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Effects of Repetitive Hypoglycemia on Neuroendocrine Response and Brain Tyrosine Hydroxylase Activity in the Rat

D.P. FIGLEWICZ[a,b,*], G. VAN DIJK[c], C.W. WILKINSON[d], P. GRONBECK[a], M. HIGGINS[a] and A. ZAVOSH[b]

[a]*Metabolism/Endocrinology (151), VA Puget Sound Health Care System, 1660 So. Columbian Way, Seattle, WA 98108, USA;* [b]*Department of Psychology, University of Washington Box 351525, Seattle, WA 98195-1525, USA;* [c]*Department of Animal Physiology, University of Groningen, Kerklaan 30, Haren 9750AA, The Netherlands;* [d]*GRECC, VA Puget Sound Health Care System, and Dept of Psychiatry Box 356560, Seattle, WA 98195-6560, USA*

Hypoglycemia-associated autonomic failure (HAAF) is a syndrome of acute adaptation to a metabolic stressor, in which neuroendocrine responses to repetitive hypoglycemic bouts are blunted. The CNS mechanisms that contribute to HAAF are unknown. In the present study, we modeled HAAF in the rat and measured the activity of tyrosine hydroxylase (TH) as an index of acute noradrenergic activation, to test the hypothesis that noradrenergic activation of the hypothalamus might be impaired. In association with a significant counter-regulatory response to a single bout of hypoglycemia (elevated corticosterone, catecholamines, and glucagon), TH activity was elevated overall in brainstem NE cell body areas and hypothalamus. With multiple hypoglycemic episodes in a 24 h period, the counter-regulatory response was blunted, and hypothalamic TH activity was comparable to that of saline-infused controls. In a similar paradigm, multiple bouts of CNS neuroglucopenia did not blunt the hyperglycemic or corticosterone responses, and were required for elevation of TH activity. This alternate response pattern suggests that insulin-induced hypoglycemia and cerebral neuroglucopenia represent somewhat different metabolic stressors at the CNS.

Keywords: Catecholamines; Glucocorticoids; Neuroglucopenia; Stress

INTRODUCTION

Repeated exposure to both physical and psychological stressors can lead to stress adaptation, in which the neuroendocrine response to the stressor is blunted (Dallman, 1993; Garcia *et al.*, 2000). One example of adaptation to a physical stressor is the phenomenon of hypoglycemia-associated autonomic failure, (HAAF) (Heller and Cryer, 1991). One component of HAAF is an impairment of the normal counter-regulatory response to hypoglycemia, including deficient output of adrenal epinephrine, glucagon from the endocrine pancreas, and adrenal glucocorticoids. This syndrome, although characterized extensively in a clinical context in insulin-requiring diabetic patients (Tamborlane and Amiel, 1992; Cryer, 1993; Veneman *et al.*, 1993; Ohkubo *et al.*, 1995), can be modeled experimentally in normal human subjects. It has been shown to occur when one or two bouts of hypoglycemia are induced within 24 h of a final (i.e. second or third) bout of hypoglycemia (Heller and Cryer, 1991; Davis *et al.*, 1996). Thus, although diabetic

complications may contribute to impaired counter-regulatory responses in patients with chronic diabetes (e.g. Bax *et al.*, 1995; Boyle *et al.*, 1995), it is clear that the onset of the syndrome is due to very acute changes in autonomic regulation of neuroendocrine responses in individuals who have no history of abnormal autonomic or endocrine pancreatic function. The mechanisms underlying this acute dysregulation have not been determined.

Recent studies have suggested that a prior history of elevation of plasma glucocorticoid levels can play a causative or strongly contributory role in the manifestation of acute HAAF (Davis *et al.*, 1996). In a two-day study design, they infused non-diabetic human subjects with cortisol on the first day, matching the plasma levels normally achieved during hypoglycemia; on the second day, patients were infused with insulin to achieve hypoglycemia. Another group received hypoglycemic insulin infusions on the first day. Davis and colleagues observed that counter-regulatory responses in the subjects who received cortisol on the first day matched or

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approximated the blunted responses observed in the group who were hypoglycemic on both the first and second days of the study. Thus, adrenal glucocorticoids appear to have an acute action to blunt activation of a normal neuroendocrine counter-regulatory response. Studies from Pacak and colleagues have shown that the adrenal glucocorticoids can act at the hypothalamus to blunt the activity of the hypothalamic-pituitary-adrenal (HPA) axis (Pacak *et al.*, 1995a), and one mechanism for this action is the inhibition of release of norepinephrine (NE) at the paraventricular nucleus of the hypothalamus (PVN). NE release and activity at the PVN are correlated with an adrenocorticotrophin (ACTH) response to a variety of stressors although the quantitative relationship between NE release and plasma ACTH is stressor-specific (Pacak *et al.*, 1995b). It is not known, however, whether this type of feedback impairment of NE activity can occur on an acute basis.

In the present study, we measured brain tyrosine hydroxylase (TH) in a rat model of acute HAAF. On a very acute basis (minutes to hours), activity of this rate-limiting synthetic enzyme for NE can be modulated at the nerve terminals in response to activation of the nerves (Goldstein, 1995; Kumer and Vrana, 1996). Here, we measured TH activity within the NE cell regions of the brainstem (A1, A2, and locus coeruleus [LC]) as well as within the hypothalamus to determine whether TH activity—presumably reflecting acute activation of NE neurons—is elevated in association with hypoglycemia; and to determine whether any activation is decreased in association with acute HAAF. Such a finding would target brainstem NE neurons as being a vulnerable neuroanatomical substrate within the brain for a hypoglycemic insult. Because lack of available glucose represents a component of insulin-induced hypoglycemia, we administered the neuroglucopenic agent, 5-thioglucose (5TG) into the cerebral ventricles to generate local CNS neuroglucopenia, and measured TH activity in the same brain areas, to determine whether this might be a factor influencing noradrenergic activation in association with recurrent hypoglycemia.

MATERIALS AND METHODS

Subjects

Male Wistar rats (Simonson, CA; 250–400 gm) were studied. Rats were maintained on a 12–12 h light-dark schedule (lights on at 7 a.m., off at 7 p.m.), with *ad libitum* access to food and water. All procedures were approved by the Institutional Animal Care and Use Committee of the VA Puget Sound Health Care System.

Surgery

Rats in the hypoglycemia study received bilateral intravenous (IV) silastic catheters according to the method of Scheurink *et al.* (1990) under ketamine/xylazine (60 mg/kg ketamine, 7.8 mg/kg xylazine) anesthesia 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 threaded to the heart. Catheters were tunneled subcutaneously and exteriorized through a midline incision in the scalp, and the neck incision was then sutured. Rats in the 5TG study received an intracerebroventricular (ICV) cannula: each rat was placed in a Kopf stereotaxic frame and a 22 gauge stainless steel guide cannula (Plastics One Inc., Roanoke, Virginia) was implanted, aimed at the lateral cerebral ventricle using the stereotaxic coordinates -1.0 mm AP from bregma, ± 1.6 mm ML, -4.0 mm DV from the dura mater. Cannula patency was verified functionally prior to the experiment by a robust 30 min drinking response following an injection of 10 ng angiotensin II (Sigma). IV catheters were held in place by acrylic cement to four skull screws. Animals received subcutaneous 1 ml Lactated Ringers solution (Baxter) and 0.2 ml Batryl antibiotic (Provet, Bayer), and were maintained on a circulating-water heating pad until they recovered from anesthesia. Catheter lines were filled with 25–30% polyvinylpyrrolidone (PVP10, Sigma, St. Louis MO)/heparin (1000 units/ml; Elkins-Sinn, NJ), and kept patent by a heparin flush (0.2 ml;100 units/ml) every three days. All animals regained their presurgery body weights prior to the experimental procedure (approximately seven days).

Hypoglycemia Procedure

All rats were studied in a two-day procedure. The protocol is shown in Fig. 1. All infusions were carried out by a programmable syringe pump (SP101i, World Precision Instruments, FL). No food was available to the rats during the infusion periods. On Day 1, rats
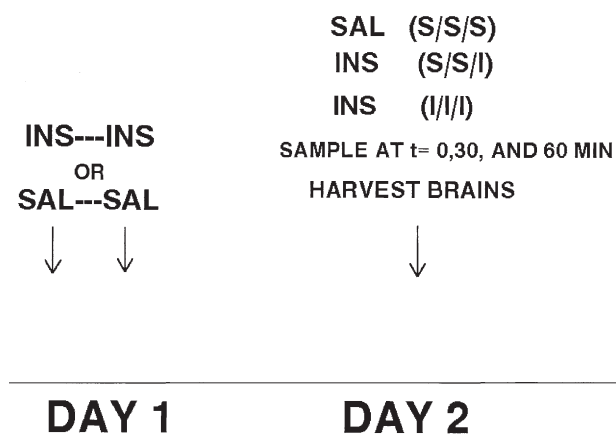

FIGURE 1 Flow chart for two-day hypoglycemia protocol. INS–INS = two insulin infusions; SAL–SAL = two saline infusions on Day 1. S/S/S = Day 1 saline infusions; Day 2 saline infusion. S/S/I = Day 1 saline infusions; Day 2 insulin infusion. I/I/I = Day 1 insulin infusions; Day 2 insulin infusion.

received two 1 h infusions of either sterile physiological saline, or 0.675 U/100 g insulin IV (Lilly Humulin, 100 U/ml stock; infusion rate, 1.145 ml/h). A 60 min interval separated the two infusions. All rats had chow pellets in the experimental chambers during this hour, and *ad libitum* food was available in the home cage, following the second infusion period. On Day 2, rats received a third infusion; blood samples (1.0 ml) were taken at 0, 30 and 60 min and replaced with donor blood drawn from unstressed rats immediately before the experiment. Rats treated with insulin on Day 1 received a third insulin infusion (I/I/I). Rats infused with saline on Day 1 received either a third saline infusion (S/S/S) or an insulin infusion (S/S/I). Immediately following the 60 min sample, rats were removed to a separate room and euthanized by decapitation. Brains were rapidly dissected (($\leq$2 min) and brain areas for measurement of TH activity were quick-frozen on dry ice and stored at $-80°C$ until assay.

5-Thioglucose (5TG) Procedure

On Day 1 of the two-day protocol, food was removed at 0600 h. Rats were weight-matched and randomly assigned to one of three injection groups. Group 1 (controls: LG/LG/LG) received two ICV injections of the non-metabolizable glucose isomer L-glucose (1.08 μmol in 1.5 μl; Aldrich Chemical Co.) 4 h apart (Day 1), and a third L-glucose injection 24 h after the first injection (Day 2). Group 2 (LG/LG/5TG) received two injections of L-glucose on Day 1, as described above, and an injection of 5TG (1.08 μmol in 1.5 μl; Sigma) on Day 2. Group 3 (5TG/5TG/5TG) received two injections of 5TG on Day 1, and a third injection of 5TG on Day 2. The 5TG dose was based upon previous studies from Ritter and colleagues (Slusser and Ritter, 1980). 60 min after the ICV injection on Day 2, rats were euthanized by decapitation and trunk blood was collected for measurement of plasma glucose and corticosterone (because these rats did not have chronic indwelling iv catheters, catecholamines were not measured). Brains were rapidly dissected and brain areas were quick-frozen on dry ice and stored at $-80°C$, for subsequent assay of TH activity.

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 (90 mg/ml:60 mg/ml; Sigma). Tubes for glucagon assays contained 1 M benzamidine (Sigma) and 1 U heparin. Blood for glucose and corticosterone was collected on EDTA. A radioenzymatic method as described in Evans *et al.* (1978) was used for determination of plasma epinephrine and norepinephrine (intra-assay variation 6%; interassay variation 12%). A radioimmunoassay procedure (intra-assay variation 7%;

interassay variation 15%) was used for plasma corticosterone measurement as described in VanDijk *et al.* (1997). Glucose was determined using a modified Trinder's reaction assay (intra-assay variation 1%; interassay variation 1.5%) and spectrophotometric quantitation with a QuadFlex instrument (Titertek). Glucagon was assayed by radioimmunoassay (Ensinck *et al.* (1972); intra-assay variation 7%, interassay variation 10%).

TH Assay

TH activity was measured in freshly prepared homogenates of the frozen tissue samples, utilizing the assay procedure of Reinhard *et al.* (1986). The biochemical basis for the assay is the generation and measurement of ^3H_2O produced by reacting ^3H-tyrosine with the enzyme present in tissue homogenates, in the presence of exogenous excess cofactor (BH$_4$). Brain regions were rapidly dissected (approximately 1 min) and immediately frozen on dry ice. Entire hypothalamus was dissected; and the brainstem was subdissected into portions that included the entire LC; Al; or A2 noradrenergic cell groups. Brainstem dissections containing the Al cell group also contained the C1 adrenergic cell group, because of the anatomical proximity of these two nuclei. Brainstem dissections were based on Levin and Sullivan (1979) and atlas plates 56, 61, 64, 74, and 75 (Paxinos and Watson, 1986). Hypothalamus dissection was based on visual landmarks as we have previously published (e.g. Baskin *et al.*, 1985). Frozen tissues were homogenized with a hand-held glass homogenizer in 500 μl of ice-cold 0.1% Triton/PBS/50 mM sodium pyrophosphate. A 70 μl aliquot of the unknown was added to 20 μl of a solution containing 1 M KH$_2$PO$_4$; catalase (3000 U/sample tube); ^3H-tyrosine at 1 μCi/sample tube (NET 127/New England Nuclear) dried under nitrogen and resuspended in 1 mM L-tyrosine hydrochloride (T-2006, Sigma) at a final specific activity of 2.5 nmol/μCi; 1 M DTT; and 1 M sodium pyrophosphate, in distilled deionized water. The reaction was started with the addition of 10 μl freshly constituted BH$_4$ (158 mg/ml) to each tube for a final volume of 100 μl. After vortexing, tubes were incubated for 30 min at 37°C. The reaction was stopped by the addition of 1 ml of 7.5% charcoal (G-60 Norit). After a rapid vortex, each tube was centrifuged 500g, 10 min. A measured amount of (2 ml) Optifluor were added to 500 μl of the clear supernatant, and cpm were counted in a beta counter. As a positive control between assays, an aliquot of a striatal homogenate pool was included in each assay. Protein was measured for each sample with a 1:12 dilution with BCA Protein Assay Reagent (Pierce) and a microplate spectrophotometer (Dynatech, MR 500). Efficiency of recovery after charcoal extraction was estimated by the cpm from samples of 100 μl ^3H_2O that had been centrifuged with or without charcoal addition. Activity was calculated according to the formula: (cpm [sample] $-$ cpm [blank]) $\times$ 1000/((cpm added/2.5) $\times$ 30 $\times$ mg protein $\times$ fraction recovered). As shown in Table I,

TABLE I   Assay of tyrosine hydroxylase activity

Tissue	*In vitro* treatment	μg Protein	Activity (pmol/mg/min)
Hypothalamus	–	8	26.6
Hypothalamus	Vehicle	21	24.9
Hypothalamus	1 mM dbcAMP (60 min)	21	75.8
Adrenal gland	–	35	182
Adrenal gland	–	70	186
Liver	–	26	0.5

assay sensitivity permits measurements of as little as 8 μg of tissue protein (#1) and demonstrates linearity with increased protein concentration over the range of 20–60 μg for the hypothalamus; the assay also demonstrates differences in activity which reflect the relative density of catecholaminergic density of the tissue being assayed (compare #1 vs. #4 in Table I). To demonstrate the potential for increased TH activity within our hypothalamic homogenate preparation, a homogenate was split and a portion pre-incubated with the cAMP analog, dibutyryl

cAMP (dbcAMP; Sigma, St. Louis MO) *in vitro* for 1 h. This treatment resulted in a three-fold increase of TH activity (compare #3 vs. #2 in Table I).

Statistical Analysis

Data from the TH assays were analyzed using repeated measures ANOVA (RMANOVA) with brain region as the repeated measure and treatment as the between factor. For all of the plasma parameters, $t.0$ baseline data were compared by t-test (S/S/S vs. S/S/I; S/S/S vs. I/I/I; S/S/I vs. I/I/I). Within-subjects changes from paired $t.0$ baseline values were calculated and Kruskal–Wallis analysis was carried out to determine whether within-subjects responses at $t.$ 30 or 60 min were statistically significant. Additionally, these response data ($t.$ 30 and $t.$ 60 time points) were compared between groups by ANOVA, to determine whether the changes in any plasma parameter in response to insulin infusion (S/S/I or I/I/I) was greater than the change which occurred in response to a control, saline
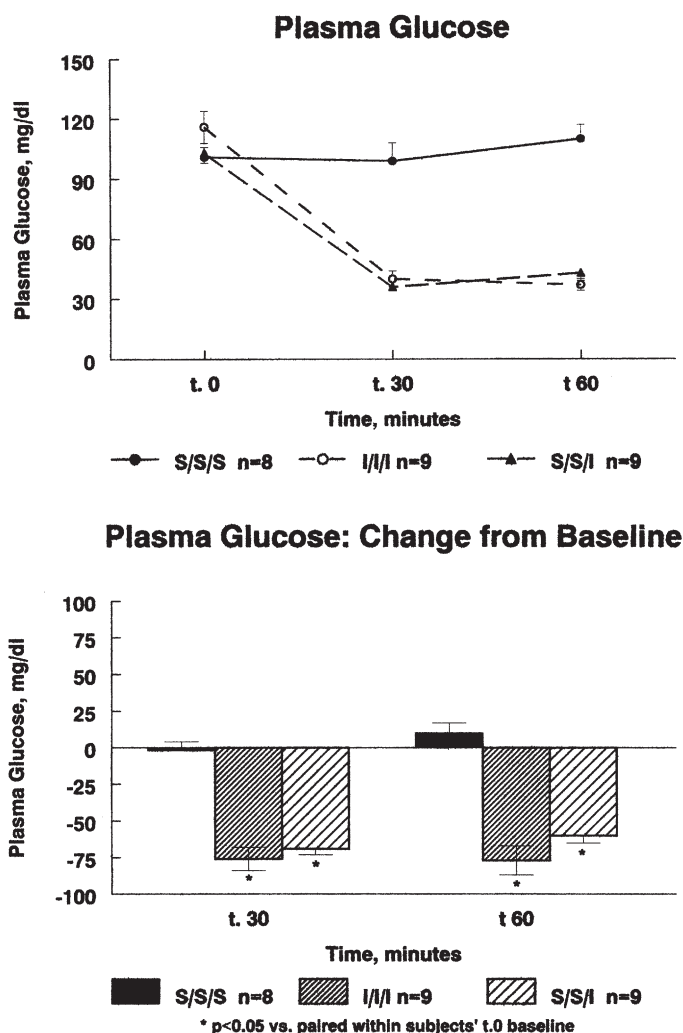

FIGURE 2   Plasma glucose levels (mg/dl) in response to Day 2 saline or insulin infusions. Upper: absolute plasma glucose values. Lower: plasma glucose response as within-subjects change (at *t.* 30 and *t.* 60) vs. paired t.0 baseline. *$p < 0.05$, significant change from *t.*0 baseline.

Plasma Epinephrine

Plasma Corticosterone

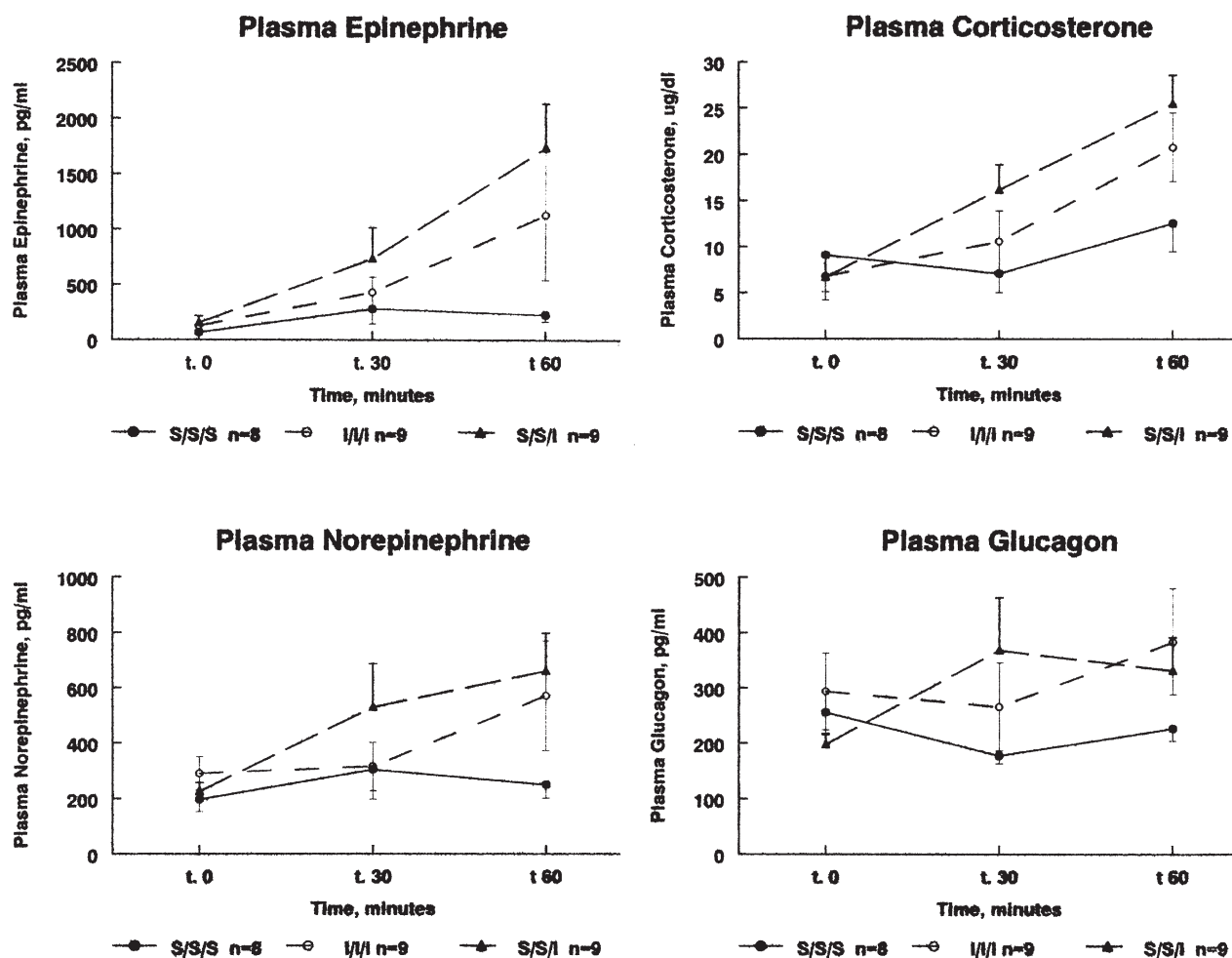## Plasma Norepinephrine

Plasma Glucagon

FIGURE 3   Absolute values for plasma glucose, corticosterone, epinephrine, glucagon, and norepinephrine on Day 2 of the two-day hypoglycemia protocol.

infusion (S/S/S). Statistical significance was defined as $p \leq 0.05$.

RESULTS

Neuroendocrine Counter-regulatory Responses

As shown in Figs. 2 and 3, Day 1 infusion of insulin had no effect on Day 2 baseline ($t.0$) values of glucose or neuroendocrine variables, compared with those of rats infused with saline on Day 1. Baseline ($t.0$) plasma glucose concentrations did not differ among the three groups. On Day 2, saline infusion in the S/S/S group of rats did not significantly alter plasma glucose levels ($p = 0.54$ overall; Fig. 2). Epinephrine ($p = 0.15$), glucagon ($p = 0.17$), corticosterone ($p = 0.54$), and norepinephrine ($p = 0.38$) levels also did not change over the 60 min infusion period, relative to each animal's $t.0$ baseline (Figs. 3 and 4). In contrast, plasma glucose levels in the S/S/I group fell significantly in response to the 60 min insulin infusion (Fig. 2; $p < 0.0001$ overall). A neuroendocrine counter-regulatory response to the

hypoglycemia was observed in the S/S/I group relative to the S/S/S controls for glucagon ($p = 0.03$); epinephrine ($p = 0.01$); norepinephrine ($p = 0.03$); and a trend for corticosterone ($p = 0.08$) (overall RMANOVA, Fig. 3). This neuroendocrine response was confirmed by within-subjects comparisons of change from individual $t.0$ baseline levels (Fig. 4), with overall significant elevations of corticosterone ($p = 0.003$); epinephrine ($p = 0.005$); glucagon ($p = 0.04$); and norepinephrine ($p = 0.02$). As shown in Fig. 4, within-subjects epinephrine, norepinephrine, and corticosterone responses were significant at both $t.30$ and $t.60$; and the within-subjects glucagon response was significant at $t.60$.

Rats which had been infused with insulin on Day 1 (I/I/I) also had a highly significant decrease in plasma glucose relative to the paired baseline (Fig. 2; $p < 0.0001$ overall) across the 60 min insulin infusion period, on Day 2. However, unlike the S/S/I group, there was no significant neuroendocrine response relative to the S/S/S control group (Fig. 3). Additionally, there was no significant within-subjects neuroendocrine response to the hypoglycemia (epinephrine, $p = 0.08$; glucagon, $p = 0.53$; norepinephrine, $p = 0.19$) with the exception
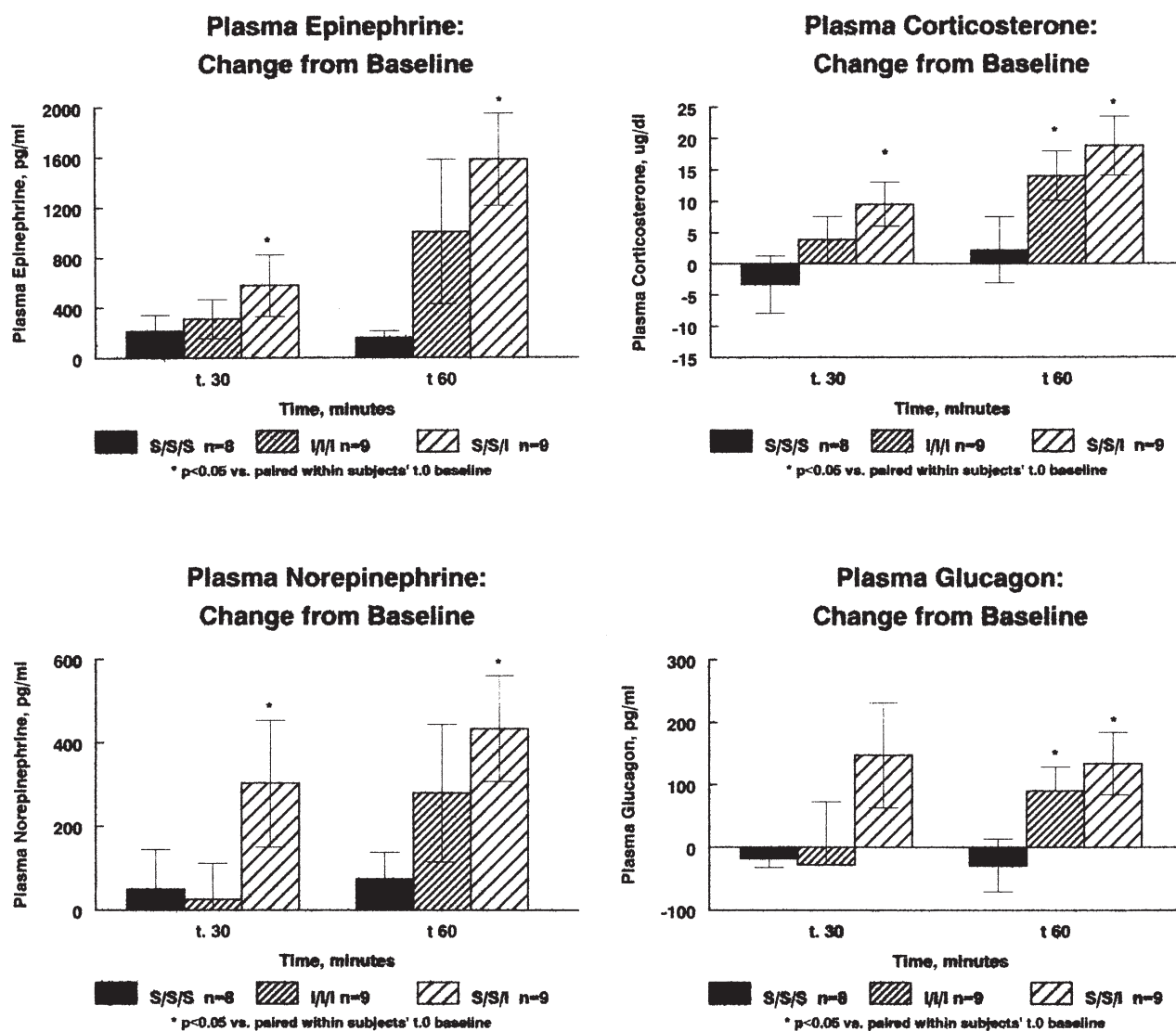
FIGURE 4 Effect of Day 2 saline or insulin infusion on within-subjects plasma corticosterone, epinephrine, glucagon and norepinephrine responses.

of corticosterone ($p = 0.03$) (Fig. 4). This pattern of blunted neuroendocrine responses, particularly the blunted glucagon and epinephrine responses, represents the HAAF phenomenon.

Between-group comparisons were also carried out for the $t.30$ and $t.60$ responses of S/S/S vs. S/S/I, and S/S/S vs. I/I/I, groups. For S/S/S vs. S/S/I comparisons, significant overall treatment effects were observed for plasma glucose ($p < 0.0001$); glucagon ($p = 0.01$); epinephrine ($p = 0.0035$); and norepinephrine ($p = 0.01$); with a non-significant trend for corticosterone ($p = 0.12$), perhaps due to some variability in the corticosterone levels within the S/S/S group. Comparisons between S/S/S and I/I/I groups revealed a significant difference in plasma glucose ($p = 0.01$). However, there was no significant treatment effect on any of the neuroendocrine parameters measured: glucagon ($p = 0.36$); epinephrine ($p = 0.17$); norepinephrine ($p = 0.29$) or corticosterone ($p = 0.36$). Because neuroendocrine values of the I/I/I group were

intermediate between those of the S/S/S controls, and the S/S/I group, they also did not differ significantly from those of the S/S/I group. Plasma glucose levels showed a trend towards a greater decrease in the I/I/I vs. S/S/I groups (Fig. 2) but this was not statistically significant.

TH Activity

Table II lists the absolute TH activity (pmol/min/mg) for the three treatment groups. For ease of comparison between treatment groups, across the four brain areas (whose basal TH activity levels vary), TH data for the S/S/I and I/I/I groups are shown in Fig. 5 (upper) normalized to the corresponding S/S/S brain area, which has been set to 100%. In response to a single bout of hypoglycemia, there was a significant overall effect of hypoglycemia to increase TH activity across all four brain regions measured (S/S/S vs. S/S/I: $p = 0.05$). Overall TH activity in the I/I/I group did not differ from the S/S/S
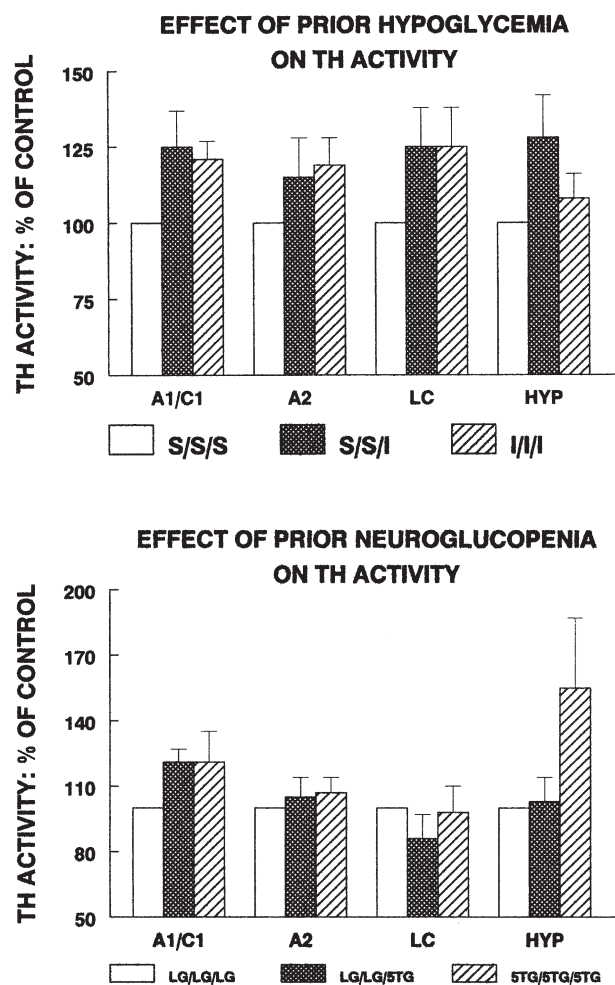
FIGURE 5 TH activity in A1, A2, LC brainstem areas and whole hypothalamus, normalized to regional TH activity in controls (set to 100%). Upper: TH activity in hypoglycemic rats (S/S/I [$n = 9$], cross-hatched bars and I/I/I [$n = 9$], hatched bars). Lower: TH activity in neuroglucopenic rats (LG/LG/5TG [$n = 6$], cross-hatched bars; and 5TG/5TG/5TG [$n = 6$], hatched bars).

TABLE II TH activity (pmol/min/mg) in response to hypoglycemia or neuroglucopenia

	Mean	SEM		Mean	SEM
Hypothalamus					
S/S/S	20.0	2.3	LG/LG/LG	11.8	0.9
I/I/I	21.6	1.6	5TG/5TG/5TG	18.4	3.5
S/S/I	25.5	2.6	LG/LG/5TG	12.2	1.4
Locus Coeruleus					
S/S/S	5.5	1	LG/LG/LG	6.7	0.7
I/I/I	6.9	0.7	5TG/5TG/5TG	6.5	0.7
S/S/I	6.9	0.7	LG/LG/5TG	4.5	0.7
A1/C1					
S/S/S	5.2	0.4	LG/LG/LG	4.4	0.6
I/I/I	6.3	0.3	5TG/5TG/5TG	5.3	0.6
S/S/I	6.5	0.6	LG/LG/5TG	5.3	0.2
A2					
S/S/S	4.7	0.5	LG/LG/LG	3.5	0.3
I/I/I	5.6	0.4	5TG/5TG/5TG	3.8	0.2
S/S/I	5.4	0.6	LG/LG/5TG	3.7	0.3

S/S/S, Day 1 saline infusions, Day 2 saline infusion $n = 8$; S/S/I, Day 1 saline infusions, Day 2 insulin infusion $n = 9$; I/I/I, Day 1 insulin infusions, Day 2 insulin infusion $n = 9$.
LG/LG/LG, Day 1 L-glucose infusions, Day 2 L-glucose infusion, $n = 6$; LG/LG/5TG, Day 1 L-glucose infusions, Day 2 5-thioglucose infusion $n = 6$; 5TG/5TG/5TG, Day 1 5-thioglucose infusions, Day 2 5-thioglucose infusion $n = 6$.

group. Increases of TH activity in all of the brain areas were modest (15–25% above S/S/S control levels). The only brain region showing a differential effect of one vs. multiple bouts of hypoglycemia was the hypothalamus, where TH activity levels of the I/I/I group were equivalent to those of controls (108 ± 7%), in contrast to the S/S/I group, where hypothalamic TH values were 128 ± 14% of the controls.

5TG Neuroglucopenia

In separate groups of rats, 5TG or control L-glucose solutions were injected into the lateral ventricles to achieve CNS neuroglucopenia. Success of the injections was observed behaviorally as a development of lethargy in the succeeding hour post-injection. Robust increases of plasma glucose and corticosterone resulted from the Day 2 5TG injections (Fig. 6): Plasma glucose and corticosterone were significantly elevated by both one ([LG/LG/5TG) glucose = 239 ± 36 mg/dl, $p = 0.026$

vs. LG/LG/LG; corticosterone = 35.2 ± 6.0 µg/dl, $p = 0.003$ vs. LG/LG/LG) or three ([5TG/5TG/5TG] glucose = 278 ± 22 mg/dl, $p = 2.67 \times 10^{-7}$ vs. LG/LG/LG; corticosterone = 46.1 ± 2.0 µg/dl, $p = 0.0002$ vs. LG/LG/LG) injections of 5TG compared with the LG/LG/LG controls (glucose = 136 ± 4 mg/dl; corticosterone = 8.4 ± 2.0 µg/dl). Glucose and corticosterone levels did not differ between rats receiving one vs. three bouts of CNS neuroglucopenia. The pattern of TH activity differed from that observed in rats that were subject to hypoglycemia (Fig. 5, lower). Rats experiencing a single bout of neuroglucopenia did not demonstrate activation of TH across the four brain areas assessed (LG/LG/LG vs. LG/LG/5TG, $p = 0.92$). However, with the third bout of neuroglucopenia, there was a significant increase of TH activity across brain areas (LG/LG/LG vs. 5TG/5TG/5TG, $p = 0.05$), that may be ascribed to increased TH activity in the A1 region and the hypothalamus (which did not show increased TH activity in response to the single injection of 5TG).

DISCUSSION

In this paper, we present a rodent model of acute HAAF. Since the phenomenon of acute HAAF has been well-documented and characterized in humans, but the underlying mechanisms remain unknown, use of animal models is needed to evaluate candidate mechanisms for acute HAAF, and for the prevention of its development. Our studies show that—similar to humans (Heller and Cryer, 1991; Davis et al., 1996)—the rat develops an impaired counter-regulatory response when studied in a two-day paradigm comparable to that which has been utilized for human studies. The rat is an experimental

EFFECT OF PRIOR 5-THIOGLUCOSE
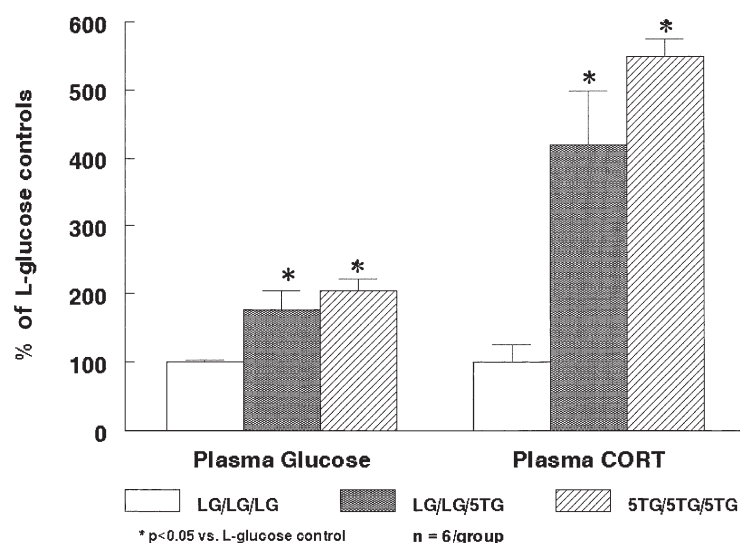## ON RESPONSE TO NEUROGLUCOPENIA

FIGURE 6　Effect of one or three bouts of neuroglucopenia on plasma glucose and CORT responses. Data are expressed as percent of L-glucose treated controls (LG/LG/LG). Cross-hatched bars represent rats receiving one bout of neuroglucopenia (LG/LG/5TG, $n = 6$); hatched bars represent rats receiving three bouts of neuroglucopenia (5TG/5TG/5TG, $n = 6$).

animal that has been well-characterized in terms of both metabolic regulation and brain function, and thus should continue to serve as an ideal model for studying HAAF.

In addition to measuring neuroendocrine responses to hypoglycemia, we measured TH activity in the brain as an index of acute activation of brainstem NE neurons. The increase of activity which we observed was relatively modest (e.g. about 28% in the whole hypothalamus). This is perhaps not surprising in light of the observation by Pacak and colleagues that the change in measurable NE release in hypothalamic PVN interstitial fluid in response to a maximal hypoglycemia stimulus is less than that observed in response to other physical stressors (Pacak et al., 1995b), and presumptively, acute stimulation of TH activity will correspond to the extent of drive for release of NE at the synaptic terminal. Similarly, Beverly et al have recently demonstrated relatively modest increases of hypothalamic NE release in their model of insulin-induced hypoglycemia (Beverly et al., 2001). It should be acknowledged that the hypothalamus also has intrinsic dopamine neurons, and receives adrenergic projections from brainstem adrenergic neurons; TH activity would reflect the composite of these three types of nerve terminals. Moore and Lookingland (1995) have summarized evidence that intrinsic hypothalamic dopaminergic activity is, in fact, suppressed in association with stress. Thus, our findings which show an overall increase of TH activity in the brain areas that contain NE cell bodies and in the hypothalamus, are supportive of the interpretation that hypoglycemia acutely increases noradrenergic (and perhaps adrenergic) signaling to the hypothalamus. The specific loci within the hypothalamus remain to

be determined since TH was measured in the entire hypothalamus; it is possible that our measurement reflects activation in very discrete areas such as the PVN, which is then masked when activity is normalized against the total protein content of the entire hypothalamus. The hypothalamic TH activity in rats with prior experience of hypoglycemia (I/I/I) was identical to that of saline-treated controls (S/S/S). This was not observed in the three NE cell body regions (A1/C1, A2, and LC) and suggests that there may be a local suppression of TH activity (and thus, catecholaminergic activation) within the hypothalamus in association with HAAF.

Use of 5TG to induce cerebral neuroglucopenia provides some insight into the mechanism(s) of acute HAAF. Specifically, both corticosterone and TH measurements in the neuroglucopenic animals suggest that the stimulus of insulin-induced hypoglycemia is not an identical stimulus to CNS neuroglucopenia. Although peripheral corticosterone levels were elevated with one bout of neuroglucopenia (the LG/LG/LG group) to a comparable degree as they were with hypoglycemia, the corticosterone response to a third bout of neuroglucopenia within a 24 h period was not impaired. The pattern of TH activity was also different between the hypoglycemia and neuroglucopenia models, both in the groups receiving only one metabolic insult, and in those receiving the recurrent insult. A recent study from Rusnak et al. (1998) reports differential effects of insulin-induced hypoglycemia and peripherally-induced neuroglucopenia on induction of TH gene expression in the LC cell bodies and in the adrenal medulla, which supports our conclusion that these two metabolic stressors are not an equivalent stimulus to the CNS. Also, patterns of NE release within the

hypothalamus differ both quantitatively and qualitatively in insulin-induced hypoglycemia and 2-deoxyglucose-induced neuroglucopenia (Beverly *et al.*, 2000; 2001). The corticosterone data in this study also suggest that although prior elevations of corticosterone may contribute to the development of acute HAAF, as proposed by Davis *et al.* (1996), corticosterone cannot be the sole mechanistic factor involved. If so, one would have predicted that the neuroendocrine response to a third bout of CNS neuroglucopenia would be blunted as is the neuroendocrine response to recurrent hypoglycemia. This conclusion is supported by our own recent observation in a slightly modified HAAF paradigm, in which intracerebral corticosterone (Day 1) did not blunt neuroendocrine responses to insulin-induced hypoglycemia on Day 2 (Evans *et al.*, 2001).

A major difference between the models in this study is the treatment route. Hypoglycemia develops peripherally and thus peripheral tissues in addition to the brain are exposed to a lack of glucose. In contrast, with CNS administration of 5TG, only the brain receives the neuroglucopenic stimulus and the peripheral tissues never experience a lack of glucose. One might conclude therefore, that peripheral afferent information plays a critical role in mediating the activation of TH in response to hypoglycemia, and the absence of ascending inputs may be responsible for the lack of stimulation observed in the hypothalamus in response to a single bout of CNS neuroglucopenia. It is well-documented that glucose-sensing neurons are present in the hypothalamus (Levin *et al.*, 1999). Thus, activation of the HPA response to specific CNS neuroglucopenia may be occurring via other pathways, perhaps being initiated directly within the hypothalamus. This is supported by the studies from Borg *et al.* (1995; 1997) that demonstrate the direct infusion of the neuroglucopenic agent 2-deoxyglucose (2DG) into the ventromedial nucleus of the hypothalamus (VMH) results in activation of a neuroendocrine counter-regulatory response; and glucose infusion into the VMH can reverse the counter-regulatory response to peripherally-induced neuroglucopenia. Peripheral administration of 2DG can result in increased c-fos immunoreactivity in brainstem catecholamine neurons (an index of neuronal activation) in brainstem catecholaminergic neurons (Ritter and Dinh, 1994; Sanders and Ritter, 2000). Insulin-induced hypoglycemia has been reported not to result in increased c-fos expression in these neurons (Baffi *et al.*, 1996). This provides further evidence, with another marker of neuronal responsivity, that insulin hypoglycemia and neuroglucopenia may represent different stimuli to the CNS.

Sanders and Ritter (2000) have recently reported that both brainstem c-fos activation and counter-regulatory responses to subcutaneous 2DG were blunted in animals pretreated with 10 daily injections of 2DG. This time course would allow for adaptive changes such as gene and protein expression in nerve terminals, or perhaps altered efficiency of glucose uptake by the CNS, as has been

reported by Doyle *et al.* (1995). The occurrence of such adaptive changes may result in a change in the signal or stimulus at the CNS, although the amount of 2DG given was held constant; thus, it might be expected that the response to a subsequent 2DG challenge would be blunted. Our study suggests that the model presented here is one of very acute HAAF, independent of chronic adaptive changes, and acute HAAF may thus represent a unique variation of the stress adaptation syndrome.

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