia promotes oxidative stress through inhibition of thioredoxin function by thioredoxin-interacting protein. *J Biol Chem* 2004;279:30369–30374
18. Lappalainen Z, Lappalainen J, Oksala NK, et al. Diabetes impairs exercise training-associated thioredoxin response and glutathione status in rat brain. *J Appl Physiol* (1985) 2009;106:461–467
19. Hotta M, Tashiro F, Ikegami H, et al. Pancreatic beta cell-specific expression of thioredoxin, an antioxidative and antiapoptotic protein, prevents autoimmune and streptozotocin-induced diabetes. *J Exp Med* 1998;188:1445–1451
20. Yamamoto M, Yamato E, Toyoda S, et al. Transgenic expression of antioxidant protein thioredoxin in pancreatic beta cells prevents progression of type 2 diabetes mellitus. *Antioxid Redox Signal* 2008;10:43–49
21. Hamada Y, Fujii H, Kitazawa R, Yodoi J, Kitazawa S, Fukagawa M. Thioredoxin-1 overexpression in transgenic mice attenuates streptozotocin-induced diabetic osteopenia: a novel role of oxidative stress and therapeutic implications. *Bone* 2009;44:936–941
22. McDermott JC, Hutber A, Tan MH, Bonen A. The use of a cell-free perfusate in the perfused rat hindquarter: methodological concerns. *Can J Physiol Pharmacol* 1989;67:1450–1454
23. Murphy BA, Fioramonti X, Jochnowitz N, et al. Fasting enhances the response of arcuate neuropeptide Y-glucose-inhibited neurons to decreased extracellular glucose. *Am J Physiol Cell Physiol* 2009;296:C746–C756
24. Ikegami H, Ono M, Fujisawa T, Hiromine Y, Kawabata Y, Yamato E. Molecular scanning of the gene for thioredoxin, an antioxidative and antiapoptotic protein, and genetic susceptibility to type 1 diabetes. *Ann N Y Acad Sci* 2008;1150:103–105
25. Benhar M, Thompson JW, Moseley MA, Stamler JS. Identification of S-nitrosylated targets of thioredoxin using a quantitative proteomic approach. *Biochemistry* 2010;49:6963–6969
26. Cryer PE. Mechanisms of hypoglycemia-associated autonomic failure in diabetes. *N Engl J Med* 2013;369:362–372
27. Campanucci V, Krishnaswamy A, Cooper E. Diabetes depresses synaptic transmission in sympathetic ganglia by inactivating nAChRs through a conserved intracellular cysteine residue. *Neuron* 2010;66:827–834
28. Munding TO, Mei Q, Foulis AK, Fligner CL, Hull RL, Taborsky GJ Jr. Human type 1 diabetes is characterized by an early, marked, sustained, and islet-selective loss of sympathetic nerves. *Diabetes* 2016;65:2322–2330
29. Zhou H, Tran PO, Yang S, et al. Regulation of alpha-cell function by the beta-cell during hypoglycemia in Wistar rats: the “switch-off” hypothesis. *Diabetes* 2004;53:1482–1487
30. Zhou H, Zhang T, Harmon JS, Bryan J, Robertson RP. Zinc, not insulin, regulates the rat alpha-cell response to hypoglycemia in vivo. *Diabetes* 2007;56:1107–1112
31. Chan O, Paranjape S, Czyzyk D, et al. Increased GABAergic output in the ventromedial hypothalamus contributes to impaired hypoglycemic counterregulation in diabetic rats. *Diabetes* 2011;60:1582–1589
32. Fan X, Ding Y, Brown S, et al. Hypothalamic AMP-activated protein kinase activation with AICAR amplifies counterregulatory responses to hypoglycemia in a rodent model of type 1 diabetes. *Am J Physiol Regul Integr Comp Physiol* 2009;296:R1702–R1708
33. Shao D, Oka S, Liu T, et al. A redox-dependent mechanism for regulation of AMPK activation by thioredoxin1 during energy starvation. *Cell Metab* 2014;19:232–245
34. Inouye KE, Yue JT, Chan O, et al. Effects of insulin treatment without and with recurrent hypoglycemia on hypoglycemic counterregulation and adrenal catecholamine-synthesizing enzymes in diabetic rats. *Endocrinology* 2006;147:1860–1870
35. Ghezzi P. Regulation of protein function by glutathionylation. *Free Radic Res* 2005;39:573–580
36. Kiba T. Changes of the expressions of multiple metabolism genes in rat pancreatic islets after ventromedial hypothalamic lesioning. *Neurosci Lett* 2015;604:64–68
37. Kiba T. Ventromedial hypothalamic lesions downregulate multiple immune signaling pathways in rat pancreatic islets. *Neurosci Lett* 2016;610:177–181
38. Kiba T, Kintaka Y, Suzuki Y, et al. Changes of neuron-specific and apoptosis gene expression levels after ventromedial hypothalamic lesions in rat intestine. *Exp Biol Med (Maywood)* 2008;233:1368–1373
39. West DB, Seino Y, Woods SC, Porte D Jr. Ventromedial hypothalamic lesions increase pancreatic sensitivity to streptozotocin in rats. *Diabetes* 1980;29:948–951
40. Coutinho EA, Okamoto S, Ishikawa AW, et al. Activation of SF1 neurons in the ventromedial hypothalamus by DREADD technology increases insulin sensitivity in peripheral tissues. *Diabetes* 2017;66:2372–2386