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Perifornical Hypothalamic Orexin and Serotonin Modulate the Counterregulatory Response to Hypoglycemic and Glucoprivic Stimuli

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COMPLICATIONS

Previous reports suggested an important role for serotonin (5-hydroxytryptamine [5-HT]) in enhancing the counterregulatory response (CRR) to hypoglycemia. To elucidate the sites of action mediating this effect, we initially found that insulin-induced hypoglycemia stimulates 5-HT release in widespread forebrain regions, including the perifornical hypothalamus (PFH; 30%), ventromedial hypothalamus (34%), paraventricular hypothalamus (34%), paraventricular thalamic nucleus (64%), and cerebral cortex (63%). Of these, we focused on the PFH because of its known modulation of diverse neurohumoral and behavioral responses. In awake, behaving rats, bilateral PFH glucoprivation with 5-thioglucose stimulated adrenal medullary epinephrine (Epi) release (3,153%) and feeding (400%), while clamping PFH glucose at postprandial brain levels blunted the Epi response to hypoglycemia by 30%. The PFH contained both glucose-excited (GE) and glucose-inhibited (GI) neurons; GE neurons were primarily excited, while GI neurons were equally excited or inhibited by 5-HT at hypoglycemic glucose levels in vitro. Also, 5-HT stimulated lactate production by cultured hypothalamic astrocytes. Depleting PFH 5-HT blunted the Epi (but not feeding) response to focal PFH (69%) and systemic glucoprivation (39%), while increasing PFH 5-HT levels amplified the Epi response to hypoglycemia by 32%. Finally, the orexin 1 receptor antagonist SB334867A attenuated both the Epi (65%) and feeding (47%) responses to focal PFH glucoprivation. Thus we have identified the PFH as a glucoregulatory region where both 5-HT and orexin modulate the CRR and feeding responses to glucoprivation.

Iatrogenic hypoglycemia is a significant clinical problem in type 1 and type 2 diabetic patients treated with exogenous insulin (1–3). The neurohumoral counterregulatory response (CRR) and awareness evoked by hypoglycemia, which typically defend against dangerously low plasma glucose levels, are progressively blunted by repeated bouts of hypoglycemia with potentially life-threatening consequences (4,5). It has been reported that treatment with selective serotonin (5-hydroxytryptamine [5-HT]) reuptake inhibitors (SSRIs) amplifies the CRR to acute hypoglycemia and prevents the blunting of the CRR after recurrent hypoglycemia in both rats (6) and humans (7,8), suggesting that 5-HT plays an important role in mediating this response. However, the role of 5-HT, per se, in the central control of the CRR is currently unknown. To explore the potential brain sites at which SSRIs might act on 5-HT metabolism to enhance the CRR, we first identified forebrain regions where hypoglycemia stimulates 5-HT release and investigated the role of 5-HT signaling at one such site, the perifornical hypothalamus (PFH), in modulating the CRR to insulin-induced hypoglycemia (IIH) in awake, behaving rats. We found that the PFH regulates adrenal medullary epinephrine (Epi) release

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and feeding in response to local and systemic glucose deficit and contains neurons that are either excited or inhibited by low glucose and/or 5-HT, that PFH 5-HT promotes the adrenomedullary response, and that PFH orexin neurons mediate both feeding and hormonal responses induced by local PFH glucoprivation.

RESEARCH DESIGN AND METHODS

Animals

Male 8-week-old Sprague-Dawley rats (250–350 g; Charles River) were used for all studies, unless otherwise noted. Animals were maintained on a conventional 12-h light/dark cycle (lights off at 2000) with food (Purina rat chow #5001) and water available ad libitum. Experimental groups contained 6–8 rats each. Experiments involving hypoglycemia and/or glucoprivation were uniformly performed during the light phase (beginning at 0800 unless otherwise noted). The animal care and experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the East Orange Veterans Affairs Medical Center.

Placement of Hypothalamic Cannulae and Vascular Catheters

For jugular venous catheters, rats were anesthetized with ketamine (60 mg/mL) and xylazine (6.5 mg/mL) at 1 mL/kg i.p. Silastic catheters (0.24 mm inner diameter) were inserted into the right jugular vein, externalized at the top of the skull, and secured with dental cement. Placement of bilateral guide cannulae for microdialysis probes or direct injection was done under isofluorane anesthesia using stereotaxic guidance (Kopf Instruments). PFH coordinates relative to bregma were A-P = −3.1, M-L = 3.0, D = 7.1–8.1 at a 15° angle. All microdialysis probe and injection cannulae placements were verified histologically.

5-HT Axon Lesion Studies

Rats were pretreated with desmethylimipramine (25 mg/kg; Sigma) to prevent damage to noradrenergic axons. Then, 2 h later, 5,7-dihydroxytryptamine (5,7-DHT; Sigma; 5 μg in 0.5 μL 0.1% ascorbic acid), a neurotoxin that selectively ablates 5-HT nerve terminals (9,10), or ascorbic acid vehicle were administered by direct bilateral infusions into the PFH via stainless steel injection cannulae over 5 min (0.06 mL/h). During the same session, venous catheters were placed in the right jugular vein. One week later, rats were assessed for their responses to IIH, and brains were collected after 2 h of hypoglycemia for determination of 5-HT and 5-hydroxyindole acetic acid (5-HIAA) from frozen brain micropunches with high-performance liquid chromatography with electrochemical detection (HPLC-ED) as described below (11).

