Participation of Noradrenergic Cell Groups A1 and A2 in Gluoregulation

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Honors Thesis
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PASS WITH DISTINCTION
TO THE UNIVERSITY HONORS COLLEGE:

As thesis advisor for Joseph Haynes,

I have read this paper and find it satisfactory.

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May 1, 2006
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Précis

The ability of the brain to respond to glucose changes in the body is vital for survival. While it is known what these changes are and how they occur within our body, the neuronal controls of these mechanisms are still poorly understood. A current belief is that both noradrenergic and adrenergic neurons in the hindbrain, which secrete norepinephrine and epinephrine, respectively, have a major control on these bodily responses such as eating more or increasing our blood glucose levels. However, there are several different noradrenergic and adrenergic cell groups distributed throughout the caudal hindbrain, and the likelihood that they each share an equal control in glucoregulation is unlikely. Also, the distinct roles of noradrenergic and adrenergic neurons in glucoregulation have not been determined.

In this experiment we examined two specific noradrenergic cell groups found within the ventral hindbrain, A1 and A2. After lesion of these cell groups with the selective neurotoxin 6-hydroxydopamine (6HD), responses normally seen in glucoprivation were tested to see any possible deficits that may have occurred due to specific cell lesions. These normal responses are increased food intake and an adrenal medullary response that increase the amount of glucose in the circulatory system. Also examined was the role of A1 or A2 cell groups in the suppression of food intake caused by cholecystokinin (CCK), a chemical signal for satiety. We found that neither A1 nor A2 lesioned cell groups were able to cause a deficit in the food intake or adrenal medullary response to glucoprivation, although A1 lesions did produce a slower blood glucose response to glucoprivation. However, A2 lesions were shown to diminish the suppression of food intake induced by CCK administration.

Overall, while it was shown that A1 and A2 cell groups did not have as great a control to bodily glucose responses as expected, the need for greater examination on this topic is needed.
While 6HD was able to cause destruction of adrenergic neurons effectively, non-specific cell damage of surrounding cell groups did occur, possibly masking the effects of the lesioning agent. Also, effective administrative techniques need to be refined so as to better lesion desired noradrenergic cell groups. By further examining these cell groups, as well as other noradrenergic cell groups, the specific role of these neurons can be further determined, allowing us to gain a better understanding of how a disease like diabetes may affect our body. In a world only now fully realizing the dangers of a disease like diabetes to and individual and society, a better understanding of the body’s normal ability to utilize glucose and respond to changes in its availability is desperately needed.
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List of Abbreviations

- 2-DG: 2-deoxy-D-glucose
- 5-TG: 5-thioglucose
- 6HD: 6-hydroxydopamine
- A1: noradrenergic cell group 1
- A2: noradrenergic cell group 2
- AA: ascorbic acid
- AP: area postrema
- C1: adrenergic cell group 1
- CCK: cholecystokinin octapeptide
- dBH: dopamine β-hydroxylase
- DSAP: anti-dopamine β-hydroxylase conjugated to the toxin saporin
- E: epinephrine
- mRNA: messenger ribonucleic acid
- NE: norepinephrine
- Nickel-DAB: nickel-intensified diaminobenzidine
- PB: phosphate buffer
- PVH: paraventricular nucleus of the hypothalamus
- TPBS: Tris sodium phosphate buffer

- NOTE: The terms noradrenergic and adrenergic are interchangeable with norepinephrine and epinephrine, respectively, as they both indicate the same type of neurons that contain either the neurotransmitters norepinephrine (noradrenaline) or epinephrine (adrenaline).
Abstract

The body’s response to glucoprivation employs such activities as increased food intake and an increased adrenal medullary response to help maintain the level of glucose in the body and brain. Noradrenergic and adrenergic neurons have been implicated in previous studies to have a role in controlling these bodily responses, but their specific roles have not been able to be picked apart. We hypothesized that lesion of the specific noradrenergic A1 and A2 neurons in the hindbrain would cause deficits in food intake tests, adrenal medullary tests and abolish the suppression of food intake controlled by satiety signals. This was tested through lesion by the neurotoxin 6-hydroxydopamine (6HD) and subsequent behavioral tests that replicated glucoprivation through the use of 2-deoxy-D-glucose (2-DG) and suppression of food intake by cholecystokinin (CCK). It was found that lesion of A1 and A2 cell groups were not able to significantly decrease food intake and the blood glucose response to 2-DG, but lesion of A1 neurons did cause a slower blood glucose response to 2-DG. Lesion of A2 cell groups were able to abolish the suppression of food intake by CCK. However, to better understand the role of these noradrenergic cell groups, a more effective method of administering 6HD, which is able to effectively lesion noradrenergic neurons, must be determined.

Introduction

Glucose is an important substrate for energy metabolism in the body and particularly in the brain. Glucose is a required energy substrate for the brain, because other potential energy-yielding substances do not cross the blood-brain barrier, are not normally available in the blood in adequate supply, or cannot be metabolized by brain cells (32). A decrease in the availability of glucose necessary for metabolism within the brain is known as glucoprivation.
Glucoprivation can be produced experimentally by administration of high insulin doses, which reduces blood glucose, or by administration of antimetabolic glucose analogues, which competitively inhibit glucose utilization by occupying the cellular enzymes required for glycolysis. Two antimetabolic glucose analogues that have been very useful research tools are 2-deoxy-D-glucose (2-DG) (3) and 5-thioglucose (5TG) (4).

Using these pharmacological tools to produce glucoprivation, it has been possible to demonstrate the mechanisms through which glucose levels are controlled. The major glucoregulatory mechanisms (or responses to glucose deficit) are increased adrenal medullary secretion, glucagon secretion, corticosterone secretion and increased food intake (9, 10, 31). These responses restore, conserve and replenish glucose stores and blood glucose concentrations.

Though the glucoregulatory responses mentioned above are now well established in physiology, the neural mechanisms through which glucose deficit is detected and the pathways necessary for elicitation of glucoregulatory responses are not fully understood. However, work attempting to elucidate these mechanisms is vital in helping to provide more effective treatments of diseases that affect the metabolic state of our body and brain, such as diabetes.

Norepinephrine (NE) and epinephrine (E) containing neurons are hindbrain catecholamine neurons that have been strongly implicated in activation of key glucoregulatory responses. Central administration of NE and E increases blood glucose levels and stimulates food intake (13, 21, 25, 33), suggesting a role for these NE and E neurons. Also, the use of the c-fos proto-oncogene has been used in conjunction with 2-DG and neuronal mapping to help localize the specific NE and E neurons that are activated by glucoprivation. The c-fos gene is induced by neuronal activation and its protein product, Fos, accumulates in the nucleus of the activated neuron and can be detected using immunohistochemical approaches. Thus Fos is a
marker of the recent activational history of the neuron (6) such as through the administration of 2-DG. 2-DG is a glycolytic inhibitor (3, 10, 30) that is able to elicit the responses seen by the body during glucoprivation (10, 30). Neuronal mapping studies examining Fos expression in response to 2-DG (23) have shown Fos expression in the ventrolateral medulla, the nucleus of the solitary tract and the locus coeruleus where NE and E neurons reside (11) and have shown that Fos is expressed in NE and E neurons themselves. These catecholamine neurons have also been previously shown to innervate the hypothalamus and intermediolateral column of the spinal cord (28, 34, 36, 37), indicating that they could influence parts of the brain and spinal cord responsible for eliciting glucoregulatory responses. However, neither the specific catecholamine neurons involved in glucoprivic responses, nor the importance of NE versus E neurons in those responses, are known.

Work dedicated to determining specific catecholamine neurons involved in glucoprivic responses has been conducted using a selective immunotoxin to produce targeted lesions of hindbrain catecholamine subgroups. The lesioning agent utilized was anti-dopamine β-hydroxylase conjugated to the toxin saporin (DSAP). The targeting agent responsible for the selectivity of this immunotoxin is a monoclonal antibody against dopamine β-hydroxylase (dβh), a NE and E biosynthetic enzyme that is found only in NE and E neurons. Thus, DSAP binds to and is selectively internalized by both NE and E neuron terminals upon injection, and is then retrogradely transported to the cell body where the saporin component of the conjugate destroys the neuron via its ribosomal toxicity (1, 38, 39).

Studies with DSAP have shown that selective lesion of NE and E groups projecting to the paraventricular nucleus of the hypothalamus (PVH), impair feeding in response to glucoprivation, without impairing the adrenal medullary response to glucoprivation. These
injections caused a complete loss or severe reduction of A1 and A2 noradrenergic and C1 adrenergic cell groups (22). DSAP injections into the spinal cord, which destroy spinally-projecting catecholamine neurons in the hindbrain, decreased the adrenal medullary response to glucoprivation, but did not impair glucoprivic feeding. Lesions resulted in a loss or reduction of A5-A6 noradrenergic and C1-C3 adrenergic neuron groups (22).

Although the DSAP data indicates an essential role for hindbrain catecholamine neurons in glucoregulation, as well as a division of labor among these neurons with respect to mediation of particular glucoregulatory responses, there are still missing pieces to the puzzle. DSAP, which is able to provide effective lesions of both NE and E cell groups, is hindered by its own strengths. By not being able to selectively lesion either NE or E neuron groups separately, the role and function of each subgroup cannot be teased apart and understood completely.

The neurotoxin 6-hydroxydopamine (6HD) has been used in past research to examine the roles of catecholamine neurons' a variety of behavioral and physiological responses. A hydroxylated analogue of dopamine (2), 6HD is able to undergo selective uptake into dopamine and noradrenergic neurons via their respective membrane transporter molecules (17). However, 6HD is not able to undergo reuptake into adrenergic neurons due to its lack of the proper transporter molecules. Once inside the neuron, 6HD induces cell death by oxidative stress and the production of free radicals (26). When injected intracerebroventricularly, 6HD caused the destruction of catecholamine neurons and impairment of glucoprivic feeding (35), although these injections destroyed dopamine neurons that produced a condition of global motor impairment similar to Parkinson's disease, calling the specificity of the feeding deficit into question. However, unlike DSAP, 6HD does not undergo retrograde transport. Therefore, it provides a lesioning agent that can destroy neurons and terminals locally at the injection site. Local
application would therefore avoid lesion to dopamine neurons and the associated Parkinsonian syndrome. Because the location of individual NE and E cell groups has been described in detail, local injections of 6HD could target these groups specifically for the purpose of determining their involvement in glucoregulatory responses.

In the experiments reported here, we examined the participation of two specific NE subgroups, known as A1 and A2, in glucoregulation using 6HD lesions. Cell group A1 is located in the ventrolateral medulla and A2 is located around the area postrema (AP) in the dorsomedial medulla. Both groups have been shown to be damaged by PVH injections of DSAP that eliminated glucoprivic feeding. After lesion of these cell groups with 6HD, we examined the feeding and adrenal medullary hyperglycemic responses of lesioned and control groups to systemic glucoprivation induced by 2-DG. We hypothesized that lesions of either A1 or A2 subgroups would cause a deficit in these responses. In addition to 6HD lesioned rats, our experiment included drug vehicle (ascorbic acid, AA) controls to examine any possible non-specific damage due to injections into the tissue sites. Ascorbic acid was used to help retard the oxidation of 6HD in its soluble form. At the conclusion of experimentation, the efficacy and selectivity of 6HD in producing clear lesions of noradrenergic cell groups was examined by immunohistochemical detection of NE and E neurons using an antibody against dopamine beta hydroxylase. By better examining specific cell groups, we hoped to resolve the enigma of what specific cell groups are involved in glucoregulation and whether different subgroups have functionally distinct roles. These studies will help us understand how our body responds to both internal and external environmental stimuli that affect our blood glucose levels and protects the supply of the brain's essential metabolic fuel.
Materials and Methods

Animals

Adult male Sprague-Dawley rats were obtained from Simonsen Laboratories (Gilroy, CA) with initial average weight of 376 ± 45 g. Each rat was housed individually in suspended wire-mesh cages under standard AAALAC-approved conditions in a temperature controlled room (21 ± 1°C) and were illuminated between 0600 and 1800 hrs. During the experiment, rats had ad libitum access to pelleted rat chow (Harlan Teklad F6 Rodent Diet W, Madison, WI) and tap water, except during blood glucose tests. Tests were conducted between the hours of 1000 and 1500 hrs and rats were handled daily during the experimental period, beginning one week prior to surgery to allow for habituation to the laboratory environment. All experimental animal protocols were approved by the Washington State University Institutional Animal Care and Use Committee, which conforms to the National Institutes of Health (NIH) rules and regulations.

Preparation of animals

Rats were anesthetized prior to surgery by intramuscular injection (0.1 ml/ 100 g BW) of a Ketaset/Rompun/Acepromazine cocktail (5 ml ketamine, 100mg/ml. Fort Dodge Animal Health, Fort Dodge, IA; 2.5 ml Rompin, 20 mg/kg. Bayer Corporation Agriculture Diversity Animal Health, Shawnee Mission, KA; 1 ml Acepromazine Maleate, 10 mg/kg, Boehringer Ingelheim Vetmedica, St. Joseph, MO; 1.5 ml 0.9% saline, Abbot Laboratories, North Chicago, IL). Anesthetic level was maintained during surgery by isofluorane (Halocarbon Products Corp., Charleston, NC) inhalation. Bilateral injections of either ascorbic acid (.1% in 0.9% saline) or 6-hydroxydopamine (6HD. Sigma, St. Louis, MO, 5 μg/100nl in .1% ascorbic acid) were then given to either A1 or A2 cell groups through a drawn glass capillary micropipette connected to a
microinjector (Picospritzer II) with polyethylene tubing. Gold colloid (Sigma, St. Louis, MO) was also added to the injection fluid to help visualize the injection site later during histological analysis. Injections were visually monitored to assure delivery of the solutions. The micropipette was inserted into the A1 cell group at a 15° angle to avoid sensitive overlying sites. Stereotaxic coordinates for the A1 cell group were 0.0 mm at suture, ± 4.0 mm lateral to midline and 9.2 mm below the dura mater. For A2 cell group injections, the foramen magnum was enlarged by removal of a portion of the occipital bone. Microinjections were then made 0.31 mm rostral to the obex, 0.4 mm ventral to the dorsal aspect of the AP. For specific injection sites in A1 and A2 cell groups, please see Figure 1. Three weeks were then allowed for the 6HD-induced degeneration of A1 or A2 neurons and for recovery from surgery before experiments were conducted.

2-DG induced feeding test

Glucoprivation was induced using 2-deoxy-D-glucose (2-DG, Sigma, St. Louis, MO, 200 mg/kg) injected subcutaneously. Pre-weighed pelleted rat chow was then placed into the rat’s food hopper immediately after injection, and 4 hrs later the remaining pellets and any chow spillage were weighed to determine the amount of food eaten. Rats were tested again in the same way, but instead by using subcutaneous injections of 0.9% saline to determine the basal (control) food intake. By subtracting the amount of food eaten in the saline test from the 2-DG test, the 2-DG induced change in feeding responses could be quantified.

Deprivation-induced feeding test

To assess the feeding response to food deprivation, food was withheld overnight and for a total of 19 hrs prior to testing. Food intake was measured in a 30 min test beginning immediately following the return of food.
Suppression of food intake by cholecystokinin.

Rats were deprived of food overnight, for a total of 19 hrs, to stimulate feeding. They were then injected intraperitoneally with 0.9% saline or cholecystokinin octapeptide (CCK, 2 μg/kg, Peptides International, Louisville, KY). Food intake (uneaten food and spillage) was measured in a 30 min test beginning immediately after the injections. Saline baseline and CCK tests were separated by two days. Suppression of food intake by the satiety peptide, CCK, was determined by comparing the amount consumed in the two tests.

2-DG Blood glucose test

Blood glucose was tested in the absence of food. For these tests, food was removed one hour prior to subcutaneous injections of 2-DG (200 mg/kg) or 0.9% saline. Tail blood (50 μl) was collected at 0, 30, 60, 90, 120 and 180 mins after injections. Saline and 2-DG tests were conducted on different days, with two intervening rest days. Blood glucose was analyzed using the glucose oxidase method (27).

Statistics

Feeding responses were analyzed using one-way repeated-measures ANOVA, followed by the Fisher’s LSD multiple-comparison procedure. The differences were considered significant if P < 0.05.

Immunohistochemistry

After completion of testing, rats were sacrificed by inhalation of halothane (Halocarbon Products Corp., Charleston, NC) and then perfused transcardially at the time of death with phosphate-buffered saline followed by 4% formalin (both pH 7.4). Brains were removed and postfixed overnight at room temperature, then cryoprotected overnight in 25% sucrose with 0.1% sodium azide. Hindbrains were sectioned coronally at 30 μm thickness and collected into three
sets and placed into 0.1 M phosphate buffer (PB, pH 7.4). One set was used for histological analysis, while the other two sets were placed in anti-freeze solution and placed in the freezer for long-term storage.

Immunohistochemical staining was done by standard avidin-biotin-peroxidase techniques describe previously (23). Sections were treated with 50% ethanol for 30 minutes, then washed (3 x 5 mins) in 0.1 M PB before being incubated for 45 minutes in 10% normal horse serum (Gibco BRL Life Technologies, Grand Island, NY) made in Tris sodium phosphate buffer (TPBS, pH 7.4) with 0.05% thimerosol. The blocking solution was removed from the tissue and sections were incubated in mouse monoclonal anti-DBH (1:100,000, Chemicon) made in 10% normal horse serum – TPBS. After 48 hrs, the primary antibody was removed and the sections were washed and incubated in biotinylated donkey anti-mouse IgG (1:500, Jackson ImmunoResearch Laboratories, West Grove, PA) made in 1% normal horse serum – TPBS. After 24 hours the tissue was washed (3 x 5 mins), incubated with Extravidin-peroxidase (1:1500 in TPBS, Sigma) for 4 hours, washed again (3 x 5 mins), and reacted through the use of nickel-intensified diaminobenzidine (nickel-DAB) for visualization of DBH-stained neurons. The sections were then mounted onto microscope slides and were cover slipped for microscopic evaluation. Histological sections were captured using a Coolsnap digital camera (Media Cybernetics, Silver Spring, MD) and Metamorph imaging software (Universal Imaging Corp., Downingtown, PA). Adobe Photoshop was utilized to assemble plates of multiple sections, as well as to match brightness of images on the same plate.

Analysis

AI and A2 cell bodies were visualized to assess the lesions from 6HD, as well as determine the site of injection. This was done by comparing anatomically equivalent sections of
AA controls and 6HD injected rats. By comparing similar sections, lesions due to 6HD, as well as non-specific damage to either drug injection or AA could be assessed.

Results

The rats recovered normally prior to testing and maintained normal body weights throughout the experimental period. Figure 2 shows body weight gain in lesioned and control rats during the study following the intracranial injections. The 6HD rats exhibited normal motor function and behavior, making them indistinguishable from AA vehicle control rats.

Immunohistochemistry

For select pictures of both A1 and A2 lesion sites, please see Figure 3. Each rat was examined for A1 or A2 lesion sites. Detailed figures of lesion locations, depicted on drawings of corresponding coronal brain sections adapted from the Paxinos and Watson rat brain atlas (18), appear in Figures 4-8. In the A1 6HD injected rats, A1 neurons were destroyed, but the damage was limited to a restricted diffusion radius. A1 neurons were not lesioned outside of this injection sphere. In A2 lesion sites, A2 neurons were effectively lesioned when a proper injection site as attained, in some cases including noradrenergic neurons of the AP. Lesions in both areas clearly produced non-specific, as well as specific damage.

2-DG feeding responses

Feeding results are shown in Figure 9. The amount of food consumed in response to 2-DG-induced glucoprivation was significantly greater for both A1 and A2 groups than the amount consumed after the saline control injection. However, the 6HD lesion did not significantly impair this response in either the A1 or the A2 group.

CCK & food deprivation feeding responses
Suppression of feeding in response to CCK is shown in Figure 10. In both A1 groups the food intake in response to CCK was significantly lower than food intake in response to saline injections, indicating effective suppression of eating (F=64.69, P<0.05). However, 6HD totally abolished the suppression of feeding by CCK in the A2-injected group. Feeding in response to 19 hr overnight food-deprivation is shown in Figure 11. There were no significant differences between groups in this response.

*Adrenal medullary hyperglycemic response*

Changes in blood glucose in response to 2-DG are shown in Figure 12. In all groups, 2-DG caused a significant increase in blood glucose above the time 0 (i.e., baseline) level. A2 injection groups did not differ in the magnitude of their glucose responses. However, 6HD injections into A1 significantly impaired the rate of glucose mobilization when compared to the AA injection group. There was a significant depression in the blood glucose levels of the A1/6HD group when compared to the A1/AA group at the 30 min time point (F=2.9, P<0.05). In comparing the blood glucose levels at each time point to the baseline blood glucose levels in the 2-DG treatment, there was a significant elevation in blood glucose noted in the A1 (F=18.5, P<0.05) and A2 (F=16.6, P<0.05) groups. However, at the 90 and 120 min time points, there is a significant difference between the A2/AA and A2/6HD group in the saline treatment (F=6.07, P=0.049).

**Discussion**

Hindbrain catecholamine neurons have been shown to be essential for glucoprivic feeding and adrenal medullary hyperglycemic responses, but these neurons are functionally heterogeneous and the particular population required for gluco regulatory responses is not known.
We tested the hypotheses that A1 and A2 cell groups are critically involved by producing localized lesions of these groups using 6HD. Results for the A2 cell group were most definitive, since the lesions for this group were the most accurately placed and the most complete. Results show that nearly complete lesions of the A2 cell group do not impair either feeding or blood glucose responses to glucoprivation. This is consistent with other results showing that, in comparison to A1 and C1, very few A2 neurons increase expression of Fos protein, a marker of cellular activation, in response to 2-DG (23). Moreover, in comparison with A1 and C1 neurons, A2 neurons show only small increases in the expression of mRNA for DBH (15), a catecholamine biosynthetic enzyme, in response to glucoprivation. Together with this data, present findings strongly suggest that A2 neurons are not critically involved in glucoregulatory feeding and adrenal medullary responses. On the other hand, some data have indicated that aspiration and electrolytic lesions of the AP and underlying nucleus of the solitary tract (the area containing the A2 cell group) severely impair glucoprivic feeding (5, 24). Our lesion, however, was less extensive than most electrolytic lesions. In addition, our lesions did not destroy the AP in most cases. Thus, differing results could be due to differences in the lesion, and additional work will be required to resolve and understand these differences.

6-hydroxydopamine lesions of A2 did impair CCK-induced suppression of deprivation induced feeding. This finding is consistent with other work showing that destruction of A2 using a catecholamine immunotoxin also impaired the response to CCK (8, 19), and that electrolytic lesions of the nucleus of the solitary tract (8), including the A2 neurons, abolish CCK satiety. Our results therefore dissociate the involvement of A2 neurons in two controls of feeding, a stimulatory control that is activated by glucoprivation and is not impaired by A2 lesions, and an inhibitory control activated by CCK that appears to require these neurons. Nevertheless, some
caution must be taken in interpreting these findings, since the 6HD lesions caused significant nonspecific damage. That is, behavioral impairment was certainly caused by lesion of the nucleus of the solitary tract, suggesting that this area of the brain contains a critical substrate for the mediation of CCK satiety, but the critical neurons may not have been A2 neurons.

An important aspect of the CCK effect observed in our experiment is that the local applications of the catecholamine toxin, 6HD, and the local application of the catecholamine-directed immunotoxin, DSAP, into the A2 cell group produced a deficit in the CCK response, while retrograde destruction of A2 neurons by PVH injection of DSAP did not cause impairment of this response (22). PVH injections destroy only about 50% of A2 neurons and destroy specifically the subpopulation that projects to the PVH. If our present findings are born out by additional work, this difference would suggest that CCK responses are mediated by a population of A2 neurons that do not project to the PVH.

An interesting anatomical observation was that 6HD did not appear to damage C2 neurons, which reside just rostral to the A2 cell group in the nucleus of the solitary tract and utilize E, as opposed to NE, for neurotransmission. The fact that the C2 neurons were spared may be due to the reported lack of catecholamine membrane transporters, required for 6HD cellular uptake, on the C2 neurons (12, 16). If so, this suggests that 6HD could be used to distinguish functional properties of these two closely associated cell populations.

Results from rats injected with 6HD into the A1 NE cell group were less definitive. Even though the injections were accurately placed, the injections destroyed only a small percentage of the total number of A1 neurons, and in two cases produced a lesion on one side of the brain only. In view of this, it is not surprising that feeding and blood glucose responses, as well as CCK satiety, remained intact in this group. Current data strongly suggests that the A1 cell group and
the partially overlapping C1 cell group are the major candidates for involvement in glucoregulation. These groups contain cells that strongly increase Fos expression and DBH mRNA in response to glucoprivation (23). In addition, a large percentage of A1 neurons co-express neuropeptide Y (14), which has been shown by gene deletion experiments to be required for glucoprivic feeding (29). Therefore, it will be important to refine the injection procedure and repeat the effort to reveal a role for this cell group in glucoregulatory responses.

With respect to A1 involvement in CCK responses, the data are less clear than for A1. No other localized A1 lesion experiments, other than the present experiment, have examined effects on CCK. However, from existing data, it seems unlikely that A1 neurons are importantly involved in CCK satiety. Although CCK increases Fos expression in some A1 neurons (7, 20), the activated neurons are a small percentage of the total number. Furthermore, retrograde lesion of hindbrain catecholamine neurons by PVH injections of DSAP destroys nearly all of the A1 neurons, but does not impair CCK satiety (22).

Blood glucose responses to glucoprivation were also largely intact in the A1 6HD lesioned rats, although there was an attenuated rate of glucose mobilization, apparent at the 30 min sampling time. The failure to find a clear deficit in A1 lesioned rats is consistent with the anatomical findings that the spinally projecting catecholamine neurons are the ones that are most critical for the blood glucose response. Spinally projecting catecholamine neurons are not present in the A1 cell group, but are located just rostral to A1, in the C1 cell group, an area that was not significantly damaged by our 6HD injections. Moreover, C1 is an E secreting cell group that, as discussed above, lacks uptake sites for 6HD and should have been less vulnerable to its toxicity. Additional work may be able to take advantage of this difference in A1 and C1 neurons to reveal the separate functional roles of the NE and E neurons in the ventrolateral medulla.
A problem with past research involving injections of 6HD was the destruction of dopaminergic neurons in the brain. These lesions caused major motor deficits similar to Parkinson’s, limiting the use of 6HD in studying other catecholaminergic neurons. However, it was shown that 6HD lesion of A1 and A2 neurons did not diminish the stimulation of feeding induced by food deprivation, shown by their similarity to control rats (see Figure 11), nor did 6HD injections diminish the ability of the lesioned rats to maintain normal body weights (see Figure 2). This indicates that injection of 6HD into the ventrolateral and dorsomedial medulla did not impair the behavioral capacity of the rats, as would be observed with injections into the forebrain dopamine-containing areas.

Histological findings with 6HD were carefully analyzed in our experiment because very few studies have attempted to use 6HD to lesion specific hindbrain cell groups by local application directly into these groups and because there is a great need for specifically targeted toxins for studies of neural function. Our results show that 6HD produced clear lesions of A1 and A2 neurons, as well as NE neurons of the area postrema (see Figures 4-8), and suggest that E neurons may be less sensitive or insensitive to the specific toxicity of this compound. Although A2 neurons were more effectively lesioned than seen A1 neurons, this appears to be due to differences in the anatomical features of the two cell groups. A1 cells are less compact and distributed over a larger area than A2 neurons. Improvements in our toxin delivery, such as increasing the dilution and volume of the injection, could both reduce the nonspecific effects of the 6HD and increase the proportion of cells in A1 that were damaged. Thus, while several aspects of glucoregulation have been examined in this study, there is still a need for reexamination and repetition of the study to gain a better understanding of the role of both A1 and A2 neurons. A need for a greater number of subjects is required, so as to increase the
number of possible successful 6HD injections and to decrease the effect of outlying data. Also, the possible nonspecific destruction of neural tissue by the drug vehicle should be examined. Although ascorbic acid is typically used as a vehicle for 6HD because it reduces oxidation of the 6HD, it seemed to produce some non-specific damage that possibly masked the effects of 6HD on A1 and A2 cell groups. Possibly decreasing the concentration of 6HD would reduce nonspecific damage, while increasing the volume, especially for A1 injections, would help to lesion more of the targeted neurons. Another important issue for future studies is the inclusion of non-injected controls. We tested two non-injected controls in the present study, but did not include their data in the results. Due to the small number of rats, the outcome was not useful.

Conclusion

In summary, we found that partial destruction of A1 neurons by localized 6HD administration retarded the development of the hyperglycemic response to 2-DG-induced glucoprivation, but did not impair the stimulation of feeding by 2-DG or the suppression of feeding by CCK. Destruction of A2 neurons by 6HD injection did not alter the hyperglycemic or feeding responses to 2-DG, but eliminated the suppression of feeding in response to CCK. However, non-specific damage was noted in both A1 and A2 injection sites for both AA and 6HD injections, and in addition, the A1 cell group was incompletely lesioned in all cases. For the role of specific NE neurons to be established and teased apart from their E counterparts, 6HD lesion techniques must be further refined. Nevertheless, the present findings have made substantial progress toward the goal of developing reliable and selective lesioning tools for assessment of the neural circuits underlying important physiological controls.
Acknowledgements

Several people have made the completion of this thesis possible. I would like to thank Patricia Duffy, Shayne Andrew, Rebecca Darling and Nathaneal Huston for their support, guidance and help in keeping me sane during stressful times. I would also like to thank Thu Dinh, whose help and support throughout my time working in the lab has made my thesis a reality. A personal thanks goes out to my mother and late father, who have helped to push me to where I am today with their love and support.

Finally, I would like to thank my thesis advisor Dr. Sue Ritter. The extensive amount of time, patience and mentoring she has given to me has helped to make this honors thesis a visible reality. She has shown me what a true scientist really is, as well as someone who cares about educating future scientists.

This research was supported by the NIH grants NS 45520 and KD 40498 to Sue Ritter.
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Fig. 1 (above). A1 & A2 injection sites
Fig. 2 (above). Weight gain of rats from post-surgery to point of sacrifice.
Fig. 3 (above). Photomicrographs showing dβh immunoreactivity (-ir) in A1 (top panel) and A2 (bottom panel) rats injected with ascorbic acid (vehicle control) or 6HD by a drawn glass capillary micropipette. Injection sites were also labeled by co-injection of colloidal gold. Top panel: 6HD produced an extreme loss of dβh-ir neurons throughout A1 areas (section B), as compared to the vehicle control rats (section A). In B, hemosiderin deposits and gliosis (see arrow) can be seen at the injection site. Bottom panel: 6HD produced a loss of dβh-ir neurons throughout middle (section B) and caudal (section D) areas of A2, indicated by the solid arrows. In some A2/6HD rats, the area postrema was also lesioned which is show by the dashed arrow in slide D. However, 6HD did not produce lesions rostral to the A2 injection site (section F), which is where C1 neurons are located.
Fig. 4 (above). A1/AA injection sites in rats 36-39, with red circles representing desired injection site and asterisks representing actual injection site. Lack of two asterisks per slide indicates an inability to locate the bilateral injection.
Fig. 5 (above). AL/6HD injection sites in rats 40-46, with the red circles representing desired AL cell group injection sites, and asterisks representing the actual injection site. Lack of two asterisks per slide indicates an inability to locate the bilateral injection.
Fig. 6 (above). A2/AA injection sites in rats 49-51, with the red triangles representing A2 cell groups and asterisks representing the actual injection site.
Fig. 7 (above). A2/6HD injection sites in rats 52-53, with empty, red circles representing desired injection sites and filled circles representing actual lesion sites in each rat.
Fig. 8 (above). A26HD injection sites in rats 54-56, with filled circles representing actual lesion sites in each rat.
Fig. 9 (above). Amount of food intake in a 4 hr test in response to 2-DG-induced glucoprivation (means ± SE). 2-DG (200 mg·kg⁻¹, 1 ml·kg⁻¹) and saline (0.9%, 1 ml·kg⁻¹) were administered subcutaneously in A1 (bottom panel) and A2 (top panel) groups. All four groups of rats increased their food intake significantly in response to 2-DG. The A2/6HD group ate less than their controls, but the difference was not significant. A2/AA, n=3; A2/6HD, n=5; A1/AA, n= 4; A1/6HD, n=5.
Fig. 10 (above). Suppression of deprivation-induced feeding in a 30 min test (means ± SE) in response to intraperitoneal injections of saline (0.9%, 1ml/kg) or CCK (2 μg/kg) in A1 (bottom panel) and A2 (top panel) groups. A2/6HD lesions significantly impaired the suppression of feeding in response to CCK, which was also seen in the A2/AA group. A1/AA lesioned rats, as well as the A1/AA control group, suppression of food intake by CCK was not impaired (* P<0.05 vs. intake after saline). A1/AA, n= 4, A1/6HD, n=5, A2/AA, n=3; A2/6HD n=5.
Fig. 11 (above). Food intake in a 60 min test following a 19 hr overnight food deprivation (mean ± SE). Deprivation-induced feeding did not differ significantly between groups. A1/AA, n = 4, A1/6HD, n=5, A2/AA, n=3; A2/6HD n=5.
Fig. 12 (above). Blood glucose levels in response to 2-DG (200 mg/kg). Mean (± SE) blood glucose levels are shown for each group. Both A1 (top panel) and A2 (bottom panel) groups exhibited a significant increase in their blood glucose levels for all TP’s when compared to the 0 min TP in the 2-DG treatment. However, the blood glucose level at the 30 min TP was significantly reduced in the A1/6HD group, compared to the A1/AA group, suggesting an impairment in that response compared to the A1/AA group (* P<0.05 vs. AA groups). A1/AA, n=4, A1/6HD, n=5, A2/AA, n=3; A2/6HD n=5.
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