1985

Studies on gold thioglucose lesion formation and glucose oxidation in the ventromedial hypothalamus

Danley Fritz Brown
Iowa State University

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Iowa State University

Ph.D. 1985

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Studies on gold thioglucose lesion formation and glucose oxidation in the ventromedial hypothalamus

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Danley Fritz Brown

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For the Graduate College

Iowa State University
Ames, Iowa

1985
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GENERAL INTRODUCTION

Brecher and Waxler (1949) were the first to discover that gold thioglucose (GTG) caused obesity in mice. In 1955, Marshall et al. found that the hyperphagia and obesity associated with GTG resulted from a ventromedial hypothalamic (VMH) lesion. In the late sixties and seventies, Debons and his colleagues pioneered the work that is now the foundation for GTG lesion formation etiology. Following the discovery by Mayer and Marshall (1956) that glucose is an essential part of GTG needed to produce GTG necrosis, Debons demonstrated that GTG lesion formation is: insulin dependent (Debons et al., 1968; 1969; 1970); abolished by glucocorticoid administration (Debons et al., 1974b); serotonin dependent (Debons et al., 1979a), prevented by glucose analog (Likuski et al., 1967), and glucose transport inhibitor (Debons et al., 1974a) treatment; and highly specific for VMH neural tissue (Debons et al., 1977; 1979b). Although Debons' laboratory showed that GTG is a specific neurotoxin for the VMH, speculation has continued over the specificity of GTG lesion formation. Liebelt and Perry (1967) suggested that GTG caused VMH destruction solely by a breakdown in the vasculature. The resultant ischemia produced the VMH necrosis. Caffyn (1971; 1972a) allegedly substantiated this phenomenon by electron microscopic observations. Additional credence has been given to this idea by circumstantial speculation by Levine and Sowinski (1982; 1983).
and Nochlin and Levine (1982).

In order to further elucidate the mechanism of GTG action in the VMH, and explore factors that may affect GTG lesion development in the VMH or VMH glucose oxidation, the ten studies comprising this dissertation were done.

Explanation of Thesis/Dissertation Format

Each chapter in this dissertation stands alone as a manuscript prepared for submission to a scientific journal. Also, every paper that comprises a chapter began as an original thought of the author. A General Introduction and Conclusions provide a continuity for the dissertation concept. Since each chapter possesses an extensive literature review, no general critical review of the literature is given to avoid redundancy.
SECTION I.

ALTERATIONS IN GLUCOSE METABOLISM
CHAPTER 1. SYSTEMIC PHLORIZIN PREVENTS GOLD THIOGLUCOSE NECROSIS IN THE VENTROMEDIAL HYPOTHALAMUS

Introduction

Phlorizin (PHL) is a well-known inhibitor of glucose transport in a variety of tissues (Lotspeich, 1961; Silverman, 1981). Infusions of small amounts of PHL into the cerebral ventricles cause hyperphagia and body weight gain (Click and Mayer, 1968). However, intraperitoneal (IP) injections of PHL have no effect on eating (Click and Mayer, 1968). Click and Mayer (1968) suggested that PHL was affecting cerebral glucoreceptors involved with the control of food intake. Others (Grossman, 1980; Stevenson, 1969) have suggested that the ventromedial hypothalamus (VMH) possesses these presumptive glucoreceptors, since this area has been implicated as a major center for food intake control.

Gold thioglucose (CTG), when injected into mice IP, will cause an obesity syndrome (Brecher and Waxler, 1949). This obesity development has been attributed to destruction of the VMH by CTG (Marshall et al., 1955). Debons et al. (1974a) demonstrated that unilateral intrahypothalamic injections of PHL into the VMH could abolish CTG lesion formation on the side of the brain receiving the PHL. These investigators also showed, by autoradiography, that cells in the VMH bound tritiated PHL (Debons et al., 1974a). These data supported the
idea that the VMH contained glucosensitive cells, which were involved with the control of food intake.

Recently, Debons and co-workers (1979b) have clearly demonstrated that GTG necrosis in the VMH is highly specific for the area, with neural components as the primary GTG target tissue. They have suggested that GTG could be used as a probe for hypothalamic function. In this study, we used the experimental method of Glick and Mayer (1968), involving parenteral injections of PHL, and increased the dose of PHL. Using GTG as a probe, changes in the VMH were observed by light microscopy. In addition, the effect of PHL on VMH glucose oxidation in vitro was assessed.

Materials and Methods

CF1 female mice, weighing 20-23 gm, were used in this study. The mice were maintained at 23°C, with a 12 hour light:12 hour dark photoperiod and given Teklad Rat and Mouse Diet and tap water freely.

