A ROLE FOR THE FOREBRAIN IN MEDIATING TIME-OF-DAY DIFFERENCES IN GLUCOCORTICOID COUNTERREGULATORY RESPONSES TO HYPOGLYCEMIA IN RATS

by

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ABSTRACT

The time of day influences the magnitude of adrenocorticotropic hormone (ACTH) and corticosterone responses to hypoglycemia. However, little is known about the mechanisms that impart these time-of-day differences on neuroendocrine CRH neurons in the hypothalamic paraventricular nucleus (PVH). Rats received 0-3U/kg of insulin (or 0.9% saline) to achieve a range of glucose nadir concentrations. Brains were processed to identify phosphorylated extracellular regulated 1/2 kinase (phospho-ERK1/2)-immunoreactive cells in the PVH and hindbrain and corticotropin-releasing hormone (CRH) heteronuclear (hn) RNA in the PVH. Hypoglycemia did not stimulate ACTH and corticosterone responses in animals unless a glucose concentration of approximately 3.15mM or below was reached. Critically, the glycemic thresholds required to stimulate ACTH and corticosterone release in the morning and night were indistinguishable. Yet, glucose concentrations below the estimated glycemic threshold correlated with a greater increase in corticosterone, ACTH, and phospho-ERK1/2-immunoreactive neurons in the PVH at night compared to morning. In these same animals, the number of phospho-ERK1/2-immunoreactive neurons in the medial part of the nucleus of the solitary tract was unchanged at both times of day. These data collectively support a model whereby changes in forebrain mechanisms alter the sensitivity of neuroendocrine CRH to the hypoglycemia-related information conveyed by ascending catecholaminergic afferents. Circadian clock driven processes together with glucose sensing elements in the forebrain would seem to be strong contenders for mediating these effects.
INTRODUCTION

Diurnal rhythmicity is a prominent feature for many components in the hypothalamic-pituitary-adrenal (HPA) axis. ACTH and glucocorticoid each show variations in basal secretory rates across the day in rodents and humans (1-3). In the hypothalamus, the rate of Crh gene transcription, the amounts of CRH mRNA present in the paraventricular nucleus of the hypothalamus (PVH), and the amount of CRH available for release in the median eminence all vary across the day (4, 5).

In rats the magnitude of the ACTH and corticosterone secretory responses to stressors also show daily variations (6-8). Yet the time-of-day when maximal secretory responses are elicited varies dramatically depending upon the nature of the applied stressor. So-called psychological or extrasensory stressors (e.g. restraint, novelty) elicit greater responses if applied during the morning (6, 7, 9), while physiological or interosensory stressors display greater responses at night (8, 10). These differences show that the magnitude of the response at a particular time-of-day is not tightly coupled to the circadian clock-driven mechanisms that alter blood levels of ACTH and corticosterone across the day (8). Instead, these differences are more a function of the way the brain processes relevant sensory information (11), which will be different for each stimulus. The neural mechanisms that impart these differences are unknown.

Blood glucose is detected by a complex and widely distributed series of glucose sensing elements that collectively control counterregulatory endocrine responses to insulin-induced hypoglycemia. The principal glucose sensing elements are located in the hepatic portal vein, hindbrain, and hypothalamus (12). A great deal of work has shown that glucose sensing mechanisms in all of these regions play crucial roles in regulating the sympathoadrenal (epinephrine and norepinephrine) and glucagon responses to hypoglycemia (13-18). The location of all the glucose sensing elements responsible for regulating ACTH release is unclear. However, evidence suggests that to stimulate corticosterone secretion glucoprivation and insulin-induced hypoglycemia engage glucose sensing mechanisms that are located outside the hypothalamus (18, 19). Neural information encoding glycemic state then converges onto neuroendocrine CRH neurons in the medial parvicellular part (mp) of the PVH to activate the HPA axis. Since catecholaminergic afferents are required for the full corticosterone response to insulin injections (19), these neurons must be integral components of the afferent set required to transmit glycemic information to the PVHmp.

The purpose of the current studies was to begin clarifying the mechanisms responsible for the previously reported diurnal variation in the ACTH and corticosterone secretory responses to insulin-induced hypoglycemia in rats (8). To do this, we determined the behavior of various components in the HPA axis following insulin-induced hypoglycemia imposed just after lights on (when secretory responses are minimal), or just before lights off (when secretory responses are maximal). When analyzing our data we also had to account for the inherent pulsatility and daily variations in basal plasma ACTH and corticosterone concentrations (20, 21) upon which any hypoglycemia-stimulated release was imposed.

At each time of day we investigated two different parts of this process: the first related to the processing and transmission of information from the sensory mechanisms encoding glycemia to CRH neurons; the second related to the way CRH neurons in the PVHmp respond to the afferents involved with encoding hypoglycemia. We hypothesized that by investigating variables associated with these two parts we would be able to discriminate between the potential mechanisms that drive these daily variations.

First, we determined if the concentration at which plasma glucose triggers an ACTH and corticosterone response (i.e. the glycemic threshold) is different in the morning and night. In healthy humans, cortisol responses occur when blood glucose reaches a concentration of between 2.9-3.2mM (53-57 mg/dl) (22). However, glycemic thresholds are not necessarily fixed and can shift toward lower glucose levels when hypoglycemic events are presented sequentially (23). Therefore, it is possible that glycemic thresholds in rats vary across the day in a way that contributes to the differential time-of-day
glucocorticoid response. It seems reasonable to assume that the mechanisms (18) responsible for sensing and transducing glycemic information are closely involved with determining the level of the glycemic thresholds.

The hindbrain contains the critical mechanisms for processing and transmitting glycemic information to the hypothalamus (19, 24). As part of our exploration of the mechanisms responsible for the time-of-day differences in ACTH and corticosterone responses that are located distal to the PVH, we also examined whether plasma glucagon and a marker for neural activation in the hindbrain also show time-of-day differences following hypoglycemia (18). We examined whether there were variations in the amount of immunoreactive (ir) phospho-p44/42 MAP kinases (ERK1/2) detectable in neurons in the medial part of the nucleus of solitary tract (NTSm) and dorsal motor nucleus of the vagus (DMX) across the day. ERK1 and ERK2 are kinases that are important signaling intermediates operating downstream from many receptors (25, 26), and are rapidly phosphorylated following a number of distinct stimuli (27-29). Thus, phospho-ERK1/2 can be used as a marker of rapid neural activation (11, 27-29). A time-of-day difference in phospho-ERK1/2-ir in hindbrain neurons would suggest that they are differentially sensitive to a hypoglycemic stimulus of equal magnitude and may trigger a commensurable response in the PVH, corticotropes, and the adrenal gland. However, no observable difference across the day would suggest that hindbrain mechanisms were not differentially responsive to a hypoglycemic stimulus.

We have recently shown that ERK1/2 phosphorylation is increased in the PVHmp following insulin-induced hypoglycemia in the morning, and is dependent on ascending catecholaminergic afferents (30). Furthermore, the majority of PVH CRH neurons exhibit rapid increases in levels of phospho-ERK1/2 following intravenous 2-deoxy-D-glucose (2DG) or insulin-induced hypoglycemia (27, 29). The second part of the present study therefore determined if cellular responses in PVHmp neurons showed differential morning and night-time responses to hypoglycemia. The experiment was designed to examine whether mechanisms proximal to PVHmp neurons could account for the different secretory responses to hypoglycemia across the day. To do this, we determined the magnitude of the Crh gene transcriptional response using in situ hybridization (ISH) and the number of phospho-ERK1/2-ir neurons in the PVHmp.

Parts of this work were presented in abstract form at the 66th Annual Scientific Sessions of the American Diabetes Association in Washington DC 2006 (31), and the Society For Neuroscience Annual Meeting in Atlanta 2006 (32).
METHODS

1) General Procedures

a) Animals

Male Sprague-Dawley rats, (Harlan, Indianapolis, IN) weighing 250-330g were housed singly in a temperature controlled vivarium with a 12:12 light/dark schedule with lights on at 08:15h [zeitgeber time (ZT) 0]. Morning experiments began 2 hours after lights-on (ZT 2); evening experiments, 1 hour before lights-off (ZT 11). Water and chow were provided ad libitum.

After 7 day acclimatization to the animal quarters, rats were anesthetized with 3:3:1 ketamine HCl, xylazine, acepromazine maleate; (0.10ml/100 g body wt), and fitted with an indwelling carotid artery catheter made from PE-50 tubing (Clay Adams). Catheters were 15cm in total length; 2.5cm was inserted into the right or left carotid artery and secured with suture thread. Catheters were exteriorized between the scapulae and trimmed to 3cm. Each animal was fitted with a non-surgically attached harness (Instech Laboratories Inc., Plymouth Meeting, PA) and given 500μl warm sterile saline ic, gentamicin (0.1ml/100g IM) and FluMeglumine (0.08ml/100g intramuscular). Patency was maintained with 0.2 ml of 500U heparin/saline solution administered post-surgery. Rats were weighed and handled daily for 7 days at their projected experiment time. On day six, catheters were flushed with 0.1ml of 1000U heparin, a volume sufficient to clear the catheter but avoid entrance into the general circulation. Catheters were extended by inserting a 27.5 gauge needle to the exteriorized portion of the indwelling catheter and attaching PE-50 tubing approximately 53cm to the needle. Extensions were threaded through a tether spring, and attached to a swivel/counterbalance system (Instech Laboratories Inc., Plymouth Meeting, PA), allowing remote access to blood sampling and infusion without non-specific handling stress. All animal procedures were approved by the Institutional Animal Care and Use Committee of University of Southern California.

b) Blood Sampling Procedure

On day seven, access to food was removed and animals were weighed two hours before insulin or 0.9% saline infusion (ZT 0 and ZT 9). Animals infused at ZT 2 were not fasted overnight to preserve food intake dependent diurnal rhythms (e.g. hormonal gut peptides), and because overnight starvation is itself a physiological stimulus that influences ACTH and corticosterone secretion (33). Rats were reattached to the PE-50 extensions, and re-tethered to the swivel and counterbalance. They then remained undisturbed for the duration of the experiment.

2) Experimental Design

The study was divided into three sets of experiments.

a) Kinetics of Plasma Glucose Following Insulin Injections at ZT 2 and ZT 11

The first experiment examined plasma glucose kinetics following a 0.5U/kg bolus of insulin given into the carotid artery (ic) either at ZT 2 or at ZT 11 to constrain the timing of subsequent sampling procedures. Because insulin sensitivity and glucose tolerance increase towards the evening in rats, (8, 34) it was important to control for the diurnal difference in stimulus intensity that may result from these changing sensitivities. Ensuring that animals received a comparable hypoglycemic stimulus regardless of time of day was a critical part of the overall experimental design.

A total of 24 animals were prepared for sampling as described in 1) General Procedures, and then injected ic with 0.5U/ml insulin (500μl) (pork Regular Iletin II, Eli Lilly, Indianapolis, IN) or 0.9% saline at ZT 2 (n=6 each for the saline and insulin injections) and ZT 11 (n=6 each for the saline and insulin injections). Blood samples (0.1ml) were taken from all animals at baseline, 5, 10, 20, 30, 60 and 120 minutes after infusion and assayed for glucose. Insulin was diluted from stock on the day of the experiment, and the same stock solution was used for all experiments.

b) Estimation of Pulsatile Hormone Release and Diurnal Variations Within the Sampling Period

The second part of the design determined the variability in plasma ACTH and corticosterone concentration deriving from pulsatility and circadian variations. We reasoned that without
accounting for these basal variations it would not be possible to resolve whether a change in plasma ACTH or corticosterone seen between 0 and 20 minutes post-injection in individual animals given insulin was a secretory episode driven by inherent pulsatility and/or diurnal variations (21), or one that was driven by the imposed hypoglycemia.

After a two-hour fast and preliminary set up as described in 1) General Procedures, animals received bolus injections of 0.5ml of 0.9% saline ic in a solution warmed to body temperature. Blood samples (0.5ml) were taken at baseline, 10, and 15 minutes after infusion (0.1ml) and collected in ethylenediaminetetraacetic acid (EDTA) coated tubes (approximately 33μl/ml of blood collected). 0.9% saline (0.2ml) was used to clear the extension and catheter after each blood sample was removed. A terminal sample (0.9ml) was taken at 20 minutes and approximately 1.5 ml of warmed 2,2,2-tribromoethanol anesthesia (3.2% in 0.9% saline) was immediately administered through the catheter. Rats were immediately sacrificed by decapitation, brains rapidly removed, fixed, and processed for immunocytochemistry and in situ hybridization as described below.

To establish whether a daily rhythm in phospho-ERK1/2 might contribute to any morning/evening response to hypoglycemia in the PVH, we also determined in a separate experiment if phospho-ERK1/2 levels change throughout the day in the PVH of unstimulated animals. Eighteen adult male Sprague-Dawley were sacrificed in groups of 3 every 3-6 hours throughout a 24-hour period. Animals were gently removed from their home cages and immediately decapitated. Brains were quickly removed and immediately fixed as described below. Tissue was processed for phospho-ERK1/2 as described below.

c) Glycemic Threshold and Response Magnitude

The third part of the experiment imposed a hypoglycemic challenge of measured intensity to which the responses of ACTH, corticosterone, and glucagon secretion were determined along with levels of CRH hnRNA and phospho-ERK1/2 in the PVH, and phospho-ERK1/2 in the hindbrain.

After a two-hour fast and preliminary set up as described in 1) General Procedures, animals had a 0.1ml blood sample taken (the 0 min sample) through the carotid catheter, which was immediately followed by bolus injections of 0.5ml saline or insulin at ZT 2 (0-3.5U/kg; mean 1.7±0.2U/kg, n=36) or ZT 11 (0-0.5U/kg; mean 0.4±0.04U/kg, n=45) in a solution warmed to body temperature.

Blood samples (0.1ml) were then taken at 10, and 15 minutes after infusion and collected in EDTA coated tubes. 0.9% saline (0.2ml) was used to clear the extension and catheter after each blood sample was removed. At 20 minutes a terminal sample was taken (0.9ml) through the catheter, immediately followed by approximately 1.5 ml of warmed 2,2,2-tribromoethanol anesthesia. Rats were immediately sacrificed by decapitation. Brains were rapidly removed, fixed, and processed for immunocytochemistry and in situ hybridization as described below.

3) Assay Procedures

a) Glucose and Hormone Determinations

All blood samples were immediately placed on ice until animal sacrifice and then centrifuged at 13,000 rpm for 20 minutes. Plasma was collected and stored at -20°C until assayed. Plasma glucose was measured immediately in duplicate using the glucose oxidase method (YSI, Yellow Springs, OH). The inter-assay coefficient of variation (cv) was 1.6%.

Plasma corticosterone and ACTH concentrations were measured in duplicate using commercially available radioimmunoassay kits (MP Biomedical, Irvine CA) with minor modifications. The sensitivity of the corticosterone and ACTH assays was 6.25ng/ml and 14pg/ml respectively. Inter- and intra-assay cv was less than 10%. Glucagon was measured in duplicate with a commercially available kit (Linco Research, St. Charles, MO). The sensitivity of glucagon was 20 pg/ml. All samples were included in one assay. The intra-assay cv was less than 10%.

b) Brain Fixation and Sectioning

Brains were fixed for further processing in one of two ways. Those from animals injected ic with saline or insulin were placed in ice-cold 4% paraformaldehyde (wt/vol) in 0.1 M borate fixative (pH 9.5) within 2.5 minutes of the end of the terminal blood sample, and kept there for 48-
52 hours. They were then cryoprotected for 24-28 hours in paraformaldehyde fixative containing 12% sucrose (wt/vol).

Alternatively, brains from the animals used to detect time-of-day variations in PVHmp phospho-ERK1/2 were rapidly removed and submerged in an ice-cold, freshly depolymerized solution of 4% paraformaldehyde buffered to pH 6.0 with sodium acetate (27). They were kept in this solution overnight at 4°C and then transferred to paraformaldehyde buffered to pH 9.5 with sodium borate, also at 4°C. Two days later they were transferred to borate-buffered paraformaldehyde containing 20% glycerol. We have already shown that both of these fixation methods are compatible with in situ hybridization and immunocytochemistry for phospho-ERK1/2 (5, 27). All brains were then frozen in hexanes that were cooled using powdered dry ice and stored at -70°C until sectioned.

Seven 1:7 series of frozen coronal serial sections (20 μm) were cut through the rostrocaudal extent of the PVH [atlas levels 22-27 of the Swanson Rat Brain Atlas (35)] using a sliding microtome. Four serial sections were collected for ISH in chilled KPBS 0.25% paraformaldehyde (wt/vol). Two series were collected for immunohistochemistry in cryoprotectant (50% 0.05M phosphate buffer, 30% ethylene glycol, 20% glycerol, pH 7.4). The final series was reserved for thionin staining. Brain sections collected for ISH were mounted immediately on Super Frost slides (Fisher Scientific, Pittsburgh, PA) and vacuum desiccated overnight. Slides were fixed with KPBS/4% paraformaldehyde (wt/vol) at room temperature for 1 hour and rinsed 5 X 5 minutes in KPBS and stored at -70°C until hybridization. Sections collected for ICC were stored at -20°C in cryoprotectant until further processing.

Five 1:5 series of frozen coronal serial sections (30 μm) were cut through the rostrocaudal extent of the nucleus of the solitary tract (NTS) corresponding to Swanson atlas levels 58-73 (35). Four series were collected in cryoprotectant and stored at -20°C. One series was collected in KPBS and reserved for thionin staining.

c) phospho-ERK1/2 Immunohistochemistry

Tris-Buffered-Saline (TBS) rinsed sections were incubated for 36-44 hours in a 1:8K dilution of a rabbit polyclonal antibody raised against the phosphorylated forms of ERK1/2 (Cell Signaling Technology cat # 9101, Beverly, MA) and a 1:3 dilution of biotinylated goat anti-rabbit secondary antibody (Kirkegaard and Perry-KPL cat # 71-00-30, Gaithersburg MD) followed by a 1:2 dilution of streptavidin-HRP (KPL cat # 71-00-38). Final reaction product was developed using a Histomark Enhanced Black solution DAB kit with peroxide (KPL cat # 71-00-08/71-00-09) as described previously (27). All sections from insulin- and 0.9% saline-treated animals were incubated in the same experimental run and separated randomly among animals so that inter-tray variability did not influence the results. Sections from the basal phospho-ERK1/2 experiment were processed separately from the saline/insulin-injected animals.

Phospho-ERK1/2-immunoreactive-(ir) cells located in the PVHmp, NTS, and the dorsal motor nucleus of the vagus (DMX) were counted blindly using a photographed transparent overlay. Digital photographs were taken of each area of interest and printed. Overhead transparencies were affixed to the printed copy and positive cells were verified by microscopic examination and counted. Adjacent thionin-stained sections were used to determine the cytoarchitectonic borders. All areas were counted bilaterally at approximate levels 26, 68-69, 69-70 of the Swanson rat brain atlas (35) for the PVHmp, NTS and DMX respectively.

d) In Situ Hybridization

Mounted sections were hybridized as described previously (36, 37). Briefly, all sections were incubated with a (35S)-UTP labeled complimentary RNA probe transcribed using a Promega Gemini kit (Promega Inc., Madison, WI) from either a 700-base pair complimentary DNA sequence coding for a portion of the mRNA of prepro CRH or the 536 base pair PvuII fragment complimentary to the CRH intron (38-40) Sections were exposed to Microvision C film for 3 or 49 days respectively. To allow direct comparisons between groups, slides for each probe were hybridized simultaneously. Autoradiographic films were scanned and quantified using IP Lab software (Signal Analytics Corp., Vienna, VA) as previously described (5).
e) Statistical Analysis

Data are expressed as mean ± S.E.M. Significance of differences between means were determined using an unpaired, two-tailed T-test for the basal conditions, or a one-way ANOVA followed by the Tukey-Kramer post-hoc test, or two-way ANOVA followed by the Bonferroni post-test where appropriate (GraphPad, Prism version 4.0c for Macintosh, GraphPad Software, San Diego CA).

4) Analytical Procedures

a) Estimation of Pulsatile Hormone Release and Diurnal Variations Within the Sampling Period

To account for the pulsatility and diurnal variations in ACTH and corticosterone release that normally occur in unstimulated rats (20, 21), we calculated the mean and standard deviations of the change in plasma ACTH corticosterone concentrations measured between 0 and 20 minutes in animals given ic 0.9% saline injections at ZT 2 or ZT 11. The incremental change in plasma ACTH or corticosterone concentration from the immediate pre-injection value to maximum post-injection value was chosen as the dependent variable to account for the variable baseline of each animal, which can be influenced by time-of-day, arousal state, or time since the last meal.

For both hormones at the two times of day, we then calculated the value equal to the mean plus 2 standard deviations of the mean (41). Any insulin-injected animal whose 0–20 minute change in plasma ACTH or corticosterone concentrations was less than this value was designated as a non-responder to the hypoglycemic challenge; any animal above this value was designated as a responder. By definition, all saline-injected animals were designated as non-responders, and the values of their dependent variables included in subsequent data analyses.

b) The Estimation of Glycemic Thresholds

Since counterregulatory responses are dependent on the absolute value of the plasma glucose concentration rather than the decrement of plasma glucose (rate of decline from basal) (41), we used the lowest blood glucose concentration of each animal in samples taken at 0, 10, 15 and 20 minutes after insulin or 0.9% saline injection (the nadir) as the independent variable. Glycemic thresholds were then estimated for ACTH and corticosterone release at each time of day (4 experimental groups) by comparing the population distribution of the non-responding animals (i.e. those that did not mount hormone secretory responses to hypoglycemia) and responding animals (i.e. those that mounted hormone secretory responses) as a function of their glycemic nadirs.

The glycemic threshold for each hormone at both times of day was considered to occur within a range of blood glucose concentrations. This range was delineated by the concentration where the percentage of the responder population first exceeded the percentage of non-responders, and the concentration above which 95% of non-responders were located. In three of the four experimental groups the threshold for stimulating ACTH or corticosterone secretion occurred within a narrow range of plasma glucose concentrations. For comparison, we found that if we used the mean, the mean plus 1 standard deviation, or the 95% confidence interval of the mean plasma corticosterone and ACTH concentrations of saline-injected animals as threshold determinants, a significant number were included in the responder group (data not shown). These methods for determining the threshold were therefore rejected in favor of the 2 standard deviations above the mean control response.

c) Post-Hoc Grouping of Animals

After estimating the glycemic threshold ranges at both times of day, hormone values for each animal were then assigned to either being above or below the glucose concentration at the lower end of this range. Post-hoc analyses of the magnitude responses of all HPA-related dependent variables were then based upon this grouping. For CRH mRNA and hnRNA in the PVHmp, and numbers of phospho-ERK1/2 labeled neurons, we used 3.15mM as the glucose nadir value for grouping data.
RESULTS

a) Kinetics of Plasma Glucose Following Insulin Injections at ZT 2 and ZT 11

Plasma glucose concentrations did not differ significantly at any time following saline injections at ZT 2 or ZT 11 (Fig. 1). Animals injected with insulin (0.5U/kg) at ZT 2 or ZT 11 showed a significant reduction in plasma glucose that reached nadir concentrations approximately 10 minutes after infusion at both times of day. Subsequent to the glycemic nadir, 20 minute plasma glucose concentrations were also not significantly different at ZT 2 and ZT 11. But from 30 minutes onwards they were significantly lower in the evening compared to the morning, most likely because of reduced glucose clearance from circulation (8). At both times of day plasma glucose concentrations were indistinguishable from saline-injected controls by 120 minutes after insulin injections. Therefore, to control for differences in stimulus intensity arising from the different plasma glucose kinetics at ZT 2 and ZT 11, measurements in all subsequent experiments were terminated 20 minutes after insulin or saline injections.

b) Estimation of Pulsatile Hormone Release and Diurnal Variations Within the Sampling Period

Table 1 shows the basal hormone concentration of glucose, ACTH, corticosterone, insulin and glucagon in catheterized animals injected with saline or insulin ic at ZT 2 and ZT 11. Note that ACTH and corticosterone hormone concentrations are within normal ranges for unstressed animals, and that the circadian rhythms are intact, confirming that animal manipulations did not alter resting HPA hormone levels. The diurnal rhythms of glucose and insulin are in contrast to previously published findings (34), possibly due to differential responses to the two-hour fast imposed at the two times of day. Plasma glucose concentrations in saline-injected animals were maintained between 5.30mM and 7.00mM at ZT 2 and 5.50mM and 7.50mM at ZT 11.

In keeping with the presence of pulsatile secretory patterns, individual animals showed variable changes in plasma ACTH (Fig. 2A) and plasma corticosterone concentrations (Fig. 2B) across the sampling period. Thus, the increments in plasma ACTH concentrations between 0 and 20 minutes after saline injections had a range of 99.2pg/mL at ZT 2, and 76.3pg/mL at ZT 11. Similarly, the plasma corticosterone increments in saline-injected controls had a range of 55.9ng/mL at ZT 2, and 316.2ng/mL at ZT 11. The much greater range of plasma corticosterone at ZT 11 compared to ZT 2 is consistent with the large pulses that drive the daily surge of plasma corticosterone at this time (21). Figures 2A and 2B also shows the values equal to the mean plus 2 standard deviations for the plasma ACTH and corticosterone variations seen between 0 and 20 minutes post-injection at both times of day. These values were subsequently used to determine whether animals did or did not respond to insulin-induced hypoglycemia.

c) Glycemic Threshold and Hormone Response Magnitudes

The values derived from the basal excursions in saline-injected animals were then used to separate the insulin-injected animals into ACTH and corticosterone non-responders and responders at both times of day. Figure 3 shows the population distribution of responders and non-responders as a function of each animal’s plasma glucose nadir. For each hormone at each time of day, the range of plasma glucose concentrations within which the percentage of responders first exceeded non-responders was considered to contain the glycemic threshold (Fig. 3), as described in the Methods. The behavior of plasma glucose, ACTH, and corticosterone derived from this analysis at each time of day are now discussed.

i) Characteristics of the Plasma Glucose Response to Insulin Given Morning or Evening

To determine whether animals at each time of day received equivalent hypoglycemic challenges, the plasma glucose concentrations of animals above and below the glycemic threshold established in Figure 3 are shown in Figure 4. (Equivalent changes in plasma glucose concentrations of saline-injected animals are shown in Figure 2A).

Figure 4A shows the plasma glucose kinetics of animals injected with insulin at ZT 2 or ZT 11 and had glucose nadirs of less than the glycemic thresholds shown in Figure 3. Insulin injections resulted in a rapid fall in plasma glucose
concentrations between 0 and 10 minutes in all animals. Plasma glucose concentrations in animals at ZT 11 tended to fall slightly more rapidly than those at ZT 2. Thereafter, plasma glucose concentrations were maintained under approximately 3.00mM, and had no discernable differences in kinetics. Critically, plasma glucose nadirs at ZT 2 and ZT 11 were statistically indistinguishable in animals that responded to the hypoglycemia with elevated ACTH and corticosterone secretion.

For animals whose nadirs were greater than the estimated thresholds for ACTH and corticosterone secretion (Fig. 4B), we found that although plasma glucose concentrations fell as rapidly immediately after insulin injections, they tended to increase more rapidly between 10 and 20 minutes at ZT 11 than at ZT 2. This is reflected in the significantly greater plasma glucose concentrations at 15 and 20 minutes after injection at ZT 11 compared to ZT 2. But as with animals whose nadirs were lower than the glycemic threshold (Fig. 4A), the mean nadir plasma glucose concentrations of animals that were above the estimated glycemic thresholds were not significantly different at ZT 2 and ZT 11 (Fig. 4B). Importantly, none of these animals showed a measurable ACTH or corticosterone response to hypoglycemia.

**ii) Characteristics of the Plasma ACTH Response to Morning and Evening Hypoglycemia**

Figure 5 shows the 20-minute ACTH increment of individual animals as a function of their nadir plasma glucose concentrations at ZT 2 (Fig. 5A) and ZT 11 (Fig. 5B). Plotting the frequency distribution of plasma ACTH concentrations of non-responding and responding animals as a function of their plasma glucose nadirs (Figs. 3A & B) showed that the estimated glycemic threshold for ACTH secretion was between 3.00 and 3.40mM at ZT 11. However, despite the fact that the percentage of responders at ZT 2 exceeded that of the non-responders at approximately 3.10mM (Fig. 3A), a glycemic threshold was more difficult to determine at this time-of-day because approximately 10% of non-responding animals at ZT 2 had glycemic nadirs of 2.5mM.

Although no animal with a plasma glucose concentration of greater than 3.10mM showed a plasma ACTH response to hypoglycemia at ZT 2 (Fig. 5A), a consistent response to hypoglycemia was seen in only about 50% of all animals with glucose concentrations below 3.10mM (Fig. 5A) compared to 95% of animals at ZT 11 (Figs. 3B & 5B). 3.10mM was used as the threshold value at ZT 2 for the purposes of post-hoc data grouping.

Figure 5C shows that although there was a clear trend towards an ACTH response to hypoglycemia in animals with glucose concentrations less than 3.10mM at ZT 2, this did not reach significance. However, the mean increase in plasma ACTH at ZT 11 was about four times greater than at ZT 2 (Fig. 5C). Two-way ANOVA revealed a significant effect of time-of-day, above and below threshold and their interaction, [F(1,77)=15.69, P<0.0005] [F(1,77)=38.37, P<0.0001], [F(1,77)=10.73, P<0.002] respectively.

**iii) Characteristics of the Plasma Corticosterone Response to Morning and Evening Hypoglycemia**

Figure 6 shows the 20-minute corticosterone increment of individual animals as a function of their nadir plasma glucose concentrations at ZT 2 (Fig. 6A) and ZT 11 (Fig. 6B). Plotting the frequency distribution of plasma corticosterone concentrations of non-responding and responding animals as a function of their plasma glucose nadirs (Figs. 3C & D) showed that the estimated glycemic thresholds for corticosterone secretion was virtually identical at ZT 2 and ZT 11; thresholds were between 3.15 and 3.25mM at ZT 2, and between 3.00 to 3.30mM at ZT 11.

Figure 6C shows the 20-minute change in mean plasma corticosterone concentration at ZT 2 and ZT 11 depending on whether animals had a nadir plasma glucose concentration above or below the estimated glycemic threshold. The mean increase in plasma corticosterone in animals with glucose concentrations below the thresholds were significantly greater than those above at both times of day. Furthermore, for animals with plasma glucose nadirs below the estimated threshold an almost twofold greater increase in the plasma corticosterone response was observed at ZT 11 than at to ZT 2. Two-way ANOVA revealed a significant effect on mean increases in plasma corticosterone of time-of-day, [F(1,78)=12.97 P<0.001], above and below threshold [F(1,78)=113.00, P<0.0001] and the interaction between them [F(1,78)=6.81, P<0.015].
Characteristics of the Plasma Glucagon Response to Morning and Evening Hypoglycemia

Plasma insufficiency meant that not all samples could be assayed for glucagon making it impossible to determine an accurate glycemic threshold (Figs. 7A & B). However, because significant plasma glucagon responses were consistently detected at glucose concentrations of 3.60mM and below, the threshold was not likely to be less than 3.60mM. Unlike plasma ACTH and corticosterone responses, there was no significant difference between the mean incremental increase in plasma glucagon from animals showing a significant response at ZT 2 and ZT 11 (Fig. 7).

Characteristics of the CRH hnRNA and mRNA Responses in the PVHmp to Morning and Evening Hypoglycemia

In agreement with our previous findings (5), CRH hnRNA levels in the PVHmp exhibited a marked diurnal variation; peak levels were seen at ZT 2 and lowest levels at ZT 11 (Fig. 8). Although there was a tendency towards an increase in CRH hnRNA levels in animals with plasma glucose concentrations less than 3.15mM plasma glucose compared to those above, it failed to reach statistical significance at either time (Fig. 8E). No significant difference in CRH mRNA levels was detected between any treatment groups (data not shown). This result was expected because there is a large pool of CRH mRNA under basal conditions (40) making short-term changes in mRNA content difficult to detect.

