

PVN activation is suppressed by repeated hypoglycemia but not antecedent corticosterone in the rat

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Evans, Scott B., Charles W. Wilkinson, Kathy Bentson, Pam Gronbeck, Aryana Zavosh, and Dianne P. Figlewicz. PVN activation is suppressed by repeated hypoglycemia but not antecedent corticosterone in the rat. *Am J Physiol Regulatory Integrative Comp Physiol* 281: R1426–R1436, 2001.—The mechanism(s) underlying hypoglycemia-associated autonomic failure (HAAF) are unknown. To test the hypothesis that the activation of brain regions involved in the counterregulatory response to hypoglycemia is blunted with HAAF, rats were studied in a 2-day protocol. Neuroendocrine responses and brain activation (c-Fos immunoreactivity) were measured during *day 2* insulin-induced hypoglycemia (0.5 U insulin·100 g body wt⁻¹·h⁻¹ iv for 2 h) after *day 1* hypoglycemia (Hypo-Hypo) or vehicle. Hypo-Hypo animals demonstrated HAAF with blunted epinephrine, glucagon, and corticosterone (Cort) responses and decreased activation of the medial hypothalamus [the paraventricular (PVN), dorsomedial (DMH), and arcuate (Arc) nuclei]. To evaluate whether increases in *day 1* Cort were responsible for the decreased hypothalamic activation, Cort was infused intracerebroventricularly (72 µg) on *day 1* and the response to *day 2* hypoglycemia was measured. Intracerebroventricular Cort infusion failed to alter the neuroendocrine response to *day 2* hypoglycemia, despite elevating both central nervous system and peripheral Cort levels. However, *day 1* Cort blunted responses in two of the same hypothalamic regions as Hypo-Hypo (the DMH and Arc) but not in the PVN. These results suggest that decreased activation of the PVN may be important in the development of HAAF and that antecedent exposure to elevated levels of Cort is not always sufficient to produce HAAF.

paraventricular nucleus; stress; c-Fos; hypoglycemia-associated autonomic failure

THE PARAVENTRICULAR NUCLEUS (PVN) of the hypothalamus plays a key role in initiating the neuroendocrine response to physiological and psychological stressors (44, 45). PVN neurons respond to stressors by increasing the synthesis and release of vasopressin and corticotropin-releasing factor, which stimulate the release of ACTH from the pituitary (62). Under the influence of

ACTH, glucocorticoids [e.g., corticosterone (Cort)] are released by the adrenal cortex. PVN neurons also project to autonomic preganglionic cells in the spinal cord (29, 40, 53, 55) and can directly activate the sympathetic nervous system. Epinephrine release from the adrenal medulla secondary to sympathetic activation, in concert with plasma Cort, represents the essential neuroendocrine response to stressors.

The neuroendocrine response may be reduced on repeated challenge with the same stressor, while enhanced or unchanged on subsequent challenge with a different stressor (6, 7, 16, 18, 27). Diminished neuroendocrine responses can be seen both for repeated physiological stressors, such as injections of hypertonic saline, as well as repeated psychological stressors, such as immobilization (27). One clinically important example of a blunted neuroendocrine response to a repeated physiological stressor is the defective counterregulatory response to repeated hypoglycemia in diabetic patients, known as hypoglycemia-associated autonomic failure (HAAF; Ref. 16). Hypoglycemia stimulates the neuroendocrine response described above, as well as glucagon release by pancreatic islet α -cells. HAAF is defined as the blunting of these responses after repeated hypoglycemic episodes such that, with repeated bouts of hypoglycemia, as can happen with intensive insulin therapy, blood glucose levels reach lower nadir values and take longer to return to the euglycemic state. However, intensive insulin therapy has been found to decrease the incidence of complications in diabetic patients (Diabetes Control and Complications Trial) and it is currently recommended by the American Diabetes Association (Clinical Practice Recommendations, 2000; Ref. 1) for patients that have health care resources and are “intellectually, emotionally, physically, and financially able to attempt tight control.” Understanding the mechanisms of HAAF and how to avoid it might make intensive insulin therapy more feasible for many patients and thus prevent chronic diabetic complications.

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The HAAF effect may involve adrenal glucocorticoids, because the feedback inhibitory effects of glucocorticoids can act at the level of protein synthesis (12, 19), which would produce a time course consistent with the HAAF effect (developing within 24 h and lasting at least several days). Additionally, an HAAF-like hormonal profile has been demonstrated in normal human subjects (18) when hypoglycemia was induced 1 day after intravenous administration of cortisol and insulin in a hypoglycemic clamp paradigm. To investigate whether increases of central nervous system (CNS) Cort are sufficient to induce HAAF-like effects, we compared the neuroendocrine responses to hypoglycemia in rats with prior exposure to either Cort or hypoglycemia. In addition to measuring the neuroendocrine responses to hypoglycemia, we mapped mid- and forebrain areas for the expression of the immediate early gene *c-fos*, a marker of neuronal activation (31). We quantified *c-Fos* immunostaining, allowing us to compare hypoglycemia-induced CNS activation alone, after antecedent Cort, or after antecedent bouts of hypoglycemia. While the pattern of neuronal activation in response to hypoglycemia has been evaluated to a limited extent (see DISCUSSION), the effect of antecedent bouts of hypoglycemia or exposure to Cort on this pattern has not. On the basis of the hypotheses presented above, we expected to observe changes in brain activation that paralleled changes in the neuroendocrine response to hypoglycemia and, specifically, decreased PVN activation (as indicated by decreased *c-Fos* expression) when the neuroendocrine response was blunted. By demonstrating the neural circuits involved in the blunted neuroendocrine response to hypoglycemia, we hope to elucidate potential CNS targets for intervention.

METHODS

Subjects. Male Wistar rats (Simonson, CA; 350–400 g) were studied. Rats were maintained on a 12–12-h light-dark schedule (lights on at 7:00 AM, off at 7:00 PM), with ad libitum access to food and water. All procedures were approved by the Animal Studies Subcommittee of the Veterans Affairs Puget Sound Health Care System Research and Development Committee.