Drugs and Dosing

The following were used: serotonin (5-HT; 10 nmol/L; Sigma), orexin 1 receptor antagonist, SB334867A (10 mg/kg; 20 mg/kg; Tocris), SSRI, sertraline (10 μmol/L for reverse microdialysis and direct injection; Toronto Biochemicals), glucose antimetabolites, 2-deoxy-D-glucose (2-DG; 200 mg/kg; Sigma), and 5-thio-D-glucose (5-TG; 60 μg in 0.5 μL per side; Sigma).

IIH

On the day prior to induction of IIH, animals were semifasted overnight (~13 g of chow). On the morning of testing, remaining food was removed, and hypoglycemia was initiated by bolus insulin (4.5 units/kg; Humulin, Lilly) injection via indwelling jugular venous catheters. Blood (0.5 mL) was collected for baseline measurements and, subsequently, at 30 min intervals over 2 h. After each blood draw, packed red blood cells were resuspended in an equal volume saline and reinfused to maintain blood volume (12).

Assays of Blood and Brain Tissues

Plasma norepinephrine (NE) and Epi were assayed by HPLC-ED on a Coulochem III system (ESA) (11,13). Glucose was determined by an automated glucose analyzer (Analox). Glucagon was assayed by commercially available radioimmunoassay (Linco). Extracellular 5-HT obtained from microdialysis samples in a pilot study by the present authors (data not shown) was below the level of detection (at baseline) by HPLC-ED. Therefore, brain 5-HT and 5-HIAA were assayed by HPLC-ED from brain micropunches isolated from fresh frozen brain slabs after homogenization and centrifugation (10,000 rpm for 10 min). The supernatant was analyzed by HPLC-ED as previously described (11); protein from micropunches was quantified with a commercially available kit and the values used to normalize HPLC results (BCA Protein Assay, Thermo).

Microdialysis

Bilateral reverse microdialysis studies were performed in awake, behaving rats using CMA 11, Harvard Instruments probes with 1 mm dialysis membranes as previously reported (14). Probes were inserted via guide cannulae, animals were rested for 2 h, and glucose (25 mmol/L), extracellular fluid (ECF), sertraline (100 μmol/L), 5-HT (100 nmol/L), or 3% DMSO/0.1% ascorbic acid were infused at a flow rate of 0.06 mL/h, and IIH was induced as described above for a total testing period of 4 h. For glucose reverse microdialysis, probe efficiency (8–12%) was calculated from glucose recovered after probe calibration in artificial ECF. For sertraline reverse microdialysis, probe efficiency was estimated as 10%.

Isolation of Primary PFH Neurons and Astrocytes

Primary neuronal and astrocytic cultures were prepared from PFH micropunches using 3-week-old male Sprague-Dawley rats, as previously described (15–17). Astrocytes were cultured for 5 days and then assessed for 5-HT–induced lactate production using previously reported culture methods (17). Lactate levels were assessed in supernatants after 5-HT exposure using a commercially available fluorometric L-lactate assay kit (Cayman).

Calcium Imaging

Calcium imaging with fura-2 calcium-sensitive dye was performed as previously described (15–17). Neurons were serially exposed to 0.5, 2.5, and 0.5 mmol/L glucose to establish their glucose responsiveness and then at 0.5 mmol/L glucose, sequentially to 50 pmol/L 5-HT, and glutamate.

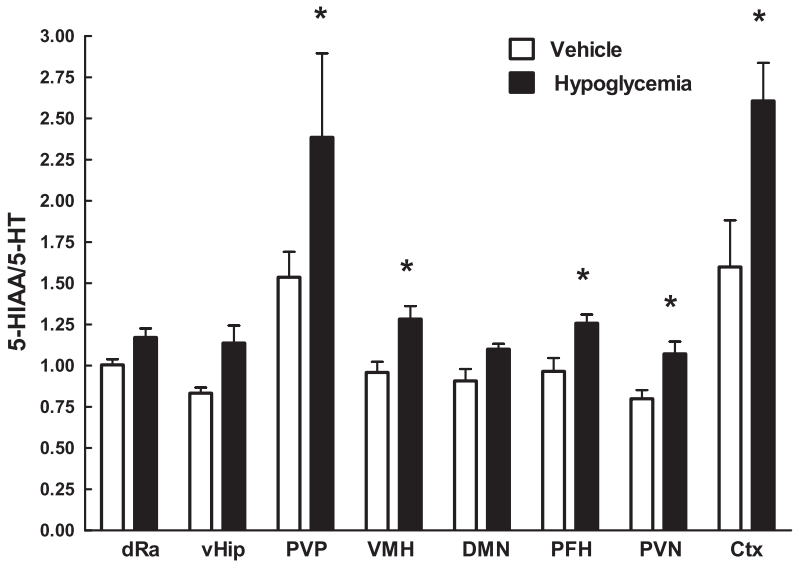

Figure 1—5-HT turnover in selected brain regions after IIH. Rats (n = 5–6/group) were given bolus insulin (4.5 units/kg s.c.) or vehicle (saline) injections, and 5-HT turnover (ratio of 5-HIAA to 5-HT) was assessed in brain micropunches after 2 h of hypoglycemia. Bars are mean ± SEM. *P = 0.05 or less between hypoglycemia and saline groups by one-way ANOVA with Tukey post hoc correction. Ctx, cerebral cortex; DMN, dorsomedial hypothalamic nucleus; dRa, dorsal raphe nucleus; vHip, ventral hippocampus.