Characteristics of the phospho-ERK1/2 Response in the PVHmp to Morning and Evening Hypoglycemia.

There was no difference in the number of phospho-ERK1/2-ir cells in the PVHmp of animals with plasma glucose concentrations greater than 3.15mM at either time of day (Figs. 9A, B). However, the number of phospho-ERK1/2-ir cells in the PVHmp was significantly greater at ZT 2 in animals with plasma glucose concentrations less than 3.15mM compared to those above 3.15mM (p<0.05; Figs. 9C, D & G). Furthermore, the number of phospho-ERK1/2-ir cells in the PVHmp of animals with plasma glucose concentrations less than 3.15mM was 59% greater at ZT 11 than at ZT 2 (Fig. 9G) (p<0.001).

The number of phospho-ERK1/2-ir cells in the PVH was low to undetectable at all times of day in non-manipulated rats (Figs. 9E, F). Two-way ANOVA yielded significance for time-of-day [F(1,50)= 11.60, P<0.002], and below threshold [F(1,50)=38.54, P<0.0001], with a significant interaction between them [F(1,50)= 4.22, P<0.05].

Characteristics of the phospho-ERK1/2 Responses in the NTS and DMX to Morning and Evening Hypoglycemia.

There was no difference in the number of phospho-ERK1/2-ir cells in the NTS of animals with plasma glucose concentrations greater than 3.15mM at either time of day (Figs. 10A, E, I & C, G, K). However, the number of phospho-ERK1/2-ir cells in the NTS was significantly greater both at ZT 2 (Figs. 10B, F, J & M; p<0.01) and ZT 11 (Figs. 10D, H, L & M) (p<0.05) in animals with plasma glucose concentrations less than 3.15mM compared to those above 3.15mM (Fig. 10M). Two-way ANOVA revealed a significant difference in the NTS of animals above or below the estimated glycemic threshold [F(1,35)=23.27 P<0.0001] but no significance was detected in the time-of-day or the interaction. In contrast to responses in the PVHmp, the number of phospho-ERK1/2-ir cells in the NTS of animals with plasma glucose concentrations less than 3.15mM was not different at either time of day (Fig. 10M). It should be noted that the majority of positive cells were found in the medial portion of the NTS (NTSm).

The number of phospho-ERK1/2-ir cells in the DMX of animals with plasma glucose concentrations above 3.15mM was significantly greater in the evening than in the morning (Figs. 10E, I, G, K & N; p<0.01). Phospho-ERK1/2-ir cells were significantly more abundant in the DMX at ZT 2 in animals with plasma glucose concentrations less than 3.15mM compared to those above 3.15mM in the morning [Figs. 10F, J, H, L & N (p<0.05)]. There was no difference in animals above and below the estimated glycemic threshold at ZT 11 and the increments were not statistically different from each other at the two times of day (Fig. 10N). There was no time of day influence on the DMX pERK response to hypoglycemia detected by Two-way ANOVA.
However, there was a significant difference in the response of animals with glycemic nadirs above and below the estimated glycemic threshold \(F(1,34)=8.68, \ P<0.01\), and their interaction \(F(1,34)=5.31, \ P<0.05\).
DISCUSSION

Our results demonstrate that the brain generates two distinct sets of responses when the same degree of hypoglycemia is imposed at ZT 2 and ZT 11. In one set are the responses of plasma glucagon and phospho-ERK1/2 [a marker of rapid neural activation (27, 28)] in the NTSm, where the magnitudes are the same at both times of day. In contrast, plasma ACTH, corticosterone, and phospho-ERK1/2 responses in the PVHmp all show significantly higher magnitudes in the evening. Before discussing the mechanistic implications of these findings, it is worth briefly reviewing what is known about the glucose sensing mechanisms that drive glucocorticoid release during rapid hypoglycemia, and how this sensory information is conveyed to the PVHmp.

Glucose sensing neurons are the principal elements that transduce local brain glucose concentrations into physiologically meaningful firing rate changes (13). Some hypothalamic neurons act as glucose sensors (13, 42-45), and much evidence supports the notion that these neurons make critical contributions to counterregulatory sympathoadrenal and glucagon responses (14, 15, 46). However, glucose sensing mechanisms located in the hypothalamus—particularly those in the region of the ventromedial and arcuate nuclei—seem much less important for the ACTH response to hypoglycemia (18, 46, 47). Moreover, no data have yet emerged showing that glucose sensing mechanisms are located proximal to CRH neurons in the PVH.

Evidence from Ritter and her colleagues supports the notion that the hindbrain rather than the hypothalamus contains the critical glucose sensing mechanisms for stimulating glucocorticoid secretion (18). Furthermore, forebrain-projecting catecholaminergic neurons are required for a full glucocorticoid response to insulin-induced hypoglycemia (19). The majority of these neurons are found in the A2/C2 group of the caudal NTS (24). Critically, a population of hindbrain catecholaminergic neurons that is separate from those driving corticosterone responses is absolutely required to drive the sympathoadrenal response to 2DG (24). These neurons are primarily located in the A5 and rostral C1 groups of the ventrolateral medulla, and the A7 group of the dorsolateral pons (24, 48). Interestingly, catecholaminergic neurons that participate in the sympathetic stimulation of glucagon secretion are located in the same hindbrain regions as those controlling epinephrine secretion (49, 50), one of the major hypoglycemic counterregulatory hormones. It is not known whether any of these catecholaminergic neurons are themselves glucose sensing.

The clear spatial separation between the glucose sensory transduction processes critical for ACTH release and the neuroendocrine motor elements in the PVHmp, together with the requirement of catecholaminergic afferents, means that there are three components required for hypoglycemic stimulation of CRH release, and subsequently ACTH and corticosterone. Each of these could contribute to the observed time-of-day differences. First, glucose sensing processes may change their sensitivity so that equivalent bouts of hypoglycemia across the day generate a stronger response in the evening. Second, the hindbrain networks responsible for transmitting critical information to CRH neurons (including catecholaminergic neurons) are more sensitive to inputs from glucose sensing mechanisms at night, and so generate a commensurately greater output to the PVH. Third, CRH neurons themselves may be differentially sensitive to inputs from the hindbrain across the day, perhaps because of locally-imposed time-of-day variations in arousal state, inputs from the circadian timing system in the suprachiasmatic nucleus, or inputs from glucose sensing mechanisms in the hypothalamus.

We will now discuss our data with respect to each of these three possibilities.

Three of our results do not support the premise that time-of-day variations in mechanisms located distal to the PVH (e.g., glucose sensory systems or their associated hindbrain processing mechanisms) are responsible for the differences in ACTH/corticosterone responses. First, we found the glycemic thresholds that triggered the different morning and evening ACTH/corticosterone responses are indistinguishable using procedures adapted from standard methods for estimating the glycemic threshold (41, 51). Interestingly, similar glycemic thresholds to those we observe here have been reported in humans (22, 52, 53) despite differences in insulin administration (bolus vs.
clamp paradigm) between studies. Our methods may not be sensitive enough to detect subtle variations in glycemic thresholds, but we do not believe these could completely account for the large differences in the nocturnal ACTH/corticosterone responses compared to the morning.

Second, the same hypoglycemic stimulus at each time of day produced plasma glucagon responses that were statistically indistinguishable. However, we do note that there was a tendency for lower plasma glucagon responses in the evening compared to the morning, which is the reverse of the ACTH and corticosterone response we observed in the same animals. A significant body of data supports an important role for central autonomic mechanisms in hypoglycemic stimulation of glucagon secretion in rats (16, 54, 55). Our results are consistent with the idea that separate hindbrain mechanisms contribute to the hypoglycemic drive of ACTH release on the one hand, and glucagon on the other. They also show that these mechanisms each respond differently following equal bouts of hypoglycemia imposed morning or evening.

Third, phospho-ERK1/2 levels were greater in the NTSm when plasma glucose fell below 3.15mM compared to nadirs above 3.15mM. However, in contrast to the responses of PVH phospho-ERK1/2, plasma ACTH, and corticosterone, no time-of-day differences were detected. We also found that neurons in the dorsal motor nucleus of the vagus of euglycemic animals showed a marked and significant increase in phospho-ERK1/2 levels in the evening compared to the morning. These neurons provide the parasympathetic pre-ganglionic innervation for a range of peripheral organs, including the liver and pancreas (16, 54, 55). Our results show that in saline-treated animals there is a daily variation in the activity of these neurons, which is perhaps related to the differing metabolic state across the day. Although we saw significant increases in phospho-ERK1/2 levels in response to hypoglycemia in the morning, no further increase above the already elevated levels was evident in the evening. These data show that hypoglycemia induced in the morning, but not the evening, changes the activity of dorsal motor nucleus neurons from that found in the euglycemic state in a way that involves phosphorylation of ERK1/2.

The absence of a time-of-day difference in these results suggests that the daily differences in the hypoglycemic response of ACTH and corticosterone derive from alterations in the magnitude of information transmission from the hindbrain, changes in CRH neuronal sensitivity driven by local hypothalamic mechanisms, or a combination of both. Recent work by Sanders et al. showed that alterations attenuating counterregulatory responses (after consecutive bouts of hypoglycemia) occur at the level of the hypothalamus rather than the hindbrain (56), thereby supporting the second of these two models.

In this regard, the changes we see in phospho-ERK1/2 levels in the PVHmp following hypoglycemia show the same time-of-day response pattern as ACTH secretory responses in animals with plasma glucose nadirs below the appropriate estimated glycemic threshold, with the greatest increases at night. The fact that phospho-ERK1/2 was undetectable at any time of day or night in unstimulated animals shows that suprachiasmatic nucleus-dependent mechanisms or changes in arousal state do not directly affect ERK1/2 phosphorylation in the PVH. Thus, alterations in the levels of phospho-ERK1/2 following hypoglycemia are a consequence of interactions between the afferent inputs engaged by hypoglycemia and local PVH mechanisms. Furthermore, greater phospho-ERK1/2 immunoreactivity, plasma ACTH, and corticosterone responses were detected at night when insulin doses and, correspondingly, plasma insulin levels were lower than in the morning. These findings strongly suggest that responses in the PVH were dependent on the absolute levels of plasma glucose and not directly to insulin. This conclusion is supported by data from Ritter et al. as well as from our own work (30), where plasma corticosterone responses to both insulin and 2DG (19), as well as phospho-ERK1/2 responses to insulin (30) were markedly attenuated by immunotoxic lesions of catecholaminergic inputs to the PVH.

There was a tendency at both times of day for elevated Crh gene transcriptional responses in
animals with glucose concentrations below 3.15mM, but at neither time was this statistically significant. We did however, confirm our previous observations that there is a significant daily rhythm in \textit{Crh} gene transcription, with highest levels occurring just after lights on (5). We have previously suggested that CRH peptide release and \textit{Crh} gene synthesis are only loosely coupled (11), and that coupling strength depends on both stimulus intensity and temporal dynamics. If synthesis and release coupling depends on stimulus attributes, intensity, and timing, then a deeper sustained hypoglycemia may be required before \textit{Crh} gene transcription is robustly activated, as occurs with the \textit{Crh} gene response to 2DG (19).

In summary, we showed that equal bouts of insulin-induced hypoglycemia led to secretory episodes of ACTH and corticosterone that were greater at night than in the morning only if plasma glucose levels fell below the appropriate estimated glycemic threshold. Phospho-ERK1/2 levels in the PVHmp showed a similar response pattern in animals whose plasma glucose concentrations fell below the estimated glycemic threshold. In contrast, no time-of-day variations were observed in the magnitude of plasma glucagon and phospho-ERK1/2 levels in the NTSm of the same animals following hypoglycemia. Importantly, time-of-day changes in the glycemic thresholds at which ACTH or corticosterone release were activated were not apparent.

These data collectively support a model whereby changes in forebrain mechanisms alter the sensitivity of neuroendocrine CRH neurons in the PVHmp to the hypoglycemia-related information conveyed by ascending catecholaminergic afferents. Strong contenders for mediating these effects are processes in the forebrain that are driven either directly or indirectly by the suprachiasmatic nucleus, perhaps in conjunction with influences from glucose sensing networks located elsewhere in the hypothalamus (13, 45, 57, 58).
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Table 1. Mean (± S.E.M.) plasma glucose, ACTH, corticosterone, insulin, and glucagon concentrations in blood samples taken from animals immediately before they were injected with 0.9% saline or insulin at ZT 2 or ZT 11.

<table>
<thead>
<tr>
<th></th>
<th>Morning (N=36)</th>
<th>Evening (N=45)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mM)</td>
<td>6.48 ± 0.06</td>
<td>6.24 ± 0.08</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>ACTH (pg/ml)</td>
<td>80.5 ± 7.4</td>
<td>107.7 ± 9.1</td>
<td>&lt;0.02</td>
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<tr>
<td>Corticosterone (ng/ml)</td>
<td>12.4 ± 2.4</td>
<td>154.2 ± 13.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>1.8 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Glucagon (pg/ml)</td>
<td>57.7 ± 2.5</td>
<td>43.5 ± 1.8</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

Figure 1. Mean (± S.E.M.) plasma glucose concentration after injection of 0.9% saline at ZT 2 (open squares, n=6) or ZT 11 (closed squares, n=7); or 0.5U/kg insulin at ZT 2 (open circles, n=7) or ZT 11 (closed circles, n=7). Insulin treated animals reached glycemic nadir concentrations approximately 10 minutes after infusion. Plasma glucose concentrations were significantly different at 30 and 60 minutes in animals given insulin at ZT 2 or ZT 11 (*, P<0.05).

Figure 2. The change in plasma ACTH (A) and corticosterone (B) concentrations between 0 and 20 minutes after 0.9% saline infusion. The longer horizontal lines indicate the mean values at each time of day. The dashed arrows and shorter horizontal lines indicate 2 standard deviations above the mean.

Figure 3. Estimated glycemic thresholds for ACTH and corticosterone release at ZT 2 (A, C) and ZT 11 (B, D) were determined by comparing the population distribution (expressed as a percentage) of the non-responding animals (i.e. those that did not mount hormone secretory responses to hypoglycemia; open circles) and responding animals (i.e. those that mounted hormone secretory responses; closed circles) as a function of their glycemic nadirs at ZT 2 and ZT 11. The shaded area in each panel denotes the range of glucose concentrations containing the glycemic threshold (see Methods for details).

Figure 4. The mean (± S.E.M) plasma glucose concentrations from samples taken at baseline, 10, 15, and 20 minutes following insulin injections at ZT 2 (open circles) or ZT 11 (closed circles). Panel A), animals with plasma glucose nadirs below the estimated glycemic threshold established in Figure 3; or B) animals with plasma glucose nadirs above the estimated glycemic threshold. The mean (± S.E.M) glucose nadirs are also indicated (*, p<0.05; **, p<0.005; ns, not significant).

Figure 5. The 0-20 minute plasma ACTH increments for individual animals injected with 0.9% saline or insulin at ZT 2 (A; open symbols) or ZT 11 (B; closed symbols) as a function of their lowest glucose concentration (glycemic nadir). Dashed horizontal lines represent 2 SD above the mean increment of the saline-injected controls at that time (see Figure 2). The vertical shaded bar in B) denotes the range of glucose concentrations containing the glycemic threshold (see Figure 3B and Methods for details). C), the mean (± S.E.M.) change in plasma ACTH concentrations in animals treated at ZT 2 (open bars) or ZT 11 (closed bars). At each time animals were grouped according to whether their glycemic nadirs were greater or less than the appropriate estimated glycemic threshold established in Figure 3B. This was 3.10mM at ZT 2 or 3.00mM at ZT 11. (**, P<0.001).

Figure 6. The 0-20 minute plasma corticosterone increments for individual animals injected with 0.9% saline or insulin at ZT 2 (A; open symbols) or ZT 11 (B; closed symbols) as a function of their lowest glucose concentration (glycemic nadir). Dashed horizontal lines represent 2 SD above the mean increment of the saline-injected controls at that time (see Figure 2). The vertical shaded bars in A) and B) denote the range of glucose concentrations containing the glycemic threshold (see Figures 3C & D, and Methods for details). C), the mean (± S.E.M,) change in plasma corticosterone concentrations in animals treated at ZT 2 (open bars) or ZT 11 (closed bars). At each time animals were grouped according to whether their glycemic nadirs were greater or less than the
appropriate estimated glycemic threshold established in Figure 3. This was 3.15mM at ZT 2 or 3.00mM at ZT 11. (**, P<0.001).

Figure 7. The 0-20 minute plasma glucagon increments for individual animals injected with 0.9% saline or insulin at ZT 2 (A; open symbols) or ZT 11 (B; closed symbols) as a function of their lowest glucose concentration (glycemic nadir). Dashed horizontal lines represent 2 SD above the mean increment of the saline-injected controls at that time. C), the mean (± S.E.M.) change in plasma glucagon concentrations in animals treated at ZT 2 (open bars) or ZT 11 (closed bars). At each time animals were grouped according to whether their glycemic nadirs were greater or less than the 3.60mM. (**, P<0.001).

Figure 8. Autoradiographs of coronal sections hybridized for CRH hnRNA in the PVHmp from representative brains with glucose concentrations above 3.15mM (A-B) or below 3.15mM (C-D) at ZT 2 and ZT 11. Scale bar indicates 100μm. Panel E shows the mean (± S.E.M.) CRH hnRNA levels in the PVHmp in animals grouped by glucose nadir concentrations. Open bars represent ZT 2 treated animals, closed bars represent animals treated at ZT 11 (**, P<0.001).

Figure 9. Photomicrographs of coronal sections showing phospho-ERK1/2-immunoreactive cells in the PVHmp from animals with glucose concentrations above 3.15mM (A-B) or below 3.15mM (C-D) at ZT 2 and ZT 11. Panel E-F shows photomicrographs of the PVHmp from unstimulated animals ZT2 and ZT11. Scale bar indicates 100μm. Panel G shows the mean (± SEM) number of phospho-ERK1/2-immunoreactive cells in the PVHmp in animals grouped by glucose concentration at ZT 2 (open bars) and ZT 11 (closed bars) (*, P<0.05; **, P<0.001). Abbreviations: pml, posterior magnocellular lateral zone; mpd, medial parvicellular part dorsal zone; pv, periventricular part; dp, dorsal parvicellular part.

Figure 10. Photomicrograph of coronal sections containing phospho-ERK1/2-immunoreactive cells through three rostro-caudal levels of the NTS and DMX from animals with glucose concentrations above 3.15mM or below during ZT 2 and ZT 11. Scale bars indicate 100μm. Panel M shows the mean (± SEM) number of phospho-ERK1/2-immunoreactive cells in the NTSm grouped by glucose concentration at ZT 2 (open bars) and ZT 11 (closed bars) (*, P<0.05; **, P<0.001). Panel N shows the mean (± SEM) number of phospho-ERK1/2-immunoreactive cells in the dorsal motor nucleus of the vagus at ZT 2 (open bars) and ZT 11 (closed bars) (*, P<0.05; **P<0.01). Abbreviations: NTSm, medial part; NTSI, lateral part; DMX, dorsal motor nucleus vagus nerve; C, central canal-spinal cord/medulla; ts, solitary tract; V4, fourth ventricle proper, ge, NTS gelatinous part; co, NTS commissural part.