Type 1 corticotropin-releasing factor receptors in the ventromedial hypothalamus promote hypoglycemia-induced hormonal counterregulation


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IATROGENIC HYPOGLYCEMIA REMAINS one of the most serious complications of insulin therapy and is the major limiting factor to optimal glycemic management in type 1 diabetes (T1DM) (8). In health, a fall in plasma glucose level is rapidly detected and a sequence of counterregulatory responses (CRRs) triggered, which mainly involves 1) suppression of insulin secretion, 2) counterregulatory hormone release, which rapidly promotes endogenous glucose production and limits peripheral glucose utilization, and 3) subjective awareness of hypoglycemia. In T1DM, these compensatory systems are disrupted at every level. Within five years of diagnosis, most patients have markedly impaired glucagon responses to hypoglycemia (31). As a result, individuals with T1DM are particularly dependent on the sympathoadrenal response to low blood glucose. However, within 10 years of diagnosis, the majority of patients develop additional impairments in sympathoadrenal and other neurohormonal responses against hypoglycemia (31). Furthermore, hormonal counterregulation can also be compromised by exposure to single or recurrent episodes of hypoglycemia (8, 10, 31). Finally, symptom awareness becomes impaired in individuals with T1DM. The term hypoglycemia-associated autonomic failure (HAAF) was introduced by Cryer to incorporate both defective glucose counterregulation and hypoglycemia unawareness (8–10). The presence of HAAF greatly increases the risk of suffering severe hypoglycemia in patients with T1DM (7, 43). Understanding the molecular mechanisms underlying glucose sensing is key to developing therapies designed to minimize the impact of severe hypoglycemia in T1DM.

Hypoglycemia must first be detected to mount protective CRRs. It is now recognized that falling glucose levels are detected by specialized glucose-sensing neurons that have been demonstrated in the brain (4, 6, 14–17, 21, 22, 28, 30, 36, 38), and periphery (11, 12, 18). Of the several brain glucose-sensing areas identified, the ventromedial hypothalamus (VMH), in particular, plays a key role in this sensing mechanism (4–6, 21, 28, 36). Borg and colleagues (5, 6) reported that lesioning the VMH significantly suppressed CRRs to hypoglycemia, whereas local VMH glucopenia triggered CRRs. The molecular mechanisms used by glucose-sensing neurons in the VMH to detect low blood glucose are incompletely understood, although roles for glucokinase (13, 21, 38) the ATP-sensitive potassium (K_{ATP}) channel (14, 26), and AMP-activated protein kinase (28, 37) have been demonstrated. However, although the focus of investigators has been to determine glucose-sensing mechanisms, it is also clear that the metabolic signal provided to glucose-sensing neurons ultimately needs to generate a neuroendocrine stress response to the hypoglycemic challenge. As the stress response is tightly regulated at both the cellular and whole body level, this introduces another possible mechanism through which CRR to hypoglycemia can be regulated and, in some conditions, impaired.

The corticotropin-releasing factor (CRF) peptide family, acting through their respective receptors (CRFRs), plays central roles in regulating both neural and endocrine responses to stress (2, 3). To date, the mammalian CRF family has been shown to consist of 1) four peptides: CRF, urocortin 1, urocortin 2, and urocortin 3; 2) two receptors: type 1 CRF receptor (CRFR1) and type 2 CRF receptor (CRFR2); and 3) a CRF-
binding protein (2, 3). Within the brain, the urocortins tend to colocalize with CRFR2, suggesting that they are the endogenous ligands for this receptor (2, 3). In contrast, CRF is thought to act primarily through CRFR1 (3). The relative balance between the two CRF pathways in the brain may be crucial in coordinating stress-coping responses and maintaining homeostasis (3). In this context, our laboratory recently demonstrated that activation of CRFR2 within the VMH has a marked suppressive effect on CRRs to acute hypoglycemia (34)(29).

Since CRFR1 and CRFR2 may operate in a functionally opposite manner in regulating stress responses, and as both subtypes are expressed in the VMH, we sought to examine the potential role of CRFR1 within the VMH. We hypothesized that, in contrast to the suppressive effect of CRFR2 activation, CRFR1 and its ligands in the VMH would act to amplify the CRR to acute hypoglycemia.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (Charles River, Richmond, VA), weighing between 250 and 350 g, were individually housed in the Yale Animal Resource Center in an environmentally controlled room with a 12:12-h light-dark cycle and fed a standard pellet diet (Prolab 3000; Agway, Syracuse, NY). The animal care and experimental protocols were reviewed and approved by the Yale Animal Care and Use Committee.

One week prior to each study, animals were anesthetized with an intraperitoneal injection (1 ml/kg) of a mixture of xylazine (Anased 20 mg/ml; Lloyd Laboratories, Shenandoah, IA) and ketamine (Ketaset 100 mg/ml; Aveco, Fort Dodge, IA) in a ratio of 1:2 (vol/vol). The animals initially underwent vascular surgery for the implantation of chronic vascular catheters, followed by the stereotaxic insertion of the catheters into the VMH or paraventricular nucleus (PVN) with intravenous pentobarbital sodium (Sleepaway, Fort Dodge, IA). The rats were then allowed to recover for 7–10 days and were subsequently studied in the overnight fasted, awake, and unrestrained state. A second group of rats, allowed to feed overnight, were rapidly killed with intravenous pentobarbital sodium (3 mg/ml; 115 NaCl, 5.9 KCl, 1.2 MgCl2, 1.2 NaH2PO4, 1.2 Na2SO4, 2.5 CaCl2, and 25 NaHCO3) and 4% bovine serum albumin, pH 7.45) and reinfused after each blood sampling to prevent volume depletion and anemia.

A second group of rats was used for additional tracer studies. In these studies, a lower dose of insulin (10 mU·kg⁻¹·min⁻¹) was used to allow for some endogenous glucose production during hypoglycemia. A constant insulin and a variable dextrose (20%) infusion were used to maintain plasma glucose levels at 2.8 mM (50 mg/dl) for 120 min. Samples for glucose, epinephrine, glucagon, and corticosterone were obtained at regular intervals during the baseline (0 min) and hypoglycemic states (60, 90, and 120 min).

Microinjection. On the morning of study, bilateral microinjection needles (Plastics One, Roanoke, VA), cut to extend 1 mm beyond the guide cannula tip, were inserted to the level of the VMH or PVN. The rats were then allowed to recover for 7–10 days and were subsequently studied in the overnight fasted, awake, and unrestrained state. A second group of rats, allowed to feed overnight, were rapidly killed with intravenous pentobarbital sodium (3 mg/ml; 115 NaCl, 5.9 KCl, 1.2 MgCl2, 1.2 NaH2PO4, 1.2 Na2SO4, 2.5 CaCl2, and 25 NaHCO3) and 4% bovine serum albumin, pH 7.45) and reinfused after each blood sampling to prevent volume depletion and anemia.

Microinjection. On the morning of study, bilateral microinjection needles (Plastics One, Roanoke, VA), cut to extend 1 mm beyond the guide cannula tip, were inserted to the level of the VMH or PVN. The rats were then microinjected over 2 min at a rate of 0.1 µl/min with vehicle [CON; 0.5% DMSO-artificial extracellular fluid (aECF), pH 6.5], CRF (1 pmol/side in 0.5% DMSO-aECF, pH 6.5), Antalarmin (500 pmol/side in 0.5% DMSO-aECF, pH 6.5), or CRF + Antalarmin (1 pmol CRF + 500 pmol Antalarmin in 0.5% DMSO-aECF, pH 6.5) using a CMA-102 infusion pump (CMA Microdialysis, North Chelmsford, MA). Following microinjection, the needles were left in place for 5 min before being removed. At the end of the study, the rats were rapidly euthanized with intravenous pentobarbital sodium (Sleepaway), and probe position was confirmed in all rats histologically. Only those animals with probes in the correct position were included in the analyses. Of the VMH surgeries performed; ~80% had probes in the correct position. Figure 1 illustrates, using 0.2 µl of Alexa 488 fluorescence dye combined with 0.5% DMSO, the relative localization of a compound to the VMH following microinjection. In addition, pilot studies were performed to compare the effects of vehicle (CON; 0.5% DMSO-aECF, pH 6.5) vs. aECF alone (pH 7.4), and no significant differences were detected (data not shown).

Human/rat CRF was obtained from Sigma (St. Louis, MO). Antalarmin was a generous gift from Dr. George Chrousos (National Institutes of Health, Bethesda, MD). The doses used were based on the results of pilot studies in smaller groups of rats and data in our previous studies (29).

Hypoglycemic clamp studies. After an overnight fast, vascular catheters were opened and connected to infusion pumps. The animals were allowed 1.5 h to recover from handling before basal hormone samples were collected. Subsequently, the rats were bilaterally microinjected into the VMH or PVN before undergoing a hyperinsulinemic hypoglycemic glucose clamp (5 min after microinjection). A constant insulin (20 mU·kg⁻¹·min⁻¹) regular human insulin; Eli Lilly, Indianapolis, IN) and a variable dextrose (20%, Abbott Laboratories, Chicago, IL) infusion were used to maintain plasma glucose levels at 2.8 mM (50 mg/dl) for 120 min. Samples for glucose, epinephrine, glucagon, and corticosterone were obtained at regular intervals during the baseline (0 min) and hypoglycemic states (60, 90, and 120 min). Red blood cells, after removal of plasma, were resuspended in an equivalent volume of sterile artificial plasma (in mM: 115 NaCl, 5.9 KCl, 1.2 MgCl2, 1.2 NaH2PO4, 1.2 Na2SO4, 2.5 CaCl2, and 25 NaHCO3 and 4% bovine serum albumin, pH 7.45) and reinfused after each blood sampling to prevent volume depletion and anemia.

A second group of rats was used for additional tracer studies. In these studies, a lower dose of insulin (10 mU·kg⁻¹·min⁻¹) was used to allow for some endogenous glucose production during hypoglycemia. A constant insulin and a variable dextrose (20%) infusion were used to maintain plasma glucose levels at 2.8 mM (50 mg/dl) for 120 min. A primed infusion of [³H]glucose was started at t = −90 min and continued throughout the hypoglycemia period. In addition, [³H]glucose was added to the 20% dextrose in an attempt to maintain plasma specific activity (SA). Effects of 1) VMH-Control, 2) VMH-CRF, 3) VMH-Antalarmin on rates of endogenous glucose production (Ra) and peripheral glucose utilization (Rd) during insulin-induced hypoglycemia were compared. Glucose turnover was calculated as previously described (39). Endogenous glucose production was calculated by subtracting the exogenous glucose infusion rate from total Ra. Using steady-state equations in this way to derive Ra can result in negative values, particularly with higher glucose infusion rate (GIR). Ra, when negative, was included as such in our analysis. For the tracer studies, hormone samples were obtained at the beginning of the clamp (i.e., baseline) and at the end of the clamp (i.e., 120 min).

Plasma glucose and hormone determination. Plasma concentrations of glucose were measured by the glucose oxidase method (GM9D Analyzer; Analox Instruments, London, UK). Plasma epi-
neuropeptide is noradrenaline concentrations were analyzed by HPLC using electrochemical detection (ESA, Acton, MA). Plasma insulin (Linco Research, St. Charles, MO; within-assay CV 4.3%), corticosterone (Diagnostic Products, Los Angeles, CA; within-assay CV 7.9%) and glucagon (Linco Research) levels were determined using commercially available RIA kits.

mRNA analysis. mRNA levels were evaluated with a Brilliant SYBR Green QRT-PCR Master Mix kit (Qiagen). Frozen microcups were taken through hypthalamic VMH or PVN regions according to the coordinates of Paxinos and Watson (35), and then total cellular RNA from punches was extracted with a PicoPure RNA isolation kit (Arcturus, CA). The forward primer for rat CRFR1 consisted of 5’-CCTTTACGGGCTTCTTG-3’ and the reverse primer consisted of 5’-GGGCATACCTCATGGCTTCT-3’ and the reverse primer consisted of 5’-GTCTGGTTATGCTTGGAAA-3’. qRT-PCR reactions were made by combining 12.5 μl of 2× SYBR RT-PCR Master Mix, 0.5 μl of upstream primer (5 μM), 0.5 μl of downstream primer (5 μM), 0.0625 μl of StrataScript RT/RNase block enzyme mixture, 1.4375 μl of RNase-free water, and 10 μl (20 ng/μl) of RNA template. No-template controls and no-RT controls were introduced in each run. The qRT-PCR was performed using DNA Engine Opticon 2 (MJ Research), in which the mixture was heated to 95°C for 30 min for reverse transcription, heated to 95°C for 10 min, and then cycled 40 times at 95°C for 30 s, 56°C for 1 min, and 72°C for 1 min. To verify the specificity of the amplification reaction, melting-curve analysis was performed. The size of the amplified product was confirmed by electrophoresis. The level of rat β-actin mRNA was determined in all the samples, and the expression of gene of interest was normalized to rat β-actin. The threshold cycle (Ct) value is taken as the fractional cycle number at which the emitted fluorescence of the sample passes a fixed threshold above the baseline. The 2–ΔΔCt method was used to calculate the relative differences between experimental and control groups as fold change in gene expression (25).

Fluorescence dye injection. To illustrate the relative localization of a compound following microinjection of a 0.2 μl to the VMH, 0.2 μl of Alexa 488 fluorescence dye (Molecular Probes) combined with 0.5% DMSO was microinjected into the VMH via microinjection guide cannula. Subsequently, the animal was anesthetized with ketamine-xylazine (80:10 mg/kg ip) and transcardially perfused with 0.5 M diethyl pyrocarbonate (DEPC)-treated (Sigma) phosphate-buffered saline (DEPC-PBS) followed by 500 ml of phosphate-buffered 10% formalin, pH 7.0 (Sigma). The brain was removed, postfixed in some fixative for 3 h, and submerged overnight in 30% sucrose in DEPC-PBS. The brain was cut on a freezing microtome at 25 μm (1.6 series). The series of coronal sections were stored at −20°C in antifreeze solution, which contained 30% ethylene, 20% glycerol and DEPC-PBS.

Immunofluorescence. The sections taken from stock were washed in PBS for 10 min. After a thorough wash, they were incubated with rabbit anti-proopiomelanocortin (POMC) serum (Phoenix Pharmaceuticals; 1:1K) overnight. The next day, they were incubated with Alexa fluor 594-conjugated streptavidin (Molecular Probes; 1:1K) for 1 h. After mounting on polylysine slides, the sections were coveredslipped with anti-fade mount medium for fluorescence (Vectorshield; Vector).

Photomicrographs. Immunofluorescence was observed under a microscope (Olympus BX-52) with appropriate filter sets for Alexa 488 (excitation, 540–552 nm, emission 575–625 nm) and Alexa 594 (excitation, 530–585 nm, emission ≥615 nm).

Statistical analysis. All data are expressed as means ± SE. Statistical analysis was by one- or two-way ANOVA, as appropriate, followed by post hoc t-test analysis using Statistica 6.0 (StatSoft, Tulsa, OK). Differences were considered significant at P < 0.05.

RESULTS

Expression of CRFR1 and CRFR2 in the VMH and the PVN. RT-PCR analysis revealed that both CRFR1 and CRFR2 were expressed within the VMH of Sprague-Dawley rats (Fig. 2A). We also detected the expression of CRFR1 and CRFR2 within the PVN region. Real-time quantitative RT-PCR demonstrated that the mRNA levels of CRFR1 were similar between the two regions [P = nonsignificant (NS), n = 13; Fig. 2B], but the level of CRFR2 within the VMH was only 54% of that within the VMH (P < 0.05, n = 13; Fig. 2B).

Effects of VMH microinjection of CRFR1 agonist and antagonist on CRF to acute hypoglycemia. We examined the effects of bilateral VMH microinjection of 1) vehicle-control (n = 7), 2) CRFR1 agonist, CRF (n = 7), 3) CRFR1 antagonist Antalarmin (n = 7), and 4) CRF + Antalarmin (n = 7) on counterregulatory responses to acute hypoglycemia. The baseline plasma hormone levels were not significantly different among the four treatment groups (Table 1). During the hyperinsulinemic hypoglycemic clamp, plasma glucose profiles (Fig. 3A) and average plasma insulin concentrations (Fig. 3B) were similar among all groups. In contrast, mean 60- to 120-min exogenous GIRs required to maintain the hypoglycemic plateau were significantly changed. VMH administration of the CRFR1 agonist CRF resulted in lower GIRs (CRF vs. Control: 9.6 ± 1.6 vs. 13.8 ± 1.5 mg·kg−1·min−1, P < 0.05), whereas CRFR1 antagonism with Antalarmin resulted in higher GIRs (Antalarmin vs. Control: 21.4 ± 1.6 vs. 13.8 ± 1.5 mg·kg−1·min−1, P < 0.05) compared with the control groups (Fig. 4A).

The epinephrine response to hypoglycemia (mean 60–120 min) was greater following VMH CRF (CRF vs. Control: 1.6 ± 0.2 vs. 13.8 ± 3.6 on November 7, 2017 http://ajpendo.physiology.org/ Downloaded from AJP-ENDO.ORG by 10.220.33.6 on November 7, 2017
In the second series of studies with glucose tracer, we examined the effect of VMH microinjection of 1) vehicle-control (n = 11), 2) the CRFR1 agonist CRF (n = 7), and 3) the CRFR1 antagonist Antalarmin (n = 7). Basal rates of Ra and Rd did not differ significantly between groups (Control vs. CRF vs. Antalarmin: 6.2 ± 0.6 vs. 5.6 ± 0.8 vs. 6.2 ± 0.8 mg·kg⁻¹·min⁻¹, P = NS). Glucose specific activity was most stable during 60–100 min of the hypoglycemic clamp (Fig. 5A). During the hypoglycemic condition (60–100 min), CRFR1 antagonism increased both the rate of peripheral glucose utilization, Rd (Antalarmin vs. Control: 12.4 ± 1.4 vs. 9.7 ± 0.7 mg·kg⁻¹·min⁻¹, P < 0.05; Fig. 5B) and significantly decreased the rate of endogenous glucose production, Ra (Antalarmin vs. Control: 0.2 ± 1.4 vs. 3.7 ± 1.0 mg·kg⁻¹·min⁻¹, P < 0.05; Fig. 5C). In comparison, CRFR1 agonism had no significant effect on Rd but did significantly lower Ra (CRF vs. Control: 6.8 ± 0.4 vs. 9.7 ± 0.7 mg·kg⁻¹·min⁻¹, P < 0.05; Fig. 5B). Epinephrine levels measured upon completion of the tracer studies showed a similar
pattern to the hormone study above, showing amplification following CRF (CRF vs. Control: 9.2 ± 1.4 vs. 6.1 ± 1.0 nM, P < 0.05). However, although Antalarmin suppressed the epinephrine response in this second group of rats, this did not reach statistical significance (Antalarmin vs. Control: 4.2 ± 1.0 vs. 6.1 ± 1.0 nM, P = 0.09), which probably reflects the use of a single time point, as this was not the main outcome measure of this study.

**Effect of PVN microinjection of CRF1 antagonist on counterregulation to acute hypoglycemia.** To determine whether the effects of CRF1 modulation are region specific, we next tested the effects of bilateral PVN microinjection of vehicle-control (n = 5) vs. CRF1 antagonist Antalarmin (n = 5). Baseline plasma hormone levels were similar between the two groups (Table 2). During the clamp, neither the mean plasma glucose (Antalarmin vs. Control: 2.78 ± 0.16 vs. 2.82 ± 0.13 mM, P = NS) nor average insulin concentrations (Antalarmin vs. Control: 5.626 ± 1,337 vs. 6,574 ± 2,090 PM, P = NS) were significantly different between the control and Antalarmin groups. In contrast to the VMH study, PVN Antalarmin had no effect on the mean GIR (Antalarmin vs. Control: 12.3 ± 1.5 vs. 14.4 ± 1.5 mg·kg⁻¹·min⁻¹, P = NS; Fig. 6A), epinephrine responses (Antalarmin vs. Control: 7.4 ± 1.0 vs. 5.5 ± 0.9 nM, P = NS; Fig. 6B), and glucagon responses (Antalarmin vs. Control: 116.7 ± 23.9 vs. 90.1 ± 13.6 ng/l, P = NS; Fig. 6C) during hypoglycemia. However, PVN microinjection of Antalarmin slightly but significantly reduced the mean corticosterone concentration (Antalarmin vs. Control: 1,415.9 ± 17.6 vs. 1,782.8 ± 156.5 nM, P < 0.05; Fig. 6D) during the hypoglycemic clamp.

**DISCUSSION**

The current study demonstrates a potential role for the VMH CRF1 system in the regulation of neuroendocrine CRRs to acute hypoglycemia. Specifically, antagonism of the CRF1 receptor in the VMH during systemic hypoglycemia suppressed both epinephrine and glucagon secretory responses, as well as increasing the rate of peripheral glucose utilization and decreasing the rate of endogenous glucose production. In the uncontrolled state, this would have the effect of markedly increasing the risk of progression to severe hypoglycemia. Conversely, VMH CRF1 agonism during systemic hypoglycemia amplified the epinephrine counterregulatory response and reduced peripheral glucose utilization, actions which together would reduce the risk of progression to severe hypoglycemia.

In contrast to urocortin, CRF is thought to be the endogenous ligand for CRF1, having an at least tenfold higher affinity for CRF1 than for CRF2 (3, 23, 34). Studies in transgenic mice have lead to the hypothesis that the two interrelated CRF receptors play essential and distinct roles in coordinating stress responses and maintaining homeostasis (3). Our finding, that in contrast to the suppressive effects of CRF2 activation CRF1 activation within the VMH (3, 29) appears to amplify CRRs to acute hypoglycemia, is consistent with this model. It is possible that the CRF1 pathway functions acutely to amplify the counterregulatory response to a given hypoglycemic stress, whereas the CRF2 pathway, the inhibitory branch, acts to contravene and modulate the stress response. The counterbalance between these two opposing CRF receptors in the VMH, a key glucose-sensing region, may play a critical role in controlling the magnitude of the counterregulatory response.

**Table 2. Baseline hormone levels for PVN microinjection studies**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Antalarmin</th>
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<tbody>
<tr>
<td>Body weight, g</td>
<td>330±10</td>
<td>332±14</td>
</tr>
<tr>
<td>Corticosterone, nM</td>
<td>252±67</td>
<td>183±30</td>
</tr>
<tr>
<td>Glucagon, ng/l</td>
<td>36±1</td>
<td>35±1</td>
</tr>
<tr>
<td>Epinephrine, nM</td>
<td>0.38±0.13</td>
<td>0.54±0.09</td>
</tr>
<tr>
<td>Norepinephrine, nM</td>
<td>0.67±0.05</td>
<td>0.64±0.14</td>
</tr>
<tr>
<td>Insulin, pM</td>
<td>44±7</td>
<td>64±16</td>
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Results are expressed as Mean ± SE; n = 5 in each group, PVN, paraventricular nucleus.
terregulatory response to hypoglycemia. Interestingly, it has been shown in other brain regions, such as the central nucleus of the amygdala and the lateral septum mediolateral nucleus, that the two CRF receptors exert opposite and counterbalancing effects and subsequently establish a homeostasis for normal excitatory glutamatergic transmission (24).

In transgenic mouse models, CRF appears to play a major permissive role in the physiological response to a variety of stressors. CRF knockout mice have impaired hypothalamic-pituitary-adrenal axis and catecholaminergic responses to tail restraint, hypovolemia and hypoglycemia (19, 20), whereas CRFR1 knockout mice show a reduced responsiveness to stressful stimuli (40). Our finding that VMH CRF amplified the CRR response is consistent with a role in the regulation of the hypoglycemic stress response. Moreover, the fact that Antalarmin by itself ameliorated the CRR response to acute hypoglycemia is consistent with the presence of an endogenous CRFR1 agonist in the VMH. The presence of CRF-immunoreactive nerve terminals in rat VMH (32, 33) would suggest that this endogenous ligand is CRF, although our study did not directly address this issue. CRF-immunoreactive neurons are also found in the PVN (32, 33) and play a role in the stimulation of ACTH in response to stress. Our finding that CRFR1 inhibition attenuated the corticosterone response to acute hypoglycemia is consistent with this role. The effect observed was modest, which may reflect less effective CRFR1 blockade in the PVN following microinjection but also could result from the well-recognized action of arginine vasopressin to at least partially compensate for CRF actions in the PVN (19, 20). However, overall it seems reasonable to conclude that our studies provide strong support for an endogenous system within the VMH, based on CRF and CRFR1, for amplifying the CRR to acute hypoglycemia.

It is also not clear exactly how the CRFR1/CRFR2 system integrates with the signaling pathways activated by low glucose within the VMH. There are several possibilities, including 1) a change in the sensitivity of VMH glucose-sensing neurons, with our previous study demonstrating that CRFR2 ligands directly alter thresholds for activation of glucose-inhibited and glucose-excited neurons in the VMH (29); 2) regulation of glucose-sensing machinery: K<sub>ATP</sub> channel gene expression has been reported to be induced by urocortin in cardiac myocytes, and the K<sub>ATP</sub> channel has been shown to play a role in VMH glucose-sensing (14, 26); 3) modulation of neurotransmitter release: activation of CRFR1/CRFR2 has been suggested to affect release of the excitatory neurotransmitters glutamate and serotonin in the amygdala (1, 24). Moreover, it is not clear what the stimulus to CRF release during hypoglycemia is. Future studies are needed to determine whether CRF neurons are in themselves glucose sensing or activated in response to hypoglycemia detected in other brain or peripheral sensors.

The lack of an available highly selective CRFR1 agonist means that we cannot exclude a potential interaction between CRF and CRFR2. However, the Antalarmin studies provide strong evidence that the effects that we have observed are CRFR1 mediated. Antalarmin is a widely used, and highly selective, nonpeptide CRFR1 antagonist. In vitro binding experiments demonstrate that Antalarmin is specific for CRFR1, since it displaced <sup>125</sup>I-labeled corticotropin-releasing hormone binding in rat pituitary, frontal cortex, and cerebellum, where CRFR1 predominates, but not in rat heart, where only CRFR2 is expressed (42). The amount of Antalarmin administered in the current study is based on a pilot dose-response curve (data not shown) and is within the amygdala microinjection dose range for its effects on fear responses (35).

In the present study, CRFR1 activation in the VMH was shown to decrease peripheral glucose utilization, whereas...
CRFR1 inhibition increased peripheral glucose utilization and suppressed endogenous glucose production during hypoglycemia. Although it is possible that these effects resulted from a direct neural action on the liver, it is perhaps more likely that the changes reflect the alterations in principal counterregulatory hormones. It is known that glucagon stimulates hepatic glucose production whereas epinephrine acts primarily to reduce glucose clearance in peripheral tissues (9). Consistent with this, VMH CRF did not alter glucagon responses or endogenous glucose production but did significantly amplify epinephrine responses and reduce peripheral glucose uptake during hypoglycemia. VMH CRFR1 antagonism amplified glucagon as well as the epinephrine CRR and affected both glucose production and uptake.

The lack of an effect of CRF on glucagon CRR was unexpected. Previously, we showed that local VMH delivery of 4 pmol CRF amplified, whereas 40 pmol CRF suppressed, both epinephrine and glucagon CRRs (29). In the present study, we chose 1 pmol CRF to try to minimize the potential interaction between CRF and CRFR2. Our data therefore suggest that a higher-dose CRF is required to amplify glucagon CRR. Why there should be this differential effect is not clear, and, although not addressed directly in the study, it is possible that this reflects the complex regulation of pancreatic α-cell secretion.

We also considered the possibility of an effect of the surgical procedure itself or of CRF spread outside the VMH. The guide cannula may damage areas of brain involved in glucose sensing (Fig. 1), and in particular in the present study it is possible that the surgery damaged a hypothalamic area associated with glucose sensing, namely the dorsomedial hypothalamus (DMH) (17). However, although the DMH has been shown to play a role in glucose sensing during hypoglycemia, chemical inactivation of the DMH with lidocaine resulted in a moderate impairment of ACTH and corticosterone responses to acute hypoglycemia only and had no effect on epinephrine and glucagon secretory responses (17). It is therefore unlikely that damage to the DMH could explain our findings. We also considered the possibility of an effect of CRF outside the VMH. Our first approach to studying this question was to microinject a green florescent dye in 0.5% DMSO (which can spread significantly from injection sites) in an identical volume to the VMH, and this showed a relatively local distribution (Fig. 1). However, the different molecular weight and solubility of the dye may have influenced its distribution; thus we cannot exclude an effect on glucose-sensing neurons in, for instance, the arcuate nucleus (Arc) or PVN. In a previous study (6), which used radiolabeled 2-deoxyglucose to perfuse the VMH, the radioactivity of tissue samples outside the VMH was not significantly greater than background, but this study used microdialysis rather than direct microinjection and cannot necessarily be extended to our study. To further address this issue, we also examined the effect of CRFR1 inhibition in the closely associated hypothalamic glucose-sensing region, the PVN (15, 16). In contrast to VMH microinjection, we found that CRFR1 inhibition in the PVN suppressed corticosterone but not glucagon or epinephrine responses to hypoglycemia, findings that are consistent with previous reports from our laboratory (14, 26–29). However, although this argues against a role for the PVN, it does not exclude the possibility that activation or suppression of CRFR1 in the Arc (41) have contributed to our findings. Overall, our data suggest that the VMH, with a possible contribution from the Arc, may be a key hypothalamic region regulating the sympathoadrenal component of the counterregulatory response to a hypoglycemic challenge.

In conclusion, the present study indicates that the CRFR1 pathway within the VMH has an important role in promoting counterregulation during acute hypoglycemia. Moreover, the counterbalance between CRFR1 and CRFR2 within the VMH may play a crucial role in modulating the magnitude of counterregulatory responses to insulin-induced hypoglycemia. Disturbance of the delicate balance between the two opposing CRF receptors in the VMH may contribute to the defective sympathoadrenal responses to hypoglycemia in type 1 diabetes.

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