Feeding Studies

Rats were fed ad libitum overnight and were then bilaterally infused with either 0.5 μL 5-TG or saline at 0900 on the day of testing. Cumulative food intake was monitored 2 h after each injection.

Statistical Analysis

All statistical analysis was carried out using Systat 8.0. One-way or two-way ANOVAs were used for determination of significance with post hoc corrections made using Tukey test. Areas under the curve were calculated using the trapezoidal rule.

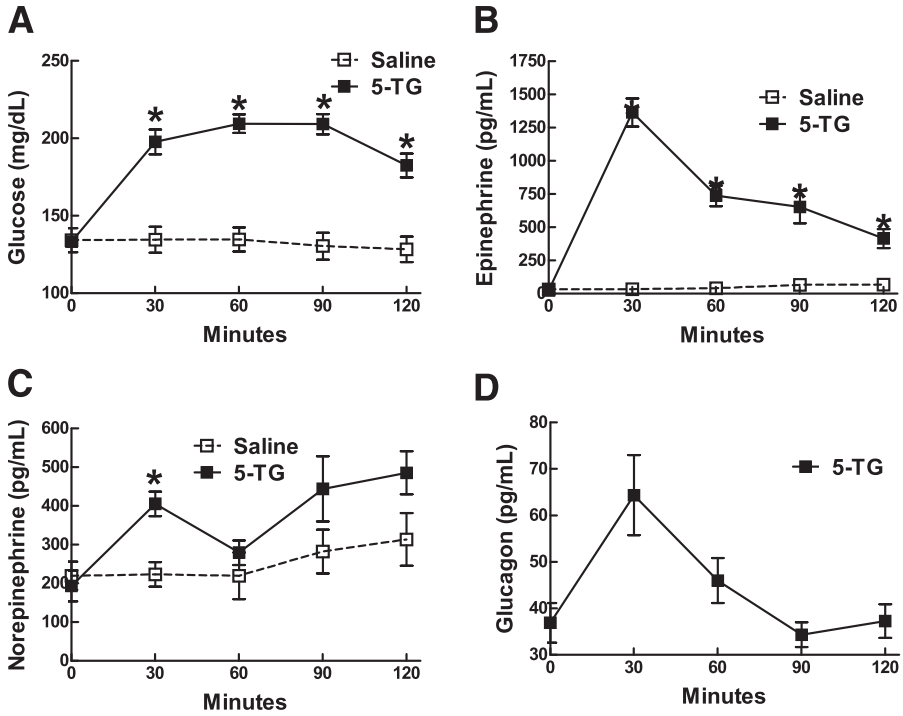

Figure 2—Effects of local PFH glucoprivation on glucoregulatory hormone release. Rats (n = 4–8/group) were infused bilaterally with 5-TG (60 μg in 0.5 μL) or vehicle (saline) in the PFH. Results are shown for blood glucose (*A*), epinephrine (*B*), norepinephrine (*C*), and glucagon (*D*). Data points are mean ± SEM. *P = 0.05 or less between 5-TG and saline groups at each time point by t test after responses over 2 h were significant by one-way repeated-measures ANOVA. Glucagon levels in saline controls (2*D*) were below assay detection limits.

RESULTS

Effect of IIH on Central 5-HT Turnover

Because others have shown that SSRIs, which alter brain 5-HT metabolism (18), amplify the CRR to IIH (6–8), we first evaluated 5-HT turnover (the ratio of 5-HIAA, the principal 5-HT metabolite, to 5-HT) (11) in response to acute IIH. Hypoglycemia significantly increased 5-HT turnover in the ventromedial hypothalamus (VMH; 34%), paraventricular hypothalamic nucleus (PVN; 34%), and PFH (30%), as well as the paraventricular thalamic nucleus (PVP; 64%) and cerebral cortex (63%) (Fig. 1).

Role of PFH Glucose Availability on the CRR

Of those areas in which hypoglycemia increased 5-HT turnover, we chose to further explore the potential role of the PFH in mediating the CRR and feeding responses to glucose deficit, because it contains glucosensing neurons (19,20), some of which project to the adrenal medulla (21). First, we used injections of 5-TG into the PFH since we (22) and others (23–25) have shown it to be highly effective in eliciting both counterregulatory and feeding responses when injected into various brain areas. Bilateral PFH 5-TG–induced glucoprivation (24,26,27) increased plasma glucose by 724%, Epi levels by 3,153%, and food intake by 400% over 2 h relative to saline-infused rats (Fig. 2*A* and *B* and Table 1). PFH 5-TG also increased 30-min peak plasma NE levels by 182%, but levels did not differ from controls when integrated over the entire 2-h test period (Fig. 2*C* and Table 1). Control plasma glucagon levels were below the level of detectability, but PFH 5-TG caused a significant 235% increase above baseline levels 30 min after PFH 5-TG infusion ($F_{4,20} = 15.608$; $P < 0.001$) (Fig. 2*D* and Table 1). To further assess the relative contribution of PFH glucoprivation-induced CRR, bilateral PFH glucose was clamped at brain levels seen following a meal (3.2 ± 0.3 mmol/L) or allowed to fall spontaneously to brain levels seen during IIH (Supplementary Fig. 1) (22,28,29). Clamping PFH glucose at ~3 mmol/L during systemic hypoglycemia affected neither the depth nor duration of hypoglycemia but did significantly reduce the plasma Epi response by 30% over 2 h of hypoglycemia relative to PFH ECF-infused rats (Fig. 3*A* and Table 1). There was no effect on NE or glucagon levels (Fig. 3*B* and *C* and Table 1).

Role of PFH 5-HT Availability in the CRR

Our initial studies suggested that hypoglycemia has a major stimulatory effect on 5-HT release (turnover) in the PFH. To test the hypothesis that 5-HT release is required for the full CRR, we first injected the PFH bilaterally with the selective 5-HT neurotoxin, 5,7-DHT. This reduced 5-HT levels below the limit for detection (less than 0.5 pg/mg protein) as determined postmortem. Prior PFH 5,7-DHT injections blunted the hyperglycemic response to systemic 2-DG by 39% over 2 h relative to sham-operated rats (Fig. 4*A*) and decreased

Table 1—Areas under the curve values for glucose, counterregulatory hormones, and food intake in response to PFH (5-TG) or systemic glucoprivation (IIH) and/or manipulation of PFH glucose levels (by reverse dialysis) or 5-HT with 5,7-DHT or sertraline and by inhibiting orexin 1 receptors with SB334867A

	PFH 5-TG 5,7-DHT		PFH Sertraline IIH			PFH 5-TG		PFH glucose IIH		PFH 5-TG SB334867A	
	0.1% AA	5,7-DHT	DMSO	Sertraline	Sertraline + 5-HT	Saline	5-TG	ECF	25 mmol/L glucose	DMSO	SB334867A
Glucose (mg/2 h)	12,078 ± 1,223	4,887 ± 628*	N/A	N/A	N/A	1,548 ± 314	11,473 ± 689*	N/A	N/A	20,150 ± 1,039	10,272 ± 1,024*
Epi (pg/2 h)	165,211 ± 9,134	51,347 ± 2,309*	168,668 ± 3,103[A]	139,217 ± 9,890[B]	223,765 ± 12,268[C]	2,581 ± 406	81,470 ± 5,279*	307,675 ± 16,871	215,849 ± 14,686*	160,331 ± 20,576	57,087 ± 7,688*
NE (pg/2 h)	23,581 ± 5,450	23,378 ± 8,365	21,659 ± 1,526	23,873 ± 3,813	19,194 ± 3,918	7,212 ± 3,519	16,486 ± 502*	28,433 ± 2,451	29,319 ± 1,909	11,796 ± 2,272	11,696 ± 931
Glucagon (pg/2 h)	N/A	N/A	14,404 ± 1,080	14,881 ± 3,066	22,377 ± 3,153	N/A	1,807 ± 556	12,045 ± 2,289	12,486 ± 3,137	5,628 ± 659	4,872 ± 710
Food intake (g/2 h)	6.3 ± 0.6	6.5 ± 1.0	N/A	N/A	N/A	1.5 ± 0.3	6.0 ± 0.3*	N/A	N/A	7.4 ± 0.6	3.5 ± 0.3*

Data are mean ± SEM. AA, ascorbic acid. *P = 0.05 or less compared with comparable controls. [A,B,C]Data with differing superscript letters differ from each other by P = 0.05 or less.

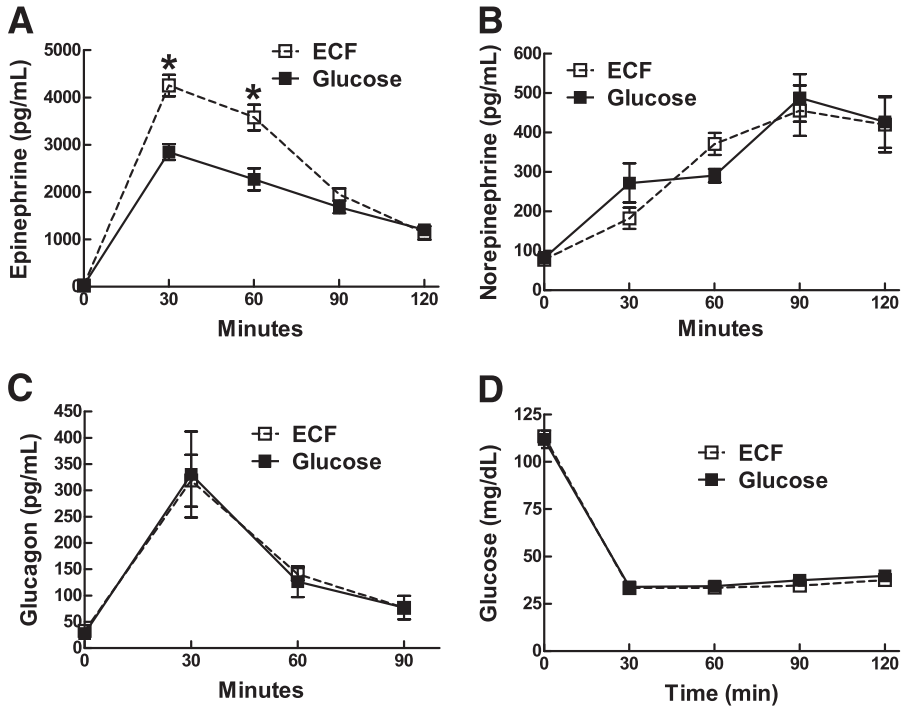

Figure 3—Effect of clamping PFH extracellular glucose at euglycemic levels on the CRR to IIH. Rats (n = 7–8/group) were given bolus insulin (4.5 units/kg i.v.) and reverse dialyzed with 25 mmol/L glucose or ECF. Results are shown for blood epinephrine (*A*), norepinephrine (*B*), glucagon (*C*), and glucose (*D*). Data points are mean ± SEM. *P = 0.05 or less between 25 mmol/L glucose and ECF groups at each time point by t test after responses over 2 h were significant by one-way repeated-measures ANOVA.

PFH 5-TG–induced hyperglycemia by 60% and plasma Epi levels by 69% over 2 h relative to PFH saline-injected controls (Fig. 4*B* and *C* and Table 1). However, PFH 5,7-DHT lesions had no effect on plasma NE levels or food intake over 2 h after PFH 5-TG (Fig. 4*D*, Table 1, and Supplementary Fig. 2). Glucagon data could not be obtained for this study because of insufficient plasma.

To test the hypothesis that increased PFH 5-HT levels would increase the CRR, we reverse dialyzed sertraline into the PFH during IIH. Unexpectedly, this reduced plasma Epi levels by 18% over 2 h relative to controls, and in a separate study, bilateral PFH sertraline dialysis (10 μmol/L in 0.5 μL) reduced the hyperglycemic effect of systemic 2-DG by 39% over 2 h (Supplementary Fig. 3). We postulated that instead of increasing synaptic 5-HT during hypoglycemia, local sertraline, by inhibiting reuptake and subsequent re-release, actually depleted synaptic 5-HT during 2 h of hypoglycemia. For that reason, we reverse dialyzed both sertraline and 5-HT bilaterally into the PFH during hypoglycemia to increase synaptic 5-HT. This combination increased plasma Epi levels by 32% over 2 h relative to controls and by 60% over 2 h relative to the PFH sertraline-dialyzed group (Fig. 5*A* and Table 1). Neither of these manipulations had a significant effect on plasma glucagon, NE, or glucose levels over 2 h of hypoglycemia (Fig. 5*B–D* and Table 1).

Given these in vivo findings, we next explored the effects of 5-HT on PFH neuronal excitability using fura-2 calcium imaging in dissociated PFH neurons (15,30). Of the PFH neurons analyzed, 11% were excited by glucose (glucose excited [GE]) and 15% were glucose inhibited (GI). When held at glucose levels comparable to those seen in the brain during hypoglycemia (0.5 mmol/L) (22,28,29), ~60% of GE neurons (which are predominantly inhibited at 0.5 mmol/L glucose [30,31]) were excited by 5-HT. Among GI neurons (which are predominantly activated at 0.5 mmol/L glucose [30,31]), ~20% were inhibited by 5-HT and ~20% were excited by 5-HT (Fig. 6).

To test the hypothesis that 5-HT might alter the release of lactate from astrocytes as an additional mechanism by which 5-HT might modulate the CRR, cultured hypothalamic astrocytes held at 0.5 mmol/L glucose were exposed to 50 pmol/L 5-HT. This increased lactate release into the culture medium by 37% at 10 min and by 68% at 30 min after exposure to 5-HT (Supplementary Fig. 4). Thus 5-HT has direct effects on PFH neuronal activity and astrocyte metabolism, both of which could potentially contribute to the observed effects on the CRR of manipulating PFH 5-HT availability during hypoglycemia in vivo.

Role of Orexin Signaling in PFH Glucoprivation-Induced Glucoregulatory Hormone Release

PFH orexin neurons project polysynaptically to the adrenal medulla (21) and are activated during hypoglycemia

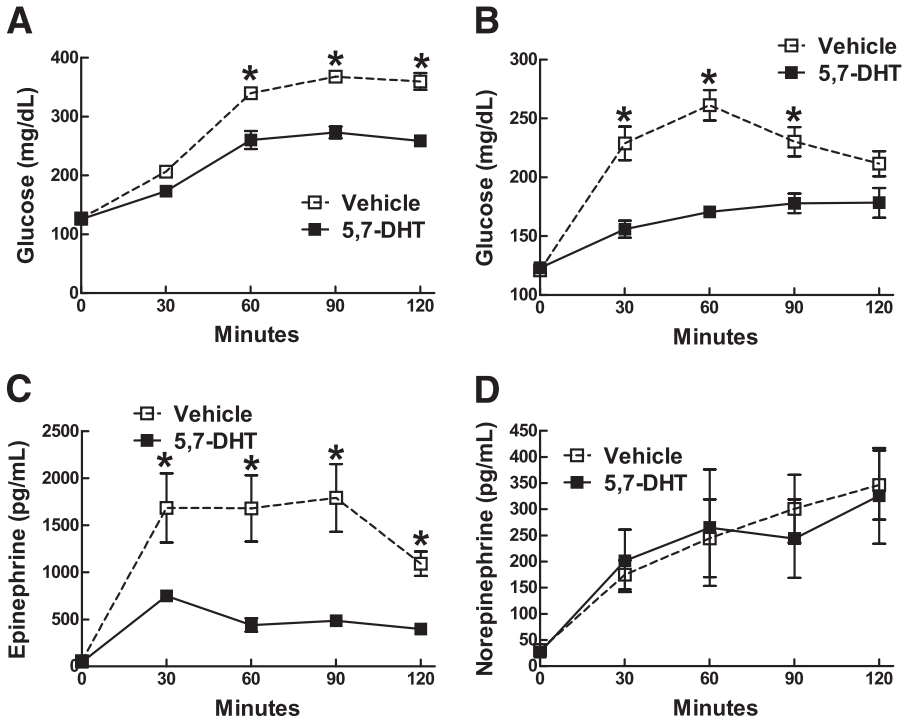

Figure 4—Effect of PFH ablation of 5-HT signaling on glucoregulatory hormone release after systemic and local PFH glucoprivation. Rats (n = 6–10/group), previously treated with 5,7-DHT (5 μg in 0.5 μL infused over 5 min, in combination with 25 mg/kg desmethylimipramine s.c.) or vehicle (0.1% ascorbic acid), were given systemic 2-DG (200 mg/kg) (*A*). In a separate group, rats (n = 5/group) with prior 5,7-DHT lesions or sham operations, were infused with 5-TG (60 μg in 0.5 μL) into the PFH (*B–D*). Data points are mean ± SEM. *P = 0.05 or less between 5,7-DHT and sham-operated groups at each time point by t test after responses over 2 h were significant by one-way repeated-measures ANOVA.

(32–37). For that reason, we postulated that these neurons might contribute to the hypoglycemic CRR. Indeed, systemic administration of the brain-penetrant orexin 1 receptor antagonist, SB334867A (20 mg/kg), given just prior to PFH 5-TG infusions, reduced PFH 5-TG–induced hyperglycemia by 50% and plasma Epi levels by 65% over 2 h relative to vehicle-treated rats (Fig. 7*A* and *B* and Table 1). While a lower dose of SB334867A (10 mg/kg) had no effect on Epi levels (data not shown), it did reduce PFH 5-TG–induced feeding by 47% over 2 h relative to vehicle-treated rats (Table 1). Neither dose affected the glucagon or NE responses to PFH 5-TG (Fig. 7*C* and *D* and Table 1).

DISCUSSION

This work provides the first demonstration that local bilateral PFH glucoprivation evokes an adrenomedullary and a feeding response in awake, behaving animals. Further, while hypoglycemia activates 5-HT neurons throughout much of the forebrain, the adrenomedullary, but not feeding, response to PFH and systemic glucoprivation are dependent upon PFH 5-HT innervation. Finally, both PFH-evoked CRR and feeding are dependent upon orexin signaling.

We initiated these studies based on the finding that SSRIs enhance the CRR to hypoglycemia (6–8) with the idea of discovering a central location at which 5-HT might act to regulate the CRR. Thus we first examined the effects of IIH on 5-HT turnover in various forebrain regions known to be involved in the CRR, which included the PVN (38,39), VMH (40–44), PVP (45), and PFH (32). We found that hypoglycemia elicited increases in the ratio of 5-HIAA to 5-HT in all of these regions. Since 5-HT release in vivo is primarily reuptake dependent and 5-HIAA is formed in presynaptic axon terminals by monoamine oxidase derived from 5-HT that is taken up from the synapse after release (46), the 5-HIAA/5-HT ratio can be used as an index of 5-HT turnover and 5-HT release (10,11). Although differences in these 5-HIAA/5-HT ratios among groups were relatively small, statistically significant differences were sufficient to identify specific areas within the brain where hypoglycemia evoked an increase, suggesting increased 5-HT release. As such, the use of this ratio served as a useful tool to identify areas where 5-HT release might modulate the CRR.

Our investigations focused on the PFH because of its known efferent connections to the adrenal medulla (21) and the fact that PFH orexin neurons, which are glucosensing (19), are activated by hypoglycemia (32–37). While this work was already in progress, Korim et al. (47) demonstrated that PFH glucoprivation increased adrenal sympathetic nerve activity and plasma metanephrine levels, while inhibition of PFH orexin neurons abolished the increased adrenal nerve response to systemic glucoprivation in anesthetized rats. We confirmed

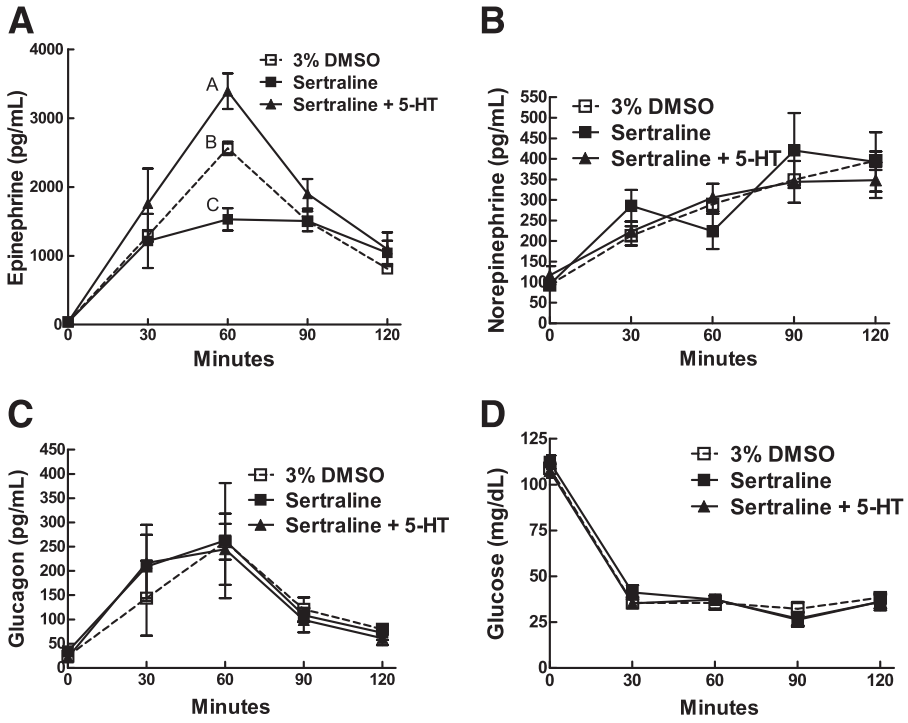

Figure 5—Effect of PFH sertraline reverse dialysis on the CRR to IIH. Rats (n = 5–6/group) were reverse dialyzed with sertraline (10 μmol/2 h), sertraline + 5-HT (10 μmol/2 h + 10 nmol/2 h, respectively), or vehicle (3% DMSO, 0.1% ascorbic acid) in combination with systemic IIH (4.5 units/kg bolus insulin i.v.). Results are shown for blood epinephrine (*A*), norepinephrine (*B*), glucagon (*C*), and glucose (*D*). Data points are mean ± SEM. Curves marked by different letters are significantly different by one-way repeated-measures ANOVA.

that local, bilateral PFH glucoprivation in awake, behaving rats was sufficient to initiate adrenal medullary Epi release but also caused feeding in satiated rats. Both of these responses were significantly attenuated by systemic administration of orexin 1 receptor antagonist SB334867A. Conversely, preventing the decline in PFH glucose during systemic hypoglycemia attenuated the adrenomedullary response. Ablation of 5-HT signaling in the PFH with 5,7-DHT attenuated, but did not completely abolish, the adrenomedullary response and had no effect on the behavioral feeding response to local PFH glucoprivation. On the other hand, increasing PFH synaptic 5-HT availability by coadministration of sertraline and 5-HT was sufficient to amplify the CRR to acute hypoglycemia.

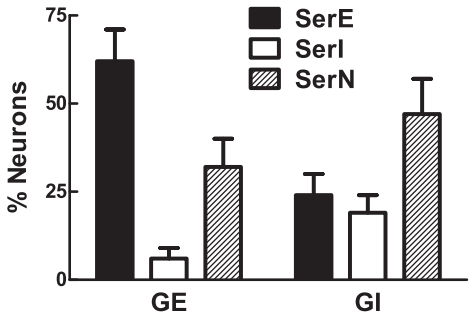

Figure 6—Effect of 5-HT on PFH glucosensing neurons in vitro. PFH neurons were classified as GE or GI and, at glucose levels comparable to those seen during hypoglycemia (0.5 mmol/L), were assessed for their responses to 50 pmol/L 5-HT. Bars are mean ± SEM. SerE, 5-HT excited; SerI, 5-HT inhibited; SerN, 5-HT nonresponsive.

Taken together, these results suggest that the PFH is an important mediator of the adrenomedullary response to IIH and confirm prior findings (47) that adrenomedullary activation induced by PFH glucoprivation is at least partially mediated by orexin signaling. Modest increments in plasma NE and glucagon after local PFH glucoprivation suggest that PFH preautonomic neurons primarily regulate (mono- or polysynaptically) adrenal medulla–projecting sympathetic preganglionic neurons (Epi release) (21) in response to local glucose availability, with less of an effect on generalized sympathetic activation (NE release) and glucagon release. On the other hand, clamping PFH glucose at postprandial brain levels (22,28,29) dampened, but did not completely abolish, the CRR. The likely reason for the only partial alteration of the CRR by focal manipulation of PFH glucose availability is that multiple sites besides the PFH are known to contribute to this response (26,27,38–44,48,49). Therefore, when hypoglycemia sensing is altered in only one of these areas, the overall CRR is not completely altered.

As a potential mechanism for the release of Epi and stimulation of feeding, we confirmed reports of others (19,20) that the PFH contains glucosensing neurons that are either excited (GE) or inhibited (GI) by rising glucose

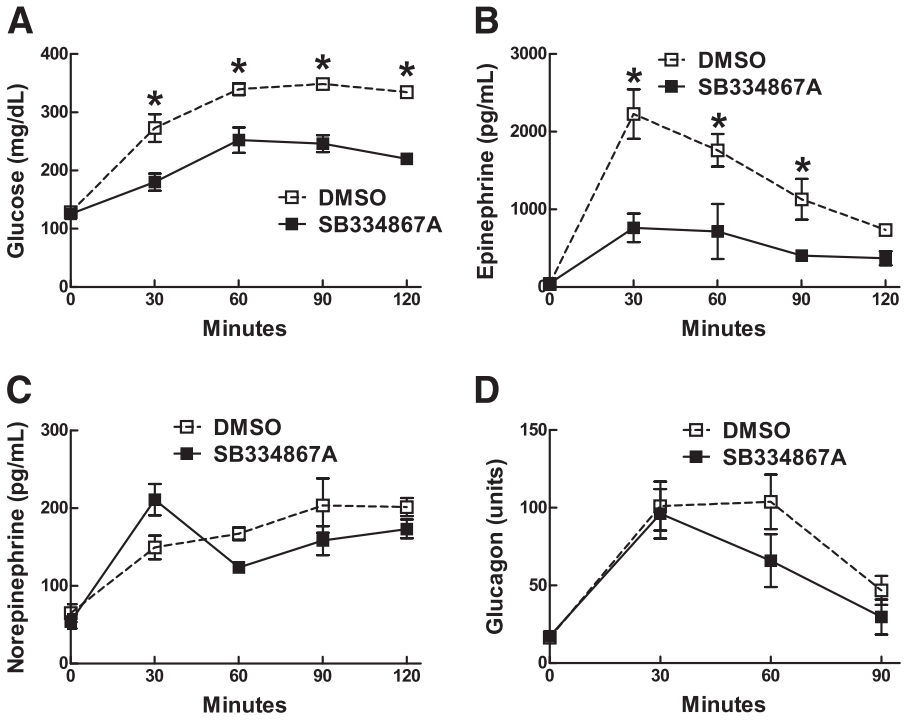

Figure 7—Effect of systemically administered brain-penetrant orexin 1 receptor antagonist SB334867A on PFH glucoprivation-induced glucoregulatory hormone release. Rats (n = 5–6/group) received bilateral PFH 5-TG infusions (60 μg in 0.5 μL) in combination with systemic administration of SB334867A (20 mg/kg i.v.) or vehicle (100% DMSO). Results are shown for blood glucose (*A*), epinephrine (*B*), norepinephrine (*C*), and glucagon (*D*). Data points are mean ± SEM. *P = 0.05 or less between SB334867A and DMSO groups at each time point by t test after responses over 2 h were significant by one-way repeated-measures ANOVA.

levels. Furthermore, our studies provide the first overall estimate of the relative percentages of GE versus GI neurons in the PFH. More importantly, at glucose levels comparable to those seen in the brain during hypoglycemia (22,28,29), we showed for the first time that GE neurons (which are largely inactivated at low glucose levels [30,31]) were predominantly activated by 5-HT, while subpopulations of GI neurons (which are largely activated at hypoglycemic levels [30,31]) were either inhibited or excited by 5-HT. Thus both glucoprivic and 5-HT activation of neighboring glutamate neurons might activate PFH orexin neurons (50). In addition, at similarly low glucose levels, 5-HT increased lactate release from cultured astrocytes. Such astrocyte-derived lactate activates orexin neurons (51), as well as PFH/lateral hypothalamic area GE (50) and ventromedial hypothalamic nucleus GE and GI neurons (52). Thus there are several ways in which local release of 5-HT in the PFH might act to alter the activity of local glucosensing and nonglucosensing neurons or astrocytes involved in the regulation of the CRR.

While the neuronal cell bodies of the 5-HT neurons in the dorsal and median raphe nuclei that innervate the PFH are not glucosensing (53), PFH orexin neurons that project to these raphe 5-HT neurons are glucosensing (19,20,54,55). This provides a circuitry by which hypoglycemia would activate dorsal and median raphe 5-HT neurons to provide positive feedback for promotion of a CRR within the PFH and other forebrain areas innervated by these 5-HT and orexin neurons that are also involved in the CRR. Given this reciprocal relationship between PFH orexin and dorsal and median raphe 5-HT neurons (54–56) and the fact that PFH orexin neurons are both glucosensing and project polysynaptically to the adrenal medulla (21), we used the orexin receptor 1 antagonist, SB334867A, to test the hypothesis that orexin signaling is involved in mounting the adrenomedullary and feeding responses to focal PFH glucoprivation. Our results in awake, behaving rats lent support to those of Korim et al. (47) in anesthetized animals, demonstrating a role for PFH orexin neurons in stimulating the adrenal nerve during PFH glucoprivation. In addition, antagonizing orexin 1 receptors also inhibited the feeding behavioral response evoked by PFH glucoprivation. The difference in the dose of SB334867A that inhibited feeding (10 mg/kg) versus the hyperglycemic adrenomedullary response (20 mg/kg) suggests that distinct pathways may mediate these branches of the CRR with differential orexin neuronal involvement. Alternatively, PFH glucoprivation might be a more potent stimulus for counterregulatory hormone release than for feeding and therefore require higher concentrations of SB334867A to overcome.

We originally postulated that local dialysis of sertraline into the PFH would increase synaptic 5-HT availability and enhance the CRR to glucoprivation. Instead, this inhibited

the CRR, suggesting that blockade of 5-HT reuptake, the major source of presynaptic 5-HT release during activation of 5-HT neurons (57), had acutely depleted extracellular 5-HT due to rapid clearance of 5-HT by postsynaptic degradation and uptake by non-serotonergic transporters (58,59). We tested this hypothesis by coinfusing sertraline and 5-HT into the PFH. As predicted, this presumptive elevation in synaptic 5-HT levels enhanced the Epi response to IIH. We were unable to measure baseline levels of 5-HT by microdialysis to confirm the basic premise of this hypothesis, but taken together with the inhibition of Epi release by local PFH 5-HT depletion with 5,7-DHT, our PFH sertraline results strongly support the idea that PFH 5-HT release during IIH is required for the full CRR.

In conclusion, we have identified a novel role for the PFH in regulating adrenomedullary and feeding responses under conditions of metabolic emergency in awake, freely behaving animals. These responses are mediated, in part, by PFH orexin neurons and 5-HT signaling. Future studies are needed to elaborate the role of the PFH, orexin neurons, and 5-HT signaling in the broader glucoregulatory network and the contribution these players make to the physiology and pathophysiology of hypoglycemia counterregulation.

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