PHL (900 mg/kg) was injected IP into mice 2 hours before GTG (300 mg/kg) was administered IP. Since PHL is only slightly soluble in cold water, the suspension was heated to solubilize the PHL and injected. Dissolving PHL in hot water does not alter the chemical structure of the compound. Hot water injections followed by GTG served as controls.

Animals were decapitated 24 hours after the GTG injection. The brains were removed from the skull and the VMH was dissected free as a cube of tissue and fixed overnight in 10% formalin. The tissues were embedded in 8% agar and sectioned at 50 um using a Smith and Farquhar
tissue sectioning. CTG-induced pathology of the VMH was rapidly determined in these sections using the Penfield modification of the del Rio-Hortega silver carbonate reaction for oligodendroglia (Humason, 1972) and light microscopy. For photographic purposes, some brains from each group were fixed in Bouin's fluid, dehydrated, embedded in paraffin, sectioned at 7 μm, stained with hematoxylin and eosin, and photographed through a light microscope.

The effect of PHL on VMH glucose oxidation in vitro was determined. Untreated animals were decapitated and the VMH was dissected free from the brain at the level of the median eminence as a cube of tissue. Two VMH tissue cubes (caps) were placed in culture vessels (25 ml Erlenmeyer flasks with a gas trap) containing 1.47 ml of medium, saline, 15, 30, 45 μg or 10.66 mg PHL in water and 7.5 μCi of uniformly labeled [14C] D-glucose. The incubating medium was similar to cerebral spinal fluid (Jones et al., 1975), and supplemented with 2.6% complete amino acid concentrate, 28.6% rat serum, and 1.3% glutathione (200 mM). Final glucose concentration was 172 mg%. Other vessels possessed no tissue and served as controls for auto-oxidation of the [14C] D-glucose. All vessels were sealed with rubber septums and gassed for 30 seconds with a 95% oxygen:5% carbon dioxide mixture. The caps were allowed to incubate with PHL or saline in the medium 15 minutes at 37°C prior to the addition of [14C] D-glucose. The incubation was continued for 90 minutes at 37°C. The reaction was stopped and the [14C] carbon dioxide released by the addition of 9.0 N sulfuric acid (0.2 ml) to the medium. Hyamine (0.5 ml) was added to the gas trap to absorb the [14C] carbon
dioxide and incubated for an additional 60 minutes. The hyamine was collected from each vessel, supplemented with 15 ml of a PPO and POPOP toluene scintillation cocktail, and [¹⁴C] emissions were counted with a Beckman 250 liquid scintillation counter. As a test for tissue viability, saline vessels with hypothalamic tissue were supplemented with 15 ul of 2.0 mM dinitrophenol in an aqueous solution and processed as described above.

Atomic absorption spectrophotometry was used to determine the gold content in urine collected from mice injected with water or PHL and subsequently with GTG. Six mice were assigned to two groups. One group was injected with water and two hours later challenged with GTG. Group two was administered PHL and given GTG two hours after the PHL injection. Urine was collected for four hours following GTG administration. The quantity of urine collected from each animal was approximately 5.0 ml. A Perkin-Elmer 503 Atomic Absorption Spectrophotometer with a 2100 Controller and a HGA-74 graphite cell was used to measure the total gold content of each urine sample. The samples were frozen, lyophylized, resuspended in 1.0 ml water and the elemental gold extracted with methyl iso-butyl ketone, using the procedures outlined by Perkin-Elmer. A standard curve was obtained using various concentrations of gold chloride. The samples were processed with the following parameters: argon gas flow--15.0 cc/minute; wavelength--243.3 nm; drying temperature--120°C for 50 seconds, charring temperature--900°C for 50 seconds, atomizing temperature--2200°C for 5 seconds, and injection volume--5 ul.
A one-way analysis of variance and Dunnett's test, and t-test were used to determine statistical significance.

Results

Control animals injected with hot water and subsequently with GTG displayed typical GTG lesions in the VMH (Figure 1A). Mice treated with PHL and GTG showed complete inhibition of GTG necrosis in the hypothalamus (Figure 1B; Table I). These brain sections were indistinguishable from untreated mouse brains.

Analysis of variance indicated that differences were present among the in vitro PHL treatments, $F(4,16)=14.27$. PHL did not inhibit glucose oxidation in the VMH at low concentrations (Figure 2). In fact, the lowest supplement of PHL to the medium caused glucose oxidation to increase significantly ($p<0.001$). This phenomenon will be discussed below. At a high concentration, PHL depressed glucose oxidation by 50% (Figure 2). This inhibition of glucose oxidation by PHL was highly significant ($p<0.001$).

Determination of glucose oxidation in normal and CMG-treated tissue cubes indicated that 75% of each tissue cube contained neuronal elements vulnerable to GTG (Table II). Histological examination confirmed this observation. In addition, injections of necrotizing GTG doses (300-800 mg/kg) generally produce lesions of equivalent size in mice (personal observation).

The presence of dinitrophenol, an uncoupler of oxidative phosphorylation, in the incubation medium caused glucose oxidation to
increase greatly \((10.8 \times 10^{-8} \text{ moles glucose oxidized/mg tissue/hour})\), a process analogous to the Pasteur effect. This response to dinitrophenol poisoning indicates that tissue viability was not impaired during incubation.

Although PHL can produce diuresis (Lotspeich, 1961; Silverman, 1981), GTG excretion apparently was not enhanced. Control animals given water and GTG excreted 187±32 (mean±SEM) μg of gold/gm body weight in the urine, over the four hour interval following the GTG injection. The PHL and GTG treated animals excreted such small amounts of gold during the same time period, that the element was essentially undetectable using our machine settings and the Perkin-Elmer protocol. Only one of the three PHL and GTG samples had any measurable gold content, 1.3 μg/gm body weight. Unexpectedly the PHL treated mice excreted significantly less gold in the urine than the controls, \(p<0.001\).

Discussion

Glick and Mayer (1968) have reported that intraventricular infusions of PHL produce a sustained hyperphagia and body weight gain. This over-eating phenomenon is probably caused by the inhibition of glucose utilization in the hypothalamus by PHL. Consistent with this idea, Debons and colleagues (1974a) have illustrated, by autoradiographic techniques, that PHL can bind to specific cells in the VMH. This area of binding is also the site of initial GTG-induced necrosis which leads to hyperphagia. In conjunction with this experiment, Debons et al. (1974a) were able to inhibit GTG lesion
formation in the VMH by intrahypothalamic PHL injections. Although the method of injection used by Debons' group has been questioned (Brown and Viles, 1980), the data presented (Debons et al., 1974a) are convincing. In this study, we have shown that an IP injection of PHL prevented CTG necrosis in the VMH (Figure 1). Also, it has been demonstrated that PHL can be a strong inhibitor of glucose oxidation in the VMH in vitro (Figure 2).

Although the in vitro VMH glucose oxidation experiments clearly demonstrated that PHL, in high concentrations, can inhibit glucose utilization, the significant increase of glucose oxidation by VMH tissue at the lowest concentration of PHL utilized, presented an enigma. Lotspeich (1961) found that PHL inhibition of kidney glucose transport could be reversed by the addition of adenine nucleotides. This result suggested that PHL may be inhibiting glucose reabsorption by affecting the ATP sensitive, Na⁺ dependent, glucose transport system. Hence, our apparent increase in VMH glucose utilization at a low PHL concentration is analogous to the dinitrophenol effect mentioned earlier. Thus, this low dose PHL phenomenon can be explained by the Pasteur effect.

Click and Mayer (1966) injected PHL parenterally; however, hyperphagia did not ensue as seen with intraventricular PHL infusions. In high concentrations, PHL is able to inhibit glucose oxidation in VMH tissue in vitro, while at a low concentration PHL significantly enhanced glucose oxidation. These results suggest the dosage Click and Mayer used (1.0 mM) may have been too small to cause hypothalamic hyperphagia. Indeed, the amount of PHL injected IP apparently is important for
altering hypothalamic function. A large toxic dose of PHL completely suppressed CTG necrosis in the VMH at the light microscopic level.

It has been postulated that glucoreceptor cells exist in the VMH, which function in the regulation of feeding behavior (Mayer, 1953). Since intrahypothalamic PHL implants appear to inhibit CTG necrosis (Debons et al., 1974a), a glucose transport mechanism may be involved with CTG lesion formation in the VMH. In other words, CTG may be prevented from interacting with the neural components possessing glucoreceptors. PHL is probably competing with CTG for the receptor sites sensitive to glucose in the VMH.

PHL may have an impact upon the blood-brain barrier (BBB). If PHL does block transport at the BBB, this phenomenon could account for the lack of CTG-induced necrosis in the VMH as a result of PHL administration. When 2-deoxyglucose was used as a reference, PHL did not effect hexose transport across the BBB (Deane and Segal, 1979). This lack of inhibition of sugar transport at the BBB indicates that PHL probably does not affect CTG transport into the brain. Thus, the PHL blockage of CTG necrosis probably occurs in the VMH.

Finally, PHL is known to produce glucosuria by preventing reabsorption of glucose from the renal tubules (Lotspeich, 1961). Prevention of CTG lesion formation in the VMH of mice treated with PHL could conceivably be due to a rapid renal excretion of CTG produced by PHL. However, the specific effects of PHL on the kidney must be considered. PHL has its greatest effect on renal glucose excretion during the initial 120 minutes after injection (Braun et al., 1957).
Subsequent time intervals show a decrease in the glucose excretion rate (Braun et al., 1957). In addition, kidneys perfused with PHL in normoglycemