Published in final edited form as:

Physiol Behav. 2007 June 8; 91(2-3): 240–249.

Hypoglycemia Activates Arousal-related Neurons and Increases Wake Time in Adult Rats

Nancy C. Tkacs¹, Yanhua Pan¹, Gagan Sawhney³, Graziella L. Mann², and Adrian R. Morrison²

¹University of Pennsylvania, School of Nursing, Philadelphia, Pennsylvania 19104-6096 USA

²University of Pennsylvania, School of Veterinary Medicine, Philadelphia, Pennsylvania 19104-6096 USA

³University of Pennsylvania, College of Arts and Sciences, Philadelphia, Pennsylvania 19104-6096 USA

Abstract

Hypoglycemia resulting from excess of exogenous or endogenous insulin elicits central nervous system activation that contributes to counterregulatory hormone secretion. In adult humans without diabetes, hypoglycemia occurring during sleep usually produces cortical activation with awakening. However, in adult humans with type 1 diabetes, hypoglycemic arousal appears blunted or absent. We hypothesized that insulin injection sufficient to produce hypoglycemia would induce awakening in adult male rats. Polysomnographic studies were carried out to characterize the effect of insulin injection on measures of sleep and waking during a circadian time of increased sleep. Compared to a baseline day, insulin treatment more than doubled the time spent awake, from $18.4 \pm 2.6\%$ after saline injection to $48.0 \pm 5.5\%$ after insulin. Insulin injection also reduced rapid eye movement sleep (REMS) from $27.3 \pm 1.8\%$ to $5.6 \pm 1.3\%$. The percent of time in non-REM sleep (NREMS) sleep was not different between saline and insulin days, however, NREMS after insulin was fragmented, with increased number and decreased duration of episodes. These electrophysiological data indicate that insulin-induced hypoglycemia is an arousing stimulus in rats, as in nondiabetic adult humans. We also studied the effect of insulin on activation of selected arousal-related neurons using immunohistochemical detection of Fos. Fos-immunoreactivity increased in orexin (OX) neurons after insulin, from $8.7 \pm 4.9\%$ after saline injection to $37 \pm 9\%$ after insulin. Basal forebrain cholinergic nuclei also showed increased Fos-immunoreactivity after insulin. These correlated behavioral and histological data provide targets for future studies of the neural pathways underlying hypoglycemic arousal.

Keywords

insulin; brain; REM sleep; arousal; hypoglycemia; orexin

1. Introduction

Hypoglycemia occurs as a result of relative excess of insulin or an increase in insulin/glucagon ratio in the context of normal or reduced dietary carbohydrate intake. Severe hypoglycemia due to relative insulin excess is a common occurrence in patients treated with exogenous insulin

Corresponding author: Nancy C. Tkacs, PhD, 420 Guardian Drive, Philadelphia, PA 19104-6096, 215-573-3045 (phone), 215-573-7492 (fax), tkacs@nursing.upenn.edu

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

for diabetes mellitus (DM). Recovery from hypoglycemia is promoted by counterregulatory hormones glucagon, epinephrine, cortisol (in humans) or corticosterone (in rats), and growth hormone [1]. The brain is involved in responses to hypoglycemia through several neural and neuroendocrine mechanisms. Hypoglycemia-induced central nervous system (CNS) activation directly mediates secretion of epinephrine, hypothalamo-pituitary-adrenal activation, and growth hormone secretion, as well as enhancing glucagon secretion. CNS activation, and particularly cortical activation, during hypoglycemia is also required for awareness of hypoglycemia, that is, for perception of hypoglycemic symptoms such as sweating, shakiness, and hunger. Finally, neural pathways stimulated by hypoglycemia induce feeding behavior [2]. Hormone responses and symptoms of hypoglycemia tend to be blunted in DM patients treated with insulin, and it is likely that deficient responses to hypoglycemia result from recurrent severe hypoglycemia sustained over many years of insulin replacement [1]

CNS mechanisms of autonomic and endocrine responses to hypoglycemia have been studied in dogs and in rodents, and these studies indicate participation of structures in brainstem as well as hypothalamus [3-12]. Although these animal studies have proven useful in elucidating aspects of neural circuitry underlying counterregulatory hormone secretion, symptom thresholds, per se, cannot be directly assessed in an animal model. One behavioral response to hypoglycemia that may correlate with hypoglycemia awareness is food intake [2]. However, eating behavior may not necessarily be equivalent to cortical activation comparable to “awareness”, in that insulin-induced feeding is preserved even in decorticate preparations [13]. Recent research indicates that hypoglycemia produces arousal from sleep in normal adult humans [14,15]. However, this hypoglycemic arousal response is blunted or absent in adults with type 1 diabetes mellitus (DM). When nocturnal polysomnography was carried out during hyperinsulinemic, stepped hypoglycemic clamps, sustained hypoglycemia (2.5 mmol/l) reduced sleep efficiency to 26% in nondiabetic adults, a significant difference from subjects with type 1 DM, who maintained 77% sleep efficiency at this level of blood glucose [14]. Due to this defective hypoglycemic arousal, in patients with intensively managed type 1 DM, episodes of severe hypoglycemia are disproportionately higher at night or during sleep compared to waking [16].

Hypoglycemia in rats strongly activates neurons containing the wake-promoting peptide orexin (OX), also known as hypocretin [17-19] however, there have been no reports to date of the effects of hypoglycemia on sleep/wake measures in rats. In this study, we sought to characterize the interaction between hypoglycemia and sleep in rats studied in the afternoon, a diurnal time associated with increased time spent in sleep in rats. We hypothesized that administering insulin to induce hypoglycemia during the afternoon in rats would produce polysomnographic signs of arousal from sleep. We also aimed to characterize activation of brain arousal-related, and putatively glucose-detecting neurons, during hypoglycemia at a depth sufficient to cause behavioral activation but avoiding severe hypoglycemia that results in obtundation or coma.

Neuronal activation was evaluated using immunohistochemical detection of Fos, a marker of neuronal activity [20,21]. Fos mapping was carried out for lateral hypothalamic orexin neurons and neurons of the pontine nucleus locus coeruleus (LC) - two neuronal groups that are implicated directly or indirectly in both arousal and glucose detection. In addition, quantification of hypoglycemia-associated Fos staining was carried out for basal forebrain and brainstem cholinergic neurons that are closely associated with cortical activation.

We propose that the increased waking we measured in rats treated with insulin injection is, in part, a consequence of activation of several groups of arousal-related neurons, including those that are directly sensitive to hypoglycemia. We further propose that polysomnographic detection of cortical activation is a novel, quantifiable, and clinically relevant marker of hypoglycemia-associated alterations in brain function in rats.

2. Methods

2.1 Subjects

Male Sprague-Dawley rats weighing 225-250g were purchased from Charles River Laboratories (Kingston, NY). During the first week after shipping rats were habituated to handling procedures. All animal procedures followed protocols approved by the University of Pennsylvania Institutional Animal Care and Use Committee. Rats were group-housed in cages in a temperature-controlled animal facility with 12h:12h light:dark cycle (lights on at 0700 h, lights off at 1900 h).

2.2 Experiment 1: The effect of moderate afternoon hypoglycemia on activation of arousal-related neurons

2.2.1 Animal preparation—Rats were prepared with indwelling jugular venous catheters under anesthesia with intramuscular ketamine/xylazine (87 mg/kg/13 mg/kg respectively). Beginning with the third postoperative day, rats were brought in their home cages to the experiment room for habituation to the recording conditions. Extension tubing made of silastic was connected to the jugular catheter and led to the outside of the cage through a plexiglass lid.

The rats were observed for 1.5-2 hours until they were quiet or sleeping. Each rat had 2-3 training sessions before the day of the experiment. On that day, rats were brought to the experiment room, food was removed from the cage, and catheter extensions were connected at 1100 hours. The rats were observed until 1300 hours, at which time they were given regular insulin, 3 U/kg subcutaneously (insulin group, n=8). Controls received saline, 1 ml/kg subcutaneously (control group, n=6).

2.2.2 Blood sampling—Blood samples (< 100 µl) for glucose and lactate were drawn using the jugular venous catheter at baseline, and then every 30 minutes after the insulin injection. The total blood volume withdrawn was less than 0.5 ml over the two-hour recording period. Samples were immediately analyzed using a YSI 2700 glucose/lactate analyzer (Yellow Springs Instrument Company, Yellow Springs, Ohio). Due to catheter malfunction and a technical problem with the glucose/lactate analyzer, glucose data shown here are based on 6 of the 8 insulin-treated rats, and lactate data are shown from 4 of the insulin-treated rats.

2.2.3 Euthanasia and perfusion—After the 120-minute blood sample, rats were deeply anesthetized with sodium pentobarbital 70-80 mg/kg intravenously and were perfused transcardially with heparinized saline followed by 10% phosphate buffered formalin (Fisher Scientific Company). Following perfusion, the brain was removed and post-fixed in buffered formalin for 24 hours, then equilibrated in 30% sucrose for 48 hours, before being frozen in -50° C isopentane.

2.2.4 Histological preparation and analyses—Forebrains and brainstems were sectioned at 40 µm on a Leica cryostat. Sections were serially saved in 6 aliquots of cryoprotectant solution and stored in tubes of cryoprotectant at -24° C until processing. Immunohistochemical detection of Fos protein was carried out using a polyclonal antibody raised in rabbit (Ab-5, originally developed by Oncogene Sciences, presently available from the Calbiochem division of EMD Biosciences; dilution 1:20,000). This antibody is raised to a synthetic peptide comprising amino acid residues 4-17 of human Fos, reacts with the 55 kDa Fos protein, and does not show cross-reactivity with other Fos-related antigens or with Jun. Primary antibody was visualized using a Vector Elite ABC kit (Vector - Burlingame, CA), following the manufacturer's instructions, with the exception that the reagents were used at one-half strength, and incubations in second antibody and ABC reagent were increased to 90

minutes. This protocol has been used in our laboratory previously to reduce background staining in immunohistochemical reactions [22]. The chromogen for Fos detection was diaminobenzidine (Sigma, St. Louis, MO), producing a brown reaction product in Fos+ neuronal nuclei. After completing Fos immunohistochemistry, the sections were placed into the next primary antibody, either a mouse monoclonal antibody to OX A (R&D monoclonal MAB763; dilution 1:8000), or a rabbit polyclonal antibody to choline acetyltransferase (ChAT - Chemicon AB5042P; dilution 1:3000). After ABC amplification with the Vector system, modified as described above, the second primary antibody was visualized with the Vector SG substrate, producing a gray-blue reaction product. Observers unaware of the treatment group of the individual rats performed the quantification of Fos staining using an Olympus microscope and 100X magnification for an initial count. Higher magnification was used to confirm double-labeled cells identified at lower magnification.

Lateral hypothalamus Staining with Fos and OX was quantified by counting cells stained for OX alone, OX + Fos, and Fos staining occurring within the boundaries of the entire region containing OX cell bodies. Unilateral counts were made on two sections with the greatest number of OX -stained profiles, corresponding to a level -3.14 through -3.0 mm caudal to bregma. These counts were summed for the total counts reported here.

Forebrain cholinergic cell groups Cells stained for ChAT alone, Fos alone, or ChAT + Fos were counted on 5 sections representing atlas levels 1.00, .48, .20, -.40 and -1.00 mm from bregma. From rostral to caudal, quantitation targeted the following cholinergic nuclei: medial septal (MS) at levels 1.00, .48, and .2 mm rostral to bregma, diagonal band of Broca (DBB) at levels .48 and .20 mm rostral to bregma, and magnocellular preoptic nucleus (MCPO) at levels -.40 and -1.00 mm caudal to bregma. Counts were made unilaterally on the side of the brain section that most closely matched the relevant atlas level. Total counts reported represent the sum of counts at the indicated rostrocaudal levels through each nucleus. Mapping and terminology (as shown in figure 1) followed the conventions used in Gritti et al., 1993 [23].

Brainstem cholinergic cell groups Sections from 9 rats (6 insulin-treated, 3 saline controls) were stained for Fos and ChAT immunoreactivity and counts of single and double-labeled cells were made at 4 levels spanning the cholinergic laterodorsal tegmental and pedunculopontine nuclei.

Locus coeruleus Counts of Fos-stained cells were made in brainstem sections by visual determination of brainstem shape, 4th ventricle orientation, and proximity to the mesencephalic nucleus of the trigeminal nerve, which is easily identifiable in Fos-stained sections. These counts were made on sections at which the 7th cranial nerve is visible but only appears in the ventral 20-30% of the brainstem. The approximate rostrocaudal level was -9.7 through -9.8 from bregma. Fos counts were made on two sections in which the LC was largest for that series, and these counts were summed, giving the totals reported here.

2.2.5 Statistical analysis—Data were analyzed by two-tailed unpaired t-test, with correction for nonhomogeneity of variance as needed (SPSS 12.0, North Carolina). Data are presented as means $\pm$ SEM.

2.3 Experiment 2: The effect of insulin-induced hypoglycemia on sleep

2.3.1 Animal preparation—Under aseptic conditions, adult, male Sprague-Dawley rats (~300g) were anesthetized with ketamine/xylazine induction (87 mg/kg/13 mg/kg, respectively) and maintained with isoflurane and were instrumented with skull electrodes for chronic recording of the cortical EEG and electromyogram (EMG) from dorsal neck muscles.

Rats were allowed to recover from the surgery for one week with daily monitoring for signs of discomfort.

2.3.2 Sleep recording—Rats were then habituated for 3 days to having their head plugs attached to a cable connected to a freely rotating swivel in the sleep-recording chamber. On the fourth day, the rat was placed in the sleep chamber at 1100h and connected for recording. At 1300h the rat was given an injection of saline, 1 ml/kg sc. Sleep monitoring continued until 1500h, after which the recording apparatus was disconnected and the rat was returned to animal housing. Two days later, the rat was again placed in the sleep chamber at 1100h. At 1300h the rat was given regular human insulin (3U/kg, sc) and sleep was again monitored until 1500h. In this study we did not randomize the order of the treatments, out of concern of a conditioning effect of prior hypoglycemia on later responses in the sleep recording environment. Blood sampling was not done during polysomnography, in order to avoid disturbing the rats in the sleep recording chamber. However, we assume that the glucose response was similar to that measured in the Fos study and in additional pilot dose-response experiments, with gradually decreasing glucose stabilizing at approximately 2.5 mmol/l in the second hour after the insulin injection.

2.3.3 Signal and sleep data processing—EEG and EMG were recorded using standard techniques, with the EEG signal filtered at 0.3-100 Hz and EMG signals at 30-1000 Hz. The signals were digitized and stored on a hard disk using a Cambridge Electronics signal interface and Spike-2 software. Somnologica software (Flaga hf. Medical Devices, Reykjavik, Iceland) was used to score the behavioral state of the animal in successive 10 s epochs as wakefulness (W), rapid eye movement sleep (REMS), and non-rapid eye movement sleep (NREMS). Scoring was based on motor activity on EMG and on EEG relative power in delta (2.0-4.5 Hz) and theta (4.5-8.0 Hz) frequencies, as well as the ratio of beta (20-25 Hz) to delta activity. The following parameters were evaluated in two successive hours after insulin administration to assess the effect of hypoglycemia on sleep and arousal: 1) total time spent in W, NREMS and REMS; 2) mean number and duration of W, NREMS and REMS episodes; and 3) percent of time spent in W, NREMS and REMS.

2.3.4 Statistical analyses—Analysis excluded the initial 30 minutes after saline or insulin injection, as rats showed varying degrees of activity immediately after the injection. Sleep data totals from 30-120 minutes after saline or insulin injection were analyzed using paired t-tests. In addition, sleep data in 30-minute bins (30-60 minutes, 60-90 minutes, and 90-120 minutes after injection) were compared with two-way repeated measures analysis of variance to determine the effect of day (saline vs insulin) and time. Post hoc testing used paired t-tests with the Bonferroni correction.

3. Results

3.1 Experiment 1: Insulin treatment induces hypoglycemia and induces Fos in brain arousal-related neurons

Glucose was measured every 30 minutes for two hours in 6 rats with chronic indwelling jugular venous catheters for blood sampling after subcutaneous insulin administration. Insulin treatment reduced blood glucose from a baseline of 6.2 ± 0.2 mmol/l, to 3.1 ± 0.2 mmol/l 30 min after injection. Blood glucose then stabilized at approximately 2.5 mmol/l in the second hour after insulin (Table 1). Lactate measurements were performed in 4 of the catheterized rats (Table 1). Lactate increased in the second hour after insulin, an indirect indicator of sympathetic activation resulting in plasma epinephrine secretion [24]. For the Fos experiments, a total of 8 rats made up the insulin-treated group, including 6 rats with indwelling jugular venous

catheters, and 2 additional rats without catheters. 6 rats were injected with saline and comprise the control group. All rats were perfused 2 hr after subcutaneous injection of insulin or saline.

Lateral hypothalamic OX cells—OX neurons showed significant activation 2 h after insulin injections, as evidenced by a significant difference in absolute numbers of Fos/OX double-labeled cells, with insulin treatment resulting in 88 ± 22 double-labeled cells and saline treatment resulting in 19 ± 11 cells (Figure 2). The number of Fos+/non-OX cells in the lateral hypothalamus was increased after insulin injection (saline: 87 ± 13 cells; insulin: 113 ± 15 cells), however, this difference did not achieve statistical significance.

Forebrain cholinergic cell groups—Significant Fos induction was observed in forebrain cholinergic regions after insulin injection, including the MS, DBB, and MCPO (Figures 2 and 3) when compared to saline injection, however the effect was modest in MS. In addition, insulin treatment was associated with a significant increase in absolute numbers of Fos+/ChAT- (presumably noncholinergic) cells in the DBB (saline 16 ± 4 ; insulin: 26 ± 3) and in the MCPO (saline: 14 ± 4 ; insulin: 44 ± 3).

Brainstem cholinergic cell groups—There were few or no double-labeled cells in the laterodorsal tegmental and pedunculopontine tegmental brainstem cholinergic cell groups in either insulin- or saline- injected rats (Figure 3). Fos counts in ChAT- cells in these cell groups showed a trend towards greater numbers after insulin treatment than after saline, however, these differences were not statistically significant (LDT: 73 ± 12 (insulin) vs 45 ± 16 (saline), $p=0.2$; PPT: 27 ± 4 (insulin) vs 13 ± 3 (saline), $p=0.06$).

Locus coeruleus—Although LC Fos staining tended to be greater in hypoglycemic brains (24 ± 9.6 Fos-labeled cells) vs control brains (4 ± 2.3), the effect did not reach statistical significance ($p=0.058$).

3.2 Experiment 2: Insulin treatment produces arousal from sleep

A paired design was used to evaluate the effect of insulin-induced hypoglycemia on sleep and wake amounts. Baseline data were collected after subcutaneous injection of saline. This measurement was made on the first day of sleep recording, to avoid a potential conditioning effect of behavioral responses to insulin [25]. On the hypoglycemia day, subcutaneous insulin injection was performed using the dose previously characterized in the Fos studies.

Behavioral responses to insulin or saline injections were assessed qualitatively by observing rats at regular intervals through the window of the sleep- recording chamber on the baseline day and after insulin injection. Brief episodes during which the rat appeared to be searching for food in the cage were noted in most, but not all of the rats during the two hour period after insulin injection. However, this exploratory activity was not sustained, and for most of the recording period rats were in a state characterized as quiet waking. They most often assumed a recumbent position, but not usually curled up in sleep in the manner of rats observed after saline injection. The EMG records corroborate these observations, showing brief periods of increased motor activity, but overall low motor activity comparable to that recorded on the baseline day.

Arousal was significantly increased after insulin injection. Qualitatively, the typical pattern of cycling between sleep and waking seen after saline injection was preserved after insulin injection. Beginning 30 minutes after insulin, however, far fewer sleep bouts were associated with REMS. Thus, the increase in W was due primarily to a profound reduction in REMS, with a modest reduction in NREMS that did not achieve statistical significance. Similar results were obtained when analyzing the percent of time spent in W, REMS, and NREMS (Figure 4).

After insulin injection, the structure of sleep was altered with respect to number of episodes of W, NREMS and REMS. During the period 30-120 minutes after insulin injection, the number of REMS episodes was reduced from 10 ± 1.2 to 3.2 ± 0.7 ($p=0.002$). On polysomnographic analysis, sleep appeared fragmented, with more rapid cycling between W and NREMS, longer W episodes, and shorter NREMS episodes. This can also be seen on a plot of absolute EEG delta power over the two hours after injection (Figure 5). After injection of saline, there are periods lasting from 5-9 minutes, characterized by high delta power and a predominance of NREMS with brief waking episodes. These episodes are separated by periods of extremely low delta power associated with REMS. After insulin injection, the total delta power does not change (summed from 30-120 minutes after injection), however, associated with the lack of REMS, there are very few brief periods of low delta power. Episodes of high delta power are broken up by many brief descents, giving an appearance of rapid cycling between waking and NREMS, which is also reflected in the hypnogram. Analysis of all of the sleep records during the time period from 30 to 120 minutes after insulin injection demonstrated that the number of W and NREMS episodes were increased with a significant decrease in the duration of NREMS episodes (Figure 6).

4. Discussion

The major new finding reported here is that insulin-induced hypoglycemia reliably increases arousal in adult male rats, similar to the effect of nocturnal hypoglycemia in adult humans. To our knowledge, the data presented here are the first to use polysomnography to characterize hypoglycemic arousal in a rodent model, correlated with functional mapping of selected chemically identified brain arousal systems. Reduced sleep during hypoglycemia was associated with a profound reduction in REM sleep. Total time in NREMS was not significantly reduced after insulin injection, however, this preservation of NREMS time was accomplished despite fragmentation of NREMS as seen by more rapid cycling of NREMS episodes of shorter duration, separated by W episodes.

Our data indicate that insulin injection results in increased time spent in waking in rats, as well as causing activation of several major wake-related neuron groups, as demonstrated by the presence of nuclear Fos-immunoreactivity in wake-related orexinergic and cholinergic neurons. The use of subcutaneous injection may be expected to result in stress-related arousal; however, it should be noted that rats in this study returned to a normal sleep pattern by 30 minutes after injection, and our data collection and comparison excluded this time period. The relative amounts of W, NREMS and REMS on the baseline day from 30-120 minutes after saline injection are comparable to a recent sleep study from our laboratory conducted from 1300-1500 hours that did not involve subcutaneous injections [26]. Although a hyperinsulinemic, euglycemic group was not included in this study, we believe that the effect of insulin treatment on arousal reported here is due to hypoglycemia, rather than hyperinsulinemia, for the following reasons: 1) Hyperinsulinemic euglycemic clamp studies in normal humans are not associated with significant autonomic activation or development of hypoglycemic symptoms [27]. 2) In the study of Banarer and Cryer, altered sleep efficiency during stepped hypoglycemic clamp was not reported for non-diabetic humans studied at the 85, 75, or 65 mg/dl steps, only during the 55 and 45 mg/dl steps [14].

As identified by other investigators and confirmed here, OX cells in the lateral hypothalamus/perifornical region have significantly increased Fos-immunoreactivity after insulin injection [17-19]. OX neuron activation is to be expected in this model due to at least two mechanisms. First, *in vitro* electrophysiological studies indicate that OX cells are directly stimulated by low glucose [28]. Second, activation of corticotrophin-releasing factor (CRF) neurons in response to stressors such as restraint and footshock is implicated in producing behavioral activation and Fos expression in OX neurons [29,30]. In our study, Fos staining was also observed in the

medial parvocellular division of the hypothalamic paraventricular nucleus, location of CRF neurons (data not shown). Intracerebroventricular injection of OX is associated with increased time in waking, profound reduction in REMS, modest reduction of NREMS, and increased motor activity [31-34]. Our results show a similar profile of changes in W and REMS, as well as fragmentation of NREMS during insulin-induced hypoglycemia, however, increased motor activity was not a consistent finding during hypoglycemia. These data suggest that increased W and reduced REMS during insulin-induced hypoglycemia may be a functional consequence of OX neuron activation, and this relationship bears further study.

Basal forebrain cholinergic neurons are thought to be a major final pathway for arousal signals ascending from the brainstem and hypothalamus [35]. We found significant activation of cholinergic cells in the medial septum, diagonal band of Broca and in the magnocellular preoptic nucleus after insulin injection. Our results are consistent with a report of increased Fos expression in basal forebrain ChAT+ cells during enforced waking, although that report did not demonstrate a significant effect of waking on medial septal cholinergic neurons [36]. In our study, medial septal cholinergic neurons also demonstrated the smallest effect of insulin treatment on Fos expression.

Brainstem cholinergic neurons are implicated in cortical arousal during REMS, and our data demonstrate that these neurons do not show increased Fos expression during hypoglycemia, when REMS is suppressed. These data are consistent with the observation of Maloney et al. [37] that behavioral states associated with low REM sleep correlate with reduced Fos-immunoreactivity in laterodorsal tegmental and pedunculopontine tegmental cholinergic neurons. Our findings differ from those of Maloney et al., in that control rats injected with saline and perfused two hours later did not have increased Fos in brainstem cholinergic cells during a circadian time that should have been characterized by increased REMS. However, our findings are consistent with another report of absence of Fos in brainstem cholinergic neurons in control animals [38].

We quantified Fos-ir in LC neurons because of prior studies indicating that LC neurons show increased action potential activity during induced hypoglycemia in cats [39]. In addition, LC neurons express relatively high levels of glucokinase, a putative mediator of neuronal glucose-sensing [40]. LC neurons showed a trend toward activation during hypoglycemia, although the difference did not achieve statistical significance in this study. The modest effect of hypoglycemia on LC Fos expression may be related to the level of hypoglycemia we induced in this behavioral study. A previous study reported that subcutaneous insulin injections that reduced blood glucose to 30 mg/dl resulted in significant Fos-immunoreactivity in LC neurons [41].

The rat has been proposed as a useful model to study sleep and pathophysiological sleep disruption [42]. The functional neuroanatomy of arousal has been extensively studied in this species [35], and there have been several Fos-mapping studies related to sleep and arousal states [32,36,37,43]. Our data as well as previous studies demonstrating that insulin-induced hypoglycemia strongly stimulates Fos production in OX neurons [17-19] predict that hypoglycemia should increase wake time in rats. In this study, we indeed found a significant increase in waking during insulin-induced hypoglycemia. The characteristics of arousal after insulin injection include a profound reduction in REM sleep as well as increased fragmentation of NREMS. These insulin-induced changes in sleep-wake cycles are similar to those observed experimentally after intra-cerebroventricular injection of OX in rats [31,33,34]. Additional experiments are needed to elucidate a potential role of OX in hypoglycemic arousal.

In addition to the sleep studies noted above, rodent models have proven useful in elucidating the neurobiological and neuropathological changes after recurrent hypoglycemia, a common

complication of insulin-treated diabetes [9,44]. A limitation of the rodent model for hypoglycemia studies is the difficulty in assessing hypoglycemic symptoms that indicate the presence of hypoglycemia awareness. In an attempt to evaluate hypoglycemic symptoms, some studies have used food intake as an indicator of hypoglycemia induced “hunger” [2,45]. Other electrophysiological and behavioral studies in rats have evaluated neuroglycopenic changes in evoked potentials or spontaneous alternation performance [46,47]. The present findings document that hypoglycemia produces cortical activation resulting in arousal from sleep in rats, correlated with functional mapping of selected chemically identified brain arousal systems. We propose measurement of hypoglycemic arousal as an informative marker of cortical activation secondary to hypoglycemia in rats. Future studies in the rodent model are indicated to further investigate the neurochemistry of arousal during hypoglycemia, and to evaluate the effect of recurrent hypoglycemia, similar to that experienced by patients with type 1 DM, on hypoglycemic arousal.

ACKNOWLEDGEMENTS

The authors thank Dr. Leszek Kubin for helpful discussions and comments on an earlier version of the manuscript.

Grants NCT: NIDDK Career Development Award (DK002899) and the University Research Foundation, University of Pennsylvania, GS: University of Pennsylvania Nassau Fund undergraduate research award.

References

- [1]. Cryer PE. Hypoglycemia: The limiting factor in the management of IDDM. *Diabetes* 1994;43:1378–1389. [PubMed: 7926315]
- [2]. Sanders NM, Figlewicz DP, Taborsky GJ, Wilkinson CW, Daumen W, Levin BE. Feeding and neuroendocrine responses after recurrent insulin-induced hypoglycemia. *Physiol Behav* 2006;87:700–706. [PubMed: 16492385]
- [3]. Beverly JL, DeVries MG, Bouman SD, Arsenau LM. Noradrenergic and GABAergic systems in the medial hypothalamus are activated during hypoglycemia. *Am J Physiol* 2001;280:R563–R569.
- [4]. Biggers DW, Myers SR, Neal D, Stinson R, Cooper NB, Jaspan JB, Williams PE, Cherrington AD, Frizzell RT. Role of brain in counterregulation of insulin-induced hypoglycemia in dogs. *Diabetes* 1989;37:7–16. [PubMed: 2642436]
- [5]. Borg WP, During MJ, Sherwin RS, Borg MA, Brines ML, Shulman GI. Ventromedial hypothalamic lesions in rats suppress counterregulatory responses to hypoglycemia. *J Clin Invest* 1994;93:1677–1682. [PubMed: 8163668]
- [6]. Borg MA, Sherwin RS, Borg WP, Tamborlane WV, Shulman GI. Local ventromedial hypothalamus glucose perfusion blocks counterregulation during systemic hypoglycemia in awake rats. *J Clin Invest* 1997;99:361–365. [PubMed: 9006005]
- [7]. Cane P, Haun CK, Evered J, Youn JH, Bergman RN. Response to deep hypoglycemia does not involve glucoreceptors in carotid perfused tissue. *Am J Physiol* 1988;255:E680–E687. [PubMed: 2847539]
- [8]. Davis SN, Dunham B, Walmsley K, Shavers C, Neal D, Williams P, Cherrington AD. Brain of the conscious dog is sensitive to physiological changes in circulating insulin. *Am J Physiol* 1997;272:E567–E575. [PubMed: 9142876]
- [9]. De Vries MG, Arsenau LM, Lawson MA, Beverly JL. Extracellular glucose in rat ventromedial hypothalamus during acute and recurrent hypoglycemia. *Diabetes* 2003;52:2767–2773. [PubMed: 14578295]
- [10]. Evans SB, Wilkinson CW, Bentson K, Gronbeck P, Zavosh A, Figlewicz DP. PVN activation is suppressed by repeated hypoglycemia but not antecedent corticosterone in the rat. *Am J Physiol* 2001;281:R1426–R1436.
- [11]. Frizzell RT, Jones EM, Davis SN, Biggers DW, Myers SR, Connolly CC, Neal DW, Jaspan JB, Cherrington AD. Counterregulation during hypoglycemia is directed by widespread brain regions. *Diabetes* 1993;42:1253–1261. [PubMed: 8349036]

- [12]. Marin-Spiotta A, Levin BE, Tkacs NC. A single episode of central glucoprivation reduces the adrenomedullary response to subsequent hypoglycemia in rats. *Neurosci Lett* 2004;360:81–84. [PubMed: 15082184]
- [13]. Flynn FW, Grill HJ. Insulin elicits ingestion in decerebrate rats. *Science* 1983;221:188–190. [PubMed: 6344221]
- [14]. Banarer S, Cryer PE. Sleep-related hypoglycemia-associated autonomic failure in Type 1 diabetes: Reduced awakening from sleep during hypoglycemia. *Diabetes* 2003;52:1195–1203. [PubMed: 12716752]
- [15]. Gais S, Born J, Peters A, Schultes B, Heindl B, Fehm HL, Kern W. Hypoglycemia counterregulation during sleep. *Sleep* 2003;26:55–59. [PubMed: 12627733]
- [16]. DCCT. Epidemiology of severe hypoglycemia in the diabetes control and complications trial. *Am J Med* 1991;90:450–459. [PubMed: 2012085]
- [17]. Moriguchi T, Sakurai T, Nambu T, Yanagisawa M, Goto K. Neurons containing orexin in the lateral hypothalamic area of the adult rat brain are activated by insulin-induced acute hypoglycemia. *Neurosci Lett* 1999;264:101–104. [PubMed: 10320024]
- [18]. Paranjape SA, Vavaiya KK, Kale AY, Briski KP. Habituation of insulin-induced hypoglycemic transcription activation of lateral hypothalamic orexin-A-containing neurons to recurring exposure. *Regulatory Peptides* 2006;135:1–6. [PubMed: 16678283]
- [19]. Cai XJ, Evans ML, Lister CA, Leslie RA, Arch JRS, Wilson S, Williams G. Hypoglycemia activates orexin neurons and selectively increases hypothalamic orexin-B levels: Responses inhibited by feeding and possibly mediated by the nucleus of the solitary tract. *Diabetes* 2001;50:105–112. [PubMed: 11147774]
- [20]. Chan RKW, Brown ER, Ericsson A, Sawchenko PE. A comparison of two immediate-early genes, *c-fos* and *NGFI-B*, as markers for functional activation in stress-related neuroendocrine circuitry. *J Neurosci* 1993;13:5126–5138. [PubMed: 8254363]
- [21]. Morgan JI, Cohen DR, Hempstead JL, Curran T. Mapping patterns of *c-fos* expression in the central nervous system after seizure. *Science* 1987;237:192–197. [PubMed: 3037702]
- [22]. Tkacs NC, Li J. Immune stimulation induces Fos expression in brainstem amygdala afferents. *Brain Res Bull* 1999;48:223–231. [PubMed: 10230714]
- [23]. Gritti I, Mainville L, Jones BE. Codistribution of GABA- with acetylcholine-synthesizing neurons in the basal forebrain of the rat. *J Comp Neurol* 1993;329:438–457. [PubMed: 8454735]
- [24]. Winder WW, MacLean PS, Chandler SL, Huang W, Mills RH. Role of epinephrine during insulin-induced hypoglycemia in fasted rats. *J Appl Physiol* 1994;77:270–276. [PubMed: 7961244]
- [25]. Sanders NM, Levin BE, Taborsky GJ, Zavosh A, Gronbeck P, Dauman W, Figlewicz DP. Prior hypoglycemia induces a conditioned anticipatory feeding response despite impaired hormonal counterregulatory responses (CRR). *Diabetes* 2005;54(suppl 1):640-P.
- [26]. Lu JW, Mann GL, Ross RJ, Morrison AR, Kubin L. Differential effect of sleep-wake states on lingual and dorsal neck muscle activity in rats. *Respiratory Physiology & Neurobiology* 2005;147:191–203. [PubMed: 15964252]
- [27]. DeRosa MA, Cryer PE. Hypoglycemia and the sympathoadrenal system: neurogenic symptoms are largely the result of sympathetic neural, rather than adrenomedullary, activation. *Am J Physiol Endo Metab* 2004;287:E32–E41.
- [28]. Burdakov D, Gerasimenko O, Verkhatsky A. Physiological changes in glucose differentially modulate the excitability of hypothalamic melanin-concentrating hormone and orexin neurons *in situ*. *J Neurosci* 2005;25:2429–2433. [PubMed: 15745970]
- [29]. Winsky-Sommerer R, Yamanaka A, Diano S, Borok E, Roberts AJ, Sakurai T, Kilduff TS, Horvath TL, de Lecea L. Interaction between the corticotropin-releasing factor system and hypocretins (orexins): a novel circuit mediating stress response. *J Neurosci* 2004;24:11439–11448. [PubMed: 15601950]
- [30]. Winsky-Sommerer R, Boutrel B, de Lecea L. Stress and Arousal: The corticotrophin-releasing factor/hypocretin circuitry. *Molecular Neurobiology* 2005;32:285–294. [PubMed: 16385142]
- [31]. Bourgin P, Huitrón-Reséndiz S, Spier AD, Fabre V, Morte B, Criado JR, Sutcliffe JG, Henriksen SJ, de Lecea L. Hypocretin-1 modulates rapid eye movement sleep through activation of locus coeruleus neurons. *J Neurosci* 2000;20:7760–7765. [PubMed: 11027239]

- [32]. España RA, Valentino RJ, Berridge CW. Fos immunoreactivity in hypocretin-synthesizing and hypocretin-1 receptor-expressing neurons: Effects of diurnal and nocturnal spontaneous waking, stress, and hypocretin-1 administration. *Neurosci* 2003;121:201–217.
- [33]. Hagan JJ, Leslie RA, Patel S, Evans ML, Wattam TA, Holmes S, Benham CD, Taylor SG, Routledge C, Hemmati P, Munton RP, Ashmeade TE, Shah AS, Hatcher JP, Hatcher PD, Jones DNC, Smith MI, Piper DC, Hunter AJ, Porter RA, Upton N. Orexin A activates locus coeruleus cell firing and increases arousal in the rat. *Proc Nat Acad Sci USA* 1999;96:10911–10916. [PubMed: 10485925]
- [34]. Piper DC, Upton N, Smith MI, Hunter AJ. The novel brain neuropeptide, orexin-A, modulates the sleep-wake cycle of rats. *Eur J Neurosci* 2000;12:726–730. [PubMed: 10712652]
- [35]. Jones BE. Arousal systems. *Front Biosci* 2003;8:438–451.
- [36]. Greco MA, Lu J, Wagner D, Shiromani PJ. c-Fos expression in the cholinergic basal forebrain after enforced wakefulness and recovery sleep. *Neuroreport* 2000;11:437–440. [PubMed: 10718290]
- [37]. Maloney KJ, Mainville L, Jones BE. Differential c-Fos expression in cholinergic, monoaminergic, and GABAergic cell groups of the pontomesencephalic tegmentum after paradoxical sleep deprivation and recovery. *J Neurosci* 1999;19:3057–3072. [PubMed: 10191323]
- [38]. Verret L, Léger L, Fort P, Luppi P-H. Cholinergic and noncholinergic brainstem neurons expressing Fos after paradoxical (REM) sleep deprivation and recovery. *Eur J Neurosci* 2005;21:2488–2504. [PubMed: 15932606]
- [39]. Morilak DA, Fornal CA, Jacobs BL. Effects of physiological manipulations on locus coeruleus neuronal activity in freely moving cats. III. Glucoregulatory challenge. *Brain Res* 1987;422:32–39. [PubMed: 3315119]
- [40]. Lynch RM, Tompkins LS, Brooks HL, Dunn-Meynell AA, Levin BE. Localization of glucokinase gene expression in the rat brain. *Diabetes* 2000;49:693–700. [PubMed: 10905475]
- [41]. Yuan P-Q, Yang H. Neuronal activation of brain vagal-regulatory pathways and upper gut enteric plexuses by insulin hypoglycemia. *Am J Physiol Endo Metab* 2002;283:E436–E448.
- [42]. Datta S, Hobson JA. The rat as an experimental model for sleep neurophysiology. *Behavioral Neuroscience* 2000;114:1239–1244. [PubMed: 11142656]
- [43]. Estabrooke IV, McCarthy MT, Ko EM, Chou TC, Chemelli RM, Yanagisawa M, Saper CB, Scammell TE. Fos expression in orexin neurons varies with behavioral state. *J Neurosci* 2001;21:1656–1662. [PubMed: 11222656]
- [44]. Tkacs NC, Dunn-Meynell AA, Levin BE. Presumed apoptosis and reduced arcuate nucleus neuropeptide Y and pro-opiomelanocortin mRNA in non-coma hypoglycemia. *Diabetes* 2000;49:820–826. [PubMed: 10905492]
- [45]. Smith GP, Epstein AN. Increased feeding in response to decreased glucose utilization in the rat and monkey. *Am J Physiol* 1969;217:1083–1087. [PubMed: 4980946]
- [46]. Jacob RJ, Weber AB, Dziura J, Morgen J, Sherwin RS. Brainstem dysfunction is provoked by a less pronounced hypoglycemic stimulus in diabetic BB rats. *Diabetes* 1995;44:900–905. [PubMed: 7621994]
- [47]. McNay EC, Sherwin RS. Effect of recurrent hypoglycemia on spatial cognition and cognitive metabolism in normal and diabetic rats. *Diabetes* 2004;53:418–425. [PubMed: 14747293]
- [48]. Paxinos, G.; Watson, C. *The Rat Brain in Stereotaxic Coordinates*. Academic Press; San Diego: 1998.

Figure 1.

Diagrams of atlas levels used for counting Fos staining in selected basal forebrain cholinergic nuclei: medial septum (MS), diagonal band of Broca (DBB), magnocellular preoptic nucleus (MCPO), based on ref. 23. Diagrams are modified from a rat brain atlas [48].

Figure 2.

Fos labeling in brain arousal-related neurons after saline injection (panels A and C) or after insulin injection (panels B and D). Panels A and B show the region of the perifornical hypothalamus in tissue prepared with double-immunohistochemical labeling for OX (blue/gray cytoplasmic stain) and Fos (brown nuclear stain). Arrows indicate double-labeled Fos+/OX+ neurons after insulin treatment. Panels C and D show the region of the magnocellular preoptic nucleus in tissue prepared with double-immunohistochemical labeling for choline acetyltransferase (ChAT - blue/gray cytoplasmic stain) and Fos (brown nuclear stain). Arrows indicate double labeled Fos+/ChAT+ neurons after insulin treatment. The calibration bar in B indicates 30 μ m in panels A and B, and 18 μ m in panels C and D.

Figure 3.

Total numbers of ChAT+/Fos+ cells in forebrain (A) and brainstem (B) cholinergic cell groups after saline or insulin treatment. In 6 saline-treated and 8 insulin-treated rats, quantification was carried out on Fos and ChAT double-labeled cells in 3 forebrain cholinergic nuclei as shown in figure 1. In 3 saline-treated and 6 insulin-treated rats, quantification was carried out in brainstem laterodorsal tegmental (LDT) and pedunculopontine tegmental (PPT) cholinergic nuclei. Insulin treatment was associated with significantly greater numbers of Fos+ cholinergic neurons only in forebrain nuclei.

Figure 4.

The effect of afternoon saline or insulin treatment on percent time in non-rapid eye movement sleep (NREMS), rapid eye movement sleep (REMS) and awake is shown as % time in stage. Data were collected from 30 minutes to 120 minutes after injection of saline or insulin and are expressed as means $\pm$ SEM. During this time after insulin injection, blood glucose is generally low and stable at 2.4-2.6 mM.

Figure 5.

Representative printout of delta power tracing and hypnogram from a polysomnography study. On the baseline day, the rat was given saline 1 ml/kg subcutaneously at 1 PM and sleep was recorded for 120 minutes. A typical sleep pattern consisting of multiple cycles between waking, NREMS, and REMS was observed. There are several episodes of prolonged increases in delta power (indicated as dark bars underneath the delta power graph), associated with a predominance of consolidated NREMS in those time periods. These are interspersed with times of greatly reduced delta power associated with REM sleep and waking. Below, the panel from the hypoglycemia day shows the effect of insulin 3 U/kg given subcutaneously. There are fewer consolidated episodes of elevated delta power and fewer REMS episodes. Cycling between W and NREMS is increased, although time in NREMS is preserved. The differences between baseline and insulin days are more pronounced during the second hour of recording, which should be the time of maximum hypoglycemia after the insulin injection.

Figure 6.

The time course of changes in W and NREMS episodes after saline or insulin injection. Repeated measures analysis of variance indicated a significant interaction of treatment (saline or insulin) and time (30-60; 60-90; 90-120 minutes after injection) in the following sleep measures: A. W episode number ($F=7.087$; $p=.026$), B. NREMS episode number ($F=9.25$; $p=.015$) and C. NREMS episode duration ($F=6.97$; $p=.027$).

Table 1
Blood glucose and lactate data before (baseline) and after insulin treatment in conscious, chronically catheterized rats

	Baseline	30 minutes	60 minutes	90 minutes	120 minutes
Glucose mmol/l (n=6)	6.2 ± 0.2	3.1 ± 0.2	2.6 ± 0.1	2.6 ± 0.3	2.4 ± 0.1
Lactate mmol/l (n=4)	0.53 ± 0.05	0.58 ± 0.04	0.76 ± 0.1	1.35 ± 0.08	1.41 ± 0.14