Surgery. All animals underwent bilateral implantation of intravenous Silastic catheters according to the method of Scheurink et al. (48) under ketamine-xylazine anesthesia (60 mg/kg ketamine, 7.8 mg/kg xylazine) with supplemental doses (25 mg/kg) of ketamine when necessary. One catheter was placed in the linguofacial vein and the other in the submaxillary vein and advanced to the heart. Catheters were tunneled subcutaneously and exteriorized through a midline incision in the scalp. Rats that received an intracerebroventricular cannula were then placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA), and a 26-gauge stainless steel guide cannula (Plastics One, Roanoke, Virginia) was implanted, aimed at the third cerebral ventricle using the stereotaxic coordinates -2.2 anteroposterior from bregma, 0.0 mediolateral, -7.5 dorsoventral from dura, as previously established in our laboratory (51). The intracerebroventricular cannula and intravenous catheters were held in place by acrylic cement to four skull screws. Animals received subcutaneous 1 ml lactated Ringer solution (Baxter)

and 0.2 ml Batryl antibiotic (Provet, Bayer) and were maintained on a circulating-water heating pad until recovery from anesthesia. Catheter lines were filled with 25–30% polyvinylpyrrolidone (PVP10, Sigma)-heparin (1,000 U/ml; Elkins-Sinn, NJ) and kept patent by a heparin (100 U/ml) flush every 3 days. All animals were allowed to reach their presurgery weights (~ 7 days) before study. In rats with an intracerebroventricular cannula, an ANG II test was performed as routinely established in our laboratory (e.g., Ref. 51) to confirm cannula placement.

Experimental procedures. Animals were divided into two groups, one receiving only intravenous catheters and the other, an intracerebroventricular cannula in addition to intravenous catheters. All animals were subjected to a 2-day procedure based on a model of HAAF in humans (18). All infusions were carried out using a programmable syringe pump (SP101i, World Precision Instruments).

On *day 1*, the intravenous group received either insulin (two 2-h infusions of $0.25 \text{ U} \cdot 100 \text{ g body wt}^{-1} \cdot \text{h}^{-1}$) or saline vehicle. In a separate study ($n = 4$), we determined that this insulin-infusion paradigm resulted in two discreet bouts of hypoglycemia (glucose fell from 109 ± 0.6 to 34 ± 3 mg/dl during the first infusion and from 148 ± 14 to 56 ± 10 mg/dl during the second infusion). On *day 2*, the animals received insulin ($0.5 \text{ U} \cdot 100 \text{ g body wt}^{-1} \cdot \text{h}^{-1}$) or saline vehicle intravenously over 120 min. Thus there were three treatment designations: Veh-Veh (intravenous vehicle on both days), Veh-Hypo (intravenous vehicle on *day 1* and intravenous insulin on *day 2*), and Hypo-Hypo (intravenous insulin on both days). Hypo-Hypo animals required supplemental glucose (in the infusate: $60 \text{ mg} \cdot 2.29 \text{ ml}^{-1} \cdot 120 \text{ min}^{-1}$) to match their plasma glucose levels to those of the Veh-Hypo rats. Blood samples (1.5 ml) were drawn every 30 min and immediately replaced with donor blood drawn from unstressed rats immediately before the experiment.

On *day 1*, the intracerebroventricular group received two 1-h infusions of either Cort (the predominant rat glucocorticoid; $36 \mu\text{g}/\text{infusion}$) or saline vehicle (93% saline, 7% propylene glycol) into the third ventricle. The dose of Cort was based on the observation that a similar dose of cortisol in humans (18) and, preliminarily, cortisone in rats (American Diabetes Association abstract, Ref. 47) produces HAAF-like effects when administered before hypoglycemic clamp. The rate of infusion was $0.25 \mu\text{l}/\text{min}$. This rate/volume has been found to be successful in effectively delivering agents intracerebroventricularly to the CNS through the cannulas used (49, 51). On *day 2*, the animals received either insulin ($0.5 \text{ U} \cdot 100 \text{ g body wt}^{-1} \cdot \text{h}^{-1}$) or physiological saline intravenously over 90 min. Thus there were three treatment designations: Veh-Veh (intracerebroventricular vehicle on *day 1* and intravenous vehicle on *day 2*), Veh-Hypo (intracerebroventricular vehicle on *day 1* and intravenous insulin on *day 2*), and Cort-Hypo (intracerebroventricular Cort on *day 1* and intravenous insulin on *day 2*). Blood samples (1.5 ml) were taken every 30 min and replaced with donor blood drawn from unstressed rats immediately before the experiment.

After the *day 2* infusion, the animals were given food and left in the experimental chambers for an additional 1.5 h. Each animal was then overdosed with pentobarbital sodium and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde. This time of perfusion was based on the work of Niimi et al. (41), examining the time course of *c-Fos* expression in the hypothalamus after insulin administration. Brains were removed, blocked into thirds (cut at approximately -0.26 mm and -8.8 mm from bregma), and placed in 4% paraformaldehyde at 4°C for 3 days. Brains were submerged in 30% sucrose followed by freezing at -80°C in

embedding media (Fisher) until sectioning at 40–50 μm . Tissue sections were stored at -20°C in cryoprotectant [30% sucrose-ethylene glycol (Sigma), 10% polyvinylpyrrolidone (Sigma) in PBS] until assay.

Plasma assays. Blood samples were obtained for the measurement of neuroendocrine responses and stored at -80°C until assayed. Blood for the catecholamine assays was collected on EGTA-glutathione (2.3:1.5 mg/ml; Sigma). Tubes for glucagon assays contained 10 μl of 1 M benzamide (Sigma) and 1 U heparin. Blood for glucose and Cort assays was collected on EDTA. A radioenzymatic method as described in Evans et al. (23) was used for determination of plasma epinephrine and norepinephrine (NE). A radioimmunoassay procedure was used for plasma Cort measurement as described in van Dijk et al. (58). Plasma glucose was measured spectrophotometrically using a glucose oxidase reaction. Glucagon was assayed by the Linco glucagon RIA kit (Linco Research). Post hoc measurements of ACTH were made using the Nichols Institute Diagnostics immunoradiometric assay kit (Nichols Institute Diagnostics, San Juan Capistrano, CA) on plasma samples that were pooled at each time point (0, 30, 60, or 90 min). For adequate volume, plasma from four to six rats was pooled. This yielded an n of two Veh-Veh, five Veh-Hypo, and three Hypo-Hypo sets of pooled plasma samples.

c-Fos immunohistochemistry and quantification. Brain sections were taken from -20°C and placed in 0.1 M PBS at room temperature. The tissue was washed for 45 min and then transferred to PBS-0.7% gelatin (Sigma)-0.25% Triton X-100 (Sigma)-3% goat serum (GIBCO), and incubated for 60 min. Primary antibody for c-Fos (Santa Cruz, sc-52) was diluted in PBS-3% goat serum at 1:2,000. Tissue was incubated in this antibody for 48 h at 4°C . The sections were then washed with PBS and placed in the secondary biotinylated antibody (Vector, BA-1000) diluted to 1:200 in PBS-3% goat serum for 60 min at room temperature. After PBS wash, the sections were developed by the avidin-biotin complex method using nickel-enhanced diaminobenzadine as the chromagen (Vector, PK-6100 and SK-4100). Sections were mounted on slides, placed under a coverslip, and numbered for counting (see below). Preincubation of the primary antibody with c-Fos protein fragments blocked staining completely using this protocol.

Images were captured on a Nikon microphot-FXA microscope with a SONY DXC-760MDR RGB camera and analyzed using MCID-M5 software (Imaging Research). Each set of sections was numbered according to a laboratory-defined standard set of sections. In this standard set, structures staining positive for c-Fos protein were given letters and outlined on atlas plates (46). An individual blind to the experimental manipulations carried out the counting of the outlined lettered areas on each plate for each animal. The number of c-Fos-positive nuclei was calculated for a structure by adding the counts across anterior-posterior (AP) plates for that structure. The AP plates used in the analysis encompassed all major structures from bregma -0.26 mm to -8.8 mm. Vehicle-infused controls were compared with insulin-treated hypoglycemic animals.

Statistical analysis. Data from the plasma assays were analyzed using repeated-measures ANOVA (RMANOVA), with time as the repeated measure and treatment (Veh-Veh, Veh-Hypo, Cort-Hypo, or Hypo-Hypo) as the between-groups factor. In the event of significant main effects or interactions, Fisher's protected least-significant difference post hoc tests were done to determine significant differences and t -tests were done where indicated. c-Fos counts from each region were analyzed by RMANOVA with brain region as the re-

peated measure and treatment (Veh-Veh, Veh-Hypo, Cort-Hypo, or Hypo-Hypo) as the between-groups factor. Significance for all tests was taken as $P \leq 0.05$. For the Veh-Hypo, Cort-Hypo, and Hypo-Hypo groups, data were excluded from the analyses if plasma glucose did not decrease to <50 mg/dl by 90 min after the start of insulin infusion on day 2. This resulted in the exclusion of three rats from the intracerebroventricular groups and five rats from the intravenous groups.

RESULTS

Counterregulatory response to hypoglycemia after previous bouts of hypoglycemia. Catecholamine and Cort levels were basal at 0 min, indicative of healthy, well-habituated (i.e., unstressed) rats (Figs. 1 and 2). With insulin infusion, glucose levels dropped to nearly 30 mg/dl by the end of insulin infusion for both Veh-Hypo and Hypo-Hypo groups. At all times, the glucose levels were significantly lower than at the start of the infusion and plasma glucose levels did not differ between the Veh-Hypo and Hypo-Hypo groups ($P > 0.1$ for Veh-Hypo vs. Hypo-Hypo for all time points). Glu-

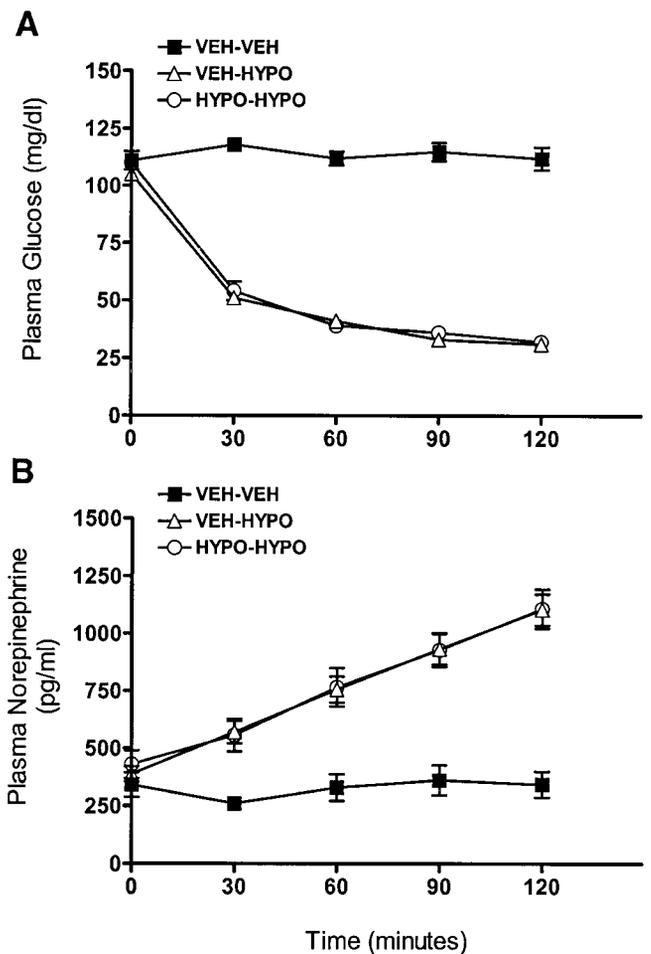


Fig. 1. A: insulin-induced decreases in plasma glucose levels were matched between the Veh-Hypo and Hypo-Hypo rats. B: norepinephrine increases in response to hypoglycemia were not altered by antecedent hypoglycemia. Hypo-Hypo, 2 bouts of hypoglycemia on day 1 followed by hypoglycemia on day 2. Veh-Hypo, 2 intravenous infusions of vehicle on day 1 followed by hypoglycemia on day 2. Veh-Veh, 2 intravenous infusions of vehicle on day 1 followed by intravenous infusion of vehicle on day 2. Error bars indicate \pm SE.

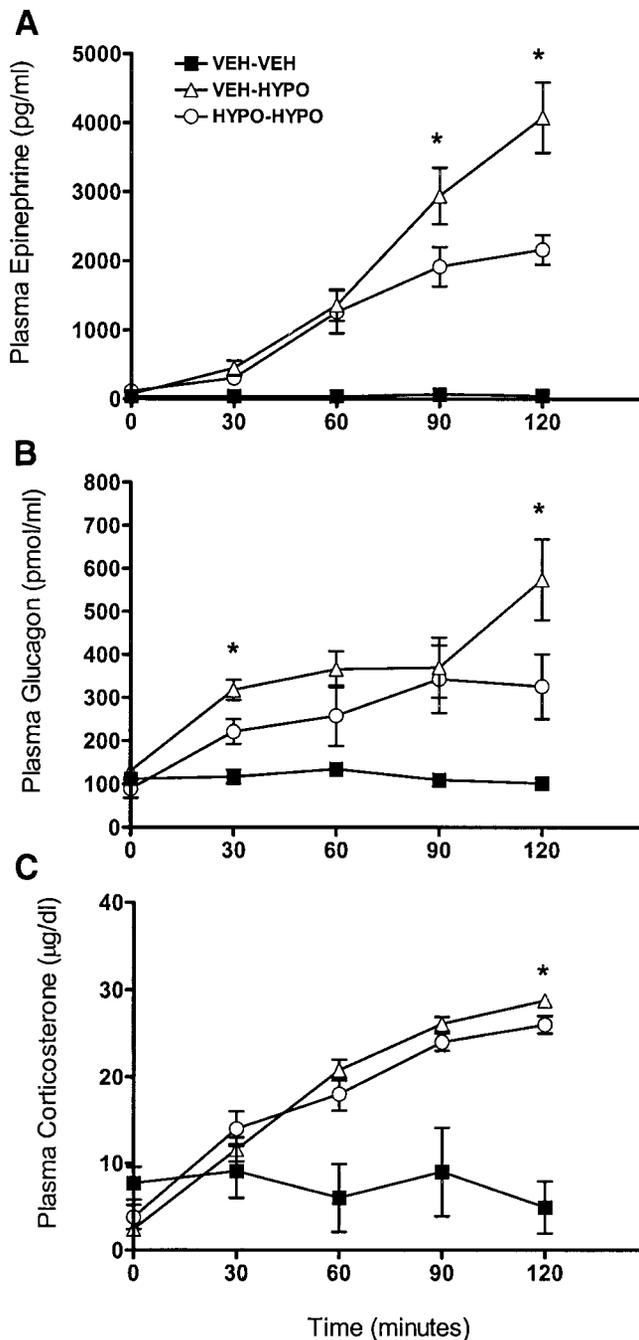


Fig. 2. *A*: increases in epinephrine were blunted by antecedent hypoglycemia at 90 and 120 min after the start of insulin infusion. Time and treatment interaction: $P < 0.0001$, $F_{8,148} = 6.3$; $*P < 0.05$, Veh-Hypo vs. Hypo-Hypo. *B*: hypoglycemia-induced increase in glucagon was blunted at 30 and 120 min by antecedent hypoglycemia. Time and treatment interaction: $P = 0.013$, $F_{8,116} = 2.6$; $*P < 0.05$, Veh-Hypo vs. Hypo-Hypo. *C*: antecedent hypoglycemia resulted in a reduction in the hypoglycemia-induced rise of corticosterone (Cort) on day 2 at 120 min. Time and treatment interaction: $P < 0.0001$, $F_{8,136} = 16$; $*P < 0.05$, Veh-Hypo vs. Hypo-Hypo. Error bars indicate \pm SE.

glucose levels did not change for the Veh-Veh control group [$P > 0.1$ for time 0 (t_0) vs. all other times], and, as a result, there was no neuroendocrine response as shown in Figs. 1 and 2.

Whether or not animals had been subjected to prior hypoglycemia on day 1, they mounted significant neuroendocrine counterregulatory responses to day 2 hypoglycemia. Both Veh-Hypo and Hypo-Hypo groups responded with increases in circulating NE, epinephrine, glucagon, and Cort. The magnitudes and time courses of these responses are shown in Figs. 1 and 2. However, two bouts of hypoglycemia on day 1 significantly blunted the counterregulatory response on day 2, such that increases of glucagon, epinephrine, and Cort were reduced (Fig. 2). Thus this experimental paradigm models HAAF in rodents.

Given that a likely mechanism of blunted Cort release is decreased ACTH release from the pituitary, ACTH levels were measured as described in METHODS. The results confirm that less ACTH is released with repeated hypoglycemia. Basal plasma ACTH levels did not differ among the three groups. In the Veh-Hypo and Hypo-Hypo groups ACTH rose to 350 ± 74 and 276 ± 101 pg/ml, respectively, at 90 min (main effect of time: $P < 0.0001$, $F_{3,21} = 12.6$). There was a significant interaction of time and treatment for the plasma ACTH changes from paired baseline ($P = 0.018$, $F_{4,14} = 4.3$). Post hoc analysis revealed that plasma ACTH was significantly different from paired t_0 levels for the Veh-Hypo group at t_{60} (vs. t_0 : $P = 0.02$) and t_{90} (vs. t_0 : $P = 0.009$). However, this was not the case for the Hypo-Hypo group, for which there was no significant elevation of ACTH vs. the paired t_0 baseline (t_{30} vs. t_0 : $P = 0.18$; t_{60} vs. t_0 : $P = 0.21$; t_{90} vs. t_0 : $P = 0.12$).

Counterregulatory response to hypoglycemia after previous exposure to Cort. The t_0 glucose and counterregulatory hormone levels were basal (Figs. 3 and 4), and plasma glucose decreases in response to day 2 insulin infusion were well matched between Veh-Hypo and Cort-Hypo groups (Fig. 3A, $P > 0.1$ for Veh-Hypo vs. Cort-Hypo at all time points). As in the intravenous groups, plasma glucose levels fell to nearly 30 mg/dl by the end of the insulin infusion in both the Veh-Hypo and Cort-Hypo groups. Glucose and counterregulatory hormone levels did not change for the Veh-Veh control group ($P > 0.1$ for t_0 vs. all other times; Figs. 3 and 4). In a pilot study, we determined that day 1 intracerebroventricular Cort infusion increased plasma Cort levels to a mean peak of 22.4 ± 2.8 μ g/dl ($n = 3$), comparable to the endogenous Cort peak after hypoglycemia of 28.8 ± 0.8 μ g/dl. However, intracerebroventricular Cort infusions on day 1 had no effect on the increases of plasma NE, epinephrine, glucagon, or Cort during day 2 hypoglycemia as documented in Figs. 3 and 4 ($P > 0.1$ for Veh-Hypo vs. Cort-Hypo at all time points for all measures).

CNS activation in response to hypoglycemia. Brain sections between -0.26 and -8.8 mm from bregma were assayed for c-Fos immunoreactivity (c-Fos-IR). On examination of the tissue from Veh-Hypo animals, a number of brain regions had c-Fos-positive nuclei. c-Fos-IR was quantified in these regions, and Veh-Hypo animals were compared with Veh-Veh animals to determine which brain regions were activated specifically in response to hypoglycemia. The levels of c-

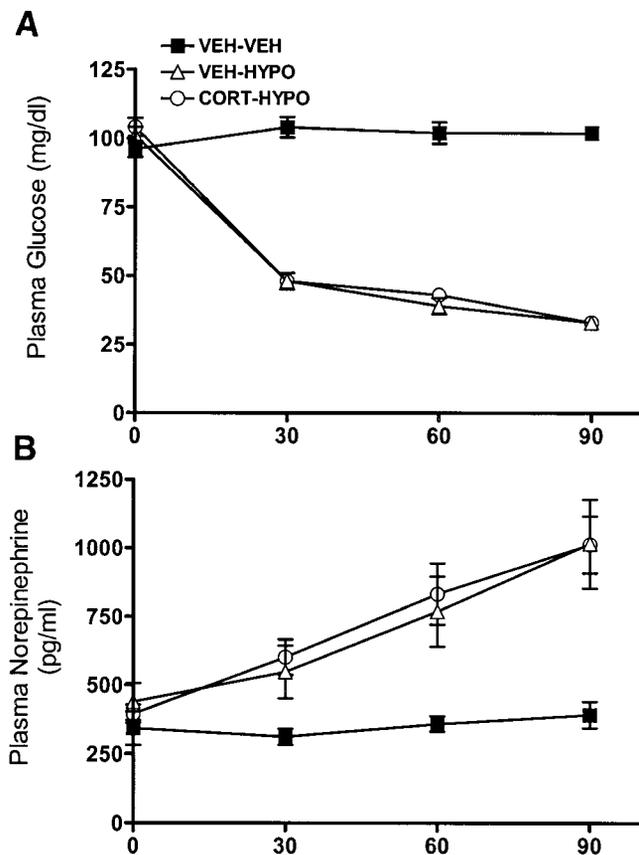


Fig. 3. A: insulin-induced decreases in plasma glucose levels were well matched between the intracerebroventricular Veh-Hypo and intracerebroventricular Cort-Hypo rats. B: norepinephrine increases in response to hypoglycemia were not altered by antecedent intracerebroventricular Cort. Cort-Hypo, 2 intracerebroventricular infusions of Cort on *day 1* followed by hypoglycemia on *day 2*. Veh-Hypo, 2 intracerebroventricular infusions of vehicle on *day 1* followed by hypoglycemia on *day 2*. Veh-Veh, 2 intracerebroventricular infusions of vehicle on *day 1* followed by intravenous infusion of vehicle on *day 2*. Error bars indicate \pm SE.

Fos-IR in all the regions examined are shown in Fig. 5. Photomicrographs of some of the brain regions consistently activated in response to hypoglycemia are shown in Fig. 6.

CNS activation in response to hypoglycemia after preexposure to hypoglycemia. Preexposure of the animals to hypoglycemia on *day 1* (Hypo-Hypo) resulted in a decrease of c-Fos-IR in response to *day 2* hypoglycemia in three brain regions (Fig. 7; region-treatment interaction: $P < 0.0001$, $F_{42,336} = 2.4$): the PVN, the arcuate nucleus (Arc), and the dorsomedial hypothalamus (DMH). c-Fos-IR in the Hypo-Hypo group did not differ from Veh-Veh controls in those three regions (see Fig. 7). c-Fos-IR in the PVN was decreased from 768 ± 122 cells in the Veh-Hypo condition to 370 ± 110 cells in the Hypo-Hypo condition. In the Arc, c-Fos-IR decreased from 212 ± 34 cells in the Veh-Hypo to 138 ± 25 cells in the Hypo-Hypo condition. The DMH showed a similar pattern, with a decrease from 523 ± 105 cells in the Veh-Hypo to 345 ± 74 cells in the Hypo-Hypo condition. Hypoglycemia-induced c-Fos-IR

in all other brain regions examined was not altered by antecedent hypoglycemia.

CNS activation in response to hypoglycemia after pretreatment with Cort. The brain regions that demonstrated decreased hypoglycemia-induced c-Fos-IR after *day 1* intracerebroventricular Cort (vs. intracerebroventricular vehicle) were the Arc, DMH, and the posterior PVN of the thalamus (ThPVP; Fig. 8; region-treatment interaction: $P < 0.0001$, $F_{20,140} = 5.1$). DMH c-Fos-IR decreased from 773 ± 145 cells in the Veh-Hypo group to 497 ± 169 cells in the Cort-Hypo group. c-Fos-IR in the Arc nucleus decreased from 589 ± 76

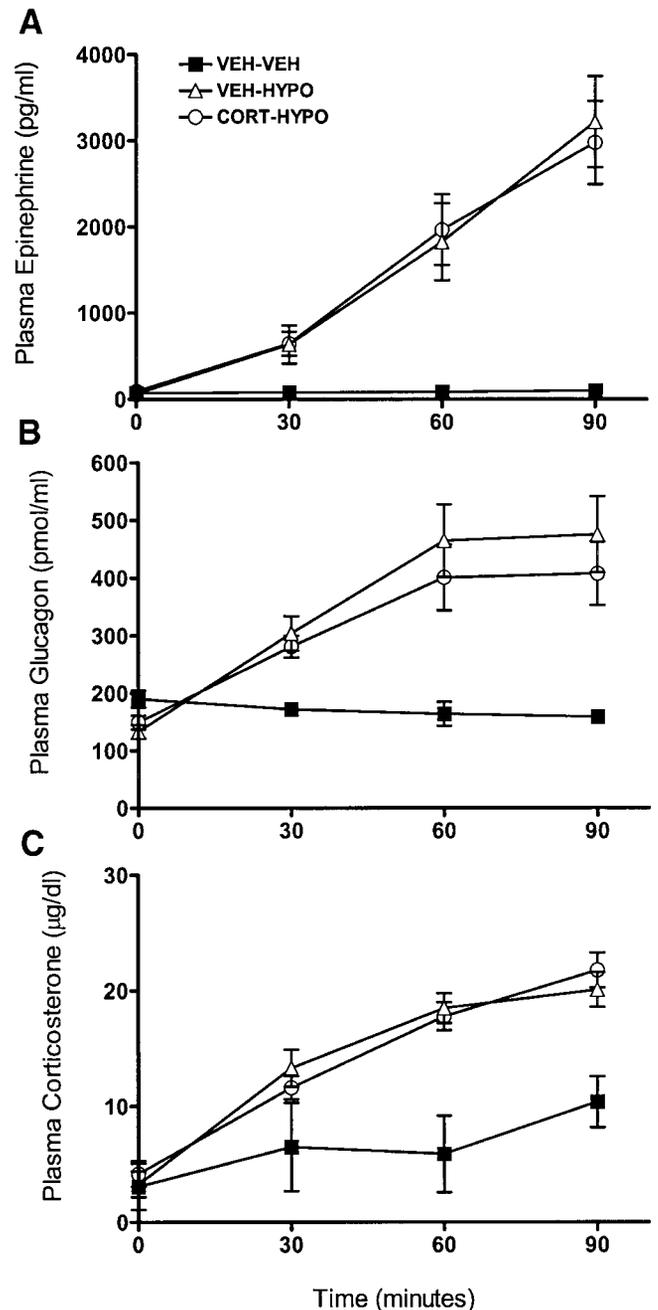


Fig. 4. Hypoglycemia-induced increases in epinephrine (A), glucagon (B), and Cort (C) were not blunted by antecedent intracerebroventricular infusions of Cort on *day 1*. Error bars indicate \pm SE.

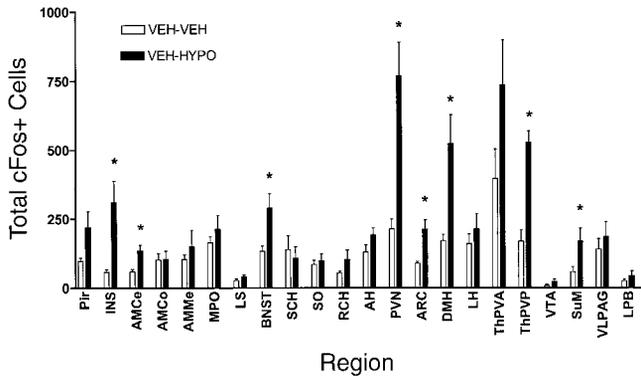


Fig. 5. Brain regions with significantly elevated c-Fos expression in response to hypoglycemia (Veh-Hypo). Error bars indicate +SE. Pir, piriform cortex; INS, insular cortex; AMCe, AMCo, and AMMe, central, cortical, and medial, respectively, nuclei of the amygdala; MPO, medial preoptic nucleus; LS, lateral septum; BNST, bed nucleus of the stria terminalis; SCH, suprachiasmatic nucleus; SO, supraoptic nucleus; RCH, retrochiasmatic nucleus; AH, anterior hypothalamic nucleus; PVN, paraventricular nucleus of the hypothalamus; ARC, arcuate nucleus; DMH, dorsomedial nucleus of the hypothalamus; LH, lateral hypothalamus; ThPVA and ThPVP, anterior and posterior, respectively, paraventricular nuclei of the thalamus; VTA, ventral tegmental nucleus; SuM, supramammillary nucleus; VLPAG, ventrolateral periaqueductal gray; LPB, lateral parabrachial nucleus. * $P < 0.05$.

cells in the Veh-Hypo group to 280 ± 85 cells in the Cort-Hypo group. The ThPVP showed decreased c-Fos-IR from 579 ± 119 cells in the Veh-Hypo group to 309 ± 76 cells in the Cort-Hypo group. Unlike antecedent hypoglycemia, antecedent intracerebroventricular Cort did not decrease hypoglycemia-induced c-Fos-IR in the PVN (Fig. 8). Hypoglycemia-induced c-Fos-IR in all other brain regions examined was not altered by antecedent Cort, nor was c-Fos-IR expression observed in any additional brain regions within the sections analyzed.

DISCUSSION

Neuroendocrine response to hypoglycemia. All animals made hypoglycemic in the current study exhibited robust counterregulatory neuroendocrine responses to hypoglycemia: activation of the hypothalamic-pituitary-adrenal (HPA) axis resulting in increased plasma ACTH and Cort, activation of the sympathetic nervous system resulting in NE release, epinephrine release from the adrenal medulla, and glucagon release from the pancreatic α -cells (Figs. 1–4). However, animals with prior exposure to hypoglycemia on *day 1* exhibited blunted glu-

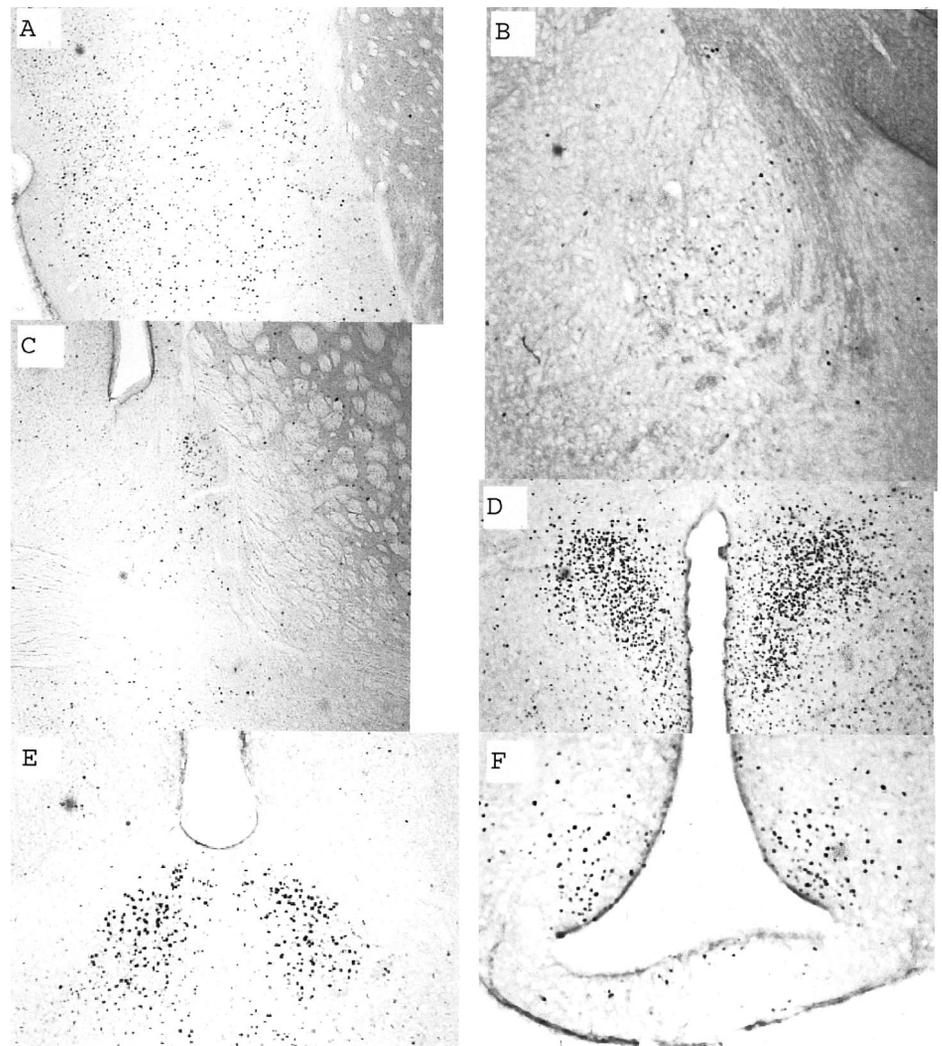


Fig. 6. Photomicrographs of some of the brain regions exhibiting increased c-Fos expression with hypoglycemia. A: INS; B: AMCe; C: BNST; D: PVN; E: ThPVP; F: ARC. Note the relative levels of c-Fos expression across regions.

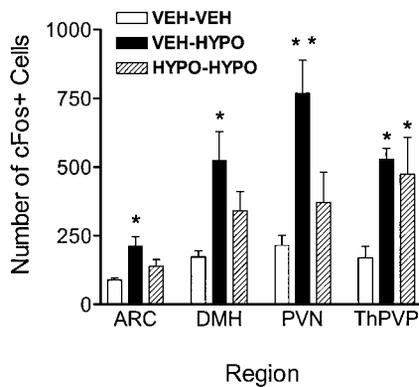


Fig. 7. Decreased c-Fos expression in 3 hypothalamic regions but not the thalamic ThPVP after antecedent hypoglycemia (Hypo-Hypo). Error bars indicate +SE. * $P < 0.05$, vs. Veh-Veh; ** $P < 0.05$, vs. Veh-Veh and Hypo-Hypo.

cagon, epinephrine, Cort, and ACTH responses (Figs. 1 and 2 and RESULTS). Thus this is a rodent model of the HAAF syndrome. It represents acute adaptation to a repeated metabolic stressor. This adaptation phenomenon has also been demonstrated with other stressors, such as restraint (50, 56).

Antecedent Cort and the neuroendocrine response to hypoglycemia. Prior exposure to intracerebroventricular Cort did not alter the hormonal response to hypoglycemia. This finding contrasts with work by Davis et al. (18) that demonstrated that prior exposure to systemic cortisol in humans blunts counterregulatory responses to hypoglycemia. However, there are some very important procedural differences between Davis's experiments and those presented here. Davis et al. used glucose clamp methodology to hold the plasma glucose levels at ~ 50 mg/dl over the entire session on *day 2*. In contrast, the average plasma glucose level in our animals continued to decrease over the 120-min *day 2* infusion, reaching ~ 30 mg/dl. Perhaps this stronger hypoglycemic stimulus is able to overcome glucocorticoid inhibition of the counterregulatory response. If so, then the more severe episodes of hypoglycemia in the current study may produce HAAF by different (or additional) mechanisms. Another obvious difference between the protocols is the route of Cort administration, intravenous vs. intracerebroventricular. However, Cort, being quite lipophilic, would be expected to diffuse through the blood-brain barrier and into the periphery after intracerebroventricular infusion. In fact, this is true; peripheral Cort levels rose significantly during the *day 1* intracerebroventricular infusion, with a mean peak of 22.4 ± 2.8 $\mu\text{g/dl}$. This is comparable to the peak after hypoglycemia of 28.8 ± 0.8 $\mu\text{g/dl}$. Therefore, our animals were exposed to high systemic and central Cort levels on *day 1*. Thus the current study demonstrates that the HAAF phenomenon at more severe levels of hypoglycemia is not likely to be solely the result of central glucocorticoid-HPA axis feedback mechanisms.

Brain activation after hypoglycemia. Hypoglycemia alone resulted in activation of brain regions that have been shown to be activated in response to other stres-

sors such as hypertonic saline (32, 50), swim stress (17), restraint (14, 63), and shock (11). These include the insular cortex; the amygdalar central nucleus (AMCe); the forebrain bed nucleus of the stria terminalis (BNST); thalamic ThPVP nucleus; the hypothalamic DMH, Arc, and PVN; and the supramammillary nucleus. Other studies have found similar patterns of hypothalamic activation with acute insulin-induced hypoglycemia (3, 39, 41). These studies did not examine areas outside the hypothalamus, and we are not aware of reports regarding activation of the extrahypothalamic areas listed above in response to insulin-induced hypoglycemia.

Interestingly, the ventromedial nucleus of the hypothalamus (VMH) and the hippocampus were not activated by hypoglycemia. Although compelling evidence exists for the role of the VMH in sensing plasma glucose levels (43, 61) and initiating counterregulatory responses (9, 10), the VMH was not activated by hypoglycemia. This was also noted by Niimi et al. (41). It is possible that the VMH is inhibited by hypoglycemia, in which case c-Fos expression would not be evident. It has been shown that NE input to the VMH is activated by hyperinsulinemia (15) as well as 2-deoxyglucose (2-DG)-induced glucoprivation (5). Beverly et al. (5) demonstrated that 2-DG-induced glucoprivation stimulates NE release in the VMH, which in turn causes the release of the inhibitory neurotransmitter GABA within the VMH. This suggests that VMH neurons might be inhibited when deprived of glucose. VMH neurons are indeed capable of expressing c-Fos, given the right stimulus, e.g., in response to cold stress (30, 38) or leptin administration (21). The hippocampus also was not activated in response to hypoglycemia but is activated and expresses immediate early gene products in response to other stressors such as restraint stress (20, 36), ether (22), and shock (11). To our knowledge, c-Fos is expressed in the hippocampus in response to hypoglycemia only in extreme circumstances, such as hypoglycemia-induced coma (28) or hypoglycemia-induced seizure (unpublished laboratory observations).

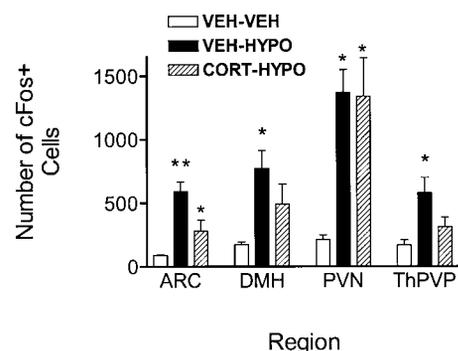


Fig. 8. Decreased c-Fos expression in 2 hypothalamic regions and the ThPVP but not in the hypothalamic PVN after antecedent corticosterone (Cort-Hypo). Error bars indicate +SE. * $P < 0.05$ vs. Veh-Veh; ** $P < 0.05$ vs. Veh-Veh and Cort-Hypo.

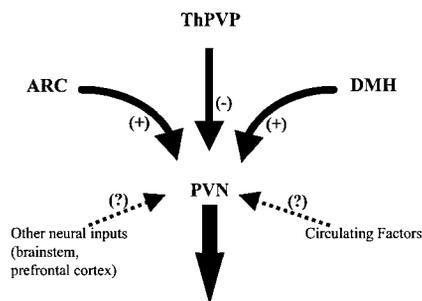
Antecedent hypoglycemia and hypoglycemia-induced brain activation. Two bouts of hypoglycemia on *day 1* resulted in a blunted neuroendocrine response to hypoglycemia on *day 2*. Quantification of c-Fos-IR demonstrated changes in brain activation as well. The activation of three structures that have been shown to be permissive or stimulatory for HPA/sympathetic activity (see discussion below), the PVN, Arc, and DMH, was blunted by prior bouts of hypoglycemia. Inhibition of potentially permissive/excitatory structures should lead to a blunted counterregulatory response to hypoglycemia, as was observed in our study. The PVN plays a pivotal role in the counterregulatory response to hypoglycemia, and this is suggested by the diminished counterregulatory response after a 52% decrease of PVN activation. The mechanism(s) of blunted PVN activation with repeated exposure to the same stressor might involve a decrease in activating input to the PVN, an increase in inhibitory input to the PVN, or both (36, 57). These inputs to the PVN are both neural afferents from other brain regions as well as direct influences of humoral factors on the activity of PVN neurons [e.g., glucocorticoids (13), but see discussion below]. Hypothalamic as well as limbic forebrain regions such as the BNST and AMCe could participate, because they modulate the activation of the PVN (24, 26, 35, 52, 59, 60). Additionally, noradrenergic and adrenergic brain stem regions that project to the PVN are known to release NE and epinephrine into the PVN in response to various stressors (44, 45). The activities of these afferent neurons could also be modulated by neural inputs and/or humoral influences (e.g., Cort).

Antecedent Cort and hypoglycemia-induced brain activation. Although the animals did not demonstrate altered counterregulatory responses to hypoglycemia with prior Cort treatment, they did demonstrate differences in CNS activation. When hypoglycemia on *day 2* was preceded by intracerebroventricular Cort infusion on *day 1*, the Arc and DMH of the hypothalamus and the ThPVP of the thalamus exhibited blunted activation. However, both the autonomic and HPA responses were normal (see above). This net lack of effect may be explained by experiments demonstrating the excitatory/permissive or inhibitory influence of these specific brain regions on the HPA axis. Pharmacological manipulation of the DMH reveals that the DMH can facilitate HPA and sympathetic responses (52). Experiments indicate that the Arc (4, 33, 34) can either potentiate or inhibit the HPA response. However, the evidence for Arc having a negative modulatory influence on the HPA axis derives chiefly from neonatally monosodium glutamate-lesioned rats (33, 34). This is a nonspecific lesion with an initial insult that causes damage to the Arc as well as all circumventricular organs, the retina, and the dentate gyrus of the hippocampus (2, 25, 37) and causes subsequent developmentally related deficits and alterations in physiology and behavior (8, 25, 37, 42, 54). Alternatively, the results of a recent study, in which the efferents of the Arc were cut in adult animals, suggest a positive or permissive role of the Arc with respect to HPA activity (4). Studies also indicate that the posterior part of the ThPVP inhibits HPA activity in repeatedly stressed animals (6, 7). Thus, in the case of intracerebroventric-

Condition 1: VEH-HYPO

Day 1: Vehicle

Day 2: Hypoglycemia (dynamic, ~30mg/dl)

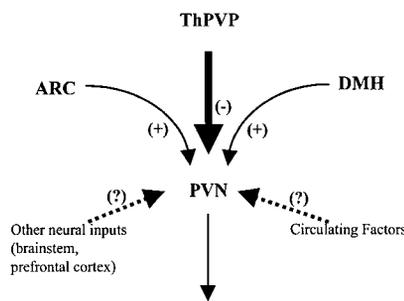


Neuroendocrine Counterregulatory Response

Condition 2: HYPO-HYPO

Day 1: Hypoglycemia (dynamic, ~30mg/dl)

Day 2: Hypoglycemia (dynamic, ~30mg/dl)

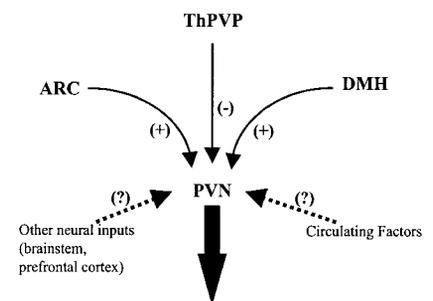


Neuroendocrine Counterregulatory Response

Condition 3: CORT-HYPO

Day 1: Corticosterone
(72µg ICV, ~23µg/dl plasma)

Day 2: Hypoglycemia (dynamic, ~30mg/dl)



Neuroendocrine Counterregulatory Response

Fig. 9. Summary of the results from the 3 experimental conditions, proposing a pivotal role for the PVN in orchestrating the counterregulatory response to hypoglycemia. In *condition 1*, hypoglycemia results in a strong neuroendocrine counterregulatory response, with strong PVN activation resulting from a balance of activating (ARC, DMH, other inputs) and inhibiting (ThPVP, other inputs) influences. In *condition 2*, measured activating influences are dampened by prior hypoglycemia, while the inhibiting influence of the ThPVP is not. Changes in other inputs may also occur. The net result is a decrease in the activation of the PVN and the neuroendocrine response. In *condition 3*, both measured activating and inhibiting influences are dampened by prior Cort infusion, changes in other inputs may also occur. The net result is no change in PVN activation or the neuroendocrine response. The unknowns in this model include the influences of circulating factors as well as neural inputs from regions not examined in the current study (e.g., brain stem, prefrontal cortex).

ular Cort, although potential HPA/sympathetic excitatory regions were inhibited (e.g., Arc, DMH), which presumably would lead to a blunted HPA/sympathetic response, a potential inhibitory region, the ThPVP, was also inhibited, which would disinhibit or increase HPA/sympathetic responses. The net result of such a combination of alterations in regional activation is no significant change in HPA/sympathetic reactivity. Of course this is a simplified portrait of very complex neuroanatomical circuitry: the inputs to the PVN probably do not sum in a simple algebraic fashion, and the timing of activation/inhibition of inputs to the PVN may be critical as well.

Figure 9 summarizes the results of the three experimental conditions: hypoglycemia (Veh-Hypo), hypoglycemia after preexposure to high Cort (Cort-Hypo), and hypoglycemia after preexposure to hypoglycemia (Hypo-Hypo). Consistent with the critical role of the PVN in the neuroendocrine response to hypoglycemia is the fact that even though the DMH and Arc hypothalamic nuclei were also inhibited by *day 1* intracerebroventricular Cort, the neuroendocrine response was blunted only in the Hypo-Hypo condition in which activation in the PVN was also inhibited. The results also suggest that the ThPVP may be important in regulating the neuroendocrine response. In the Cort-Hypo condition the ThPVP was inhibited, whereas in the Hypo-Hypo condition it was not. This is interesting in light of work by Bhatnagar and Dallman (6), which suggests a potential inhibitory role of ThPVP on HPA activity only under repeated (cold) stress conditions. Although it is not yet clear how this relates to repeated hypoglycemia, inhibitory influences of ThPVP on the HPA would be consistent with the lack of effect of antecedent intracerebroventricular Cort.

Perspectives

Although intensive insulin therapy has been shown to decrease the complications of hyperglycemia in diabetic patients, it also leads to an increase in the incidence of hypoglycemic episodes. Unfortunately, repeated hypoglycemia may induce HAAF. We have shown here that the neuroendocrine response and brain activation in response to severe, dynamic hypoglycemia are not blunted by prior increases in Cort in contrast to less severe, steady-state hypoglycemia (18). Thus HAAF may be induced by different mechanisms at different levels of hypoglycemia. We also demonstrate blunted activation in several hypothalamic regions in a rodent model of HAAF. These data suggest that decreased activation of the PVN may be necessary for the induction of HAAF during severe hypoglycemia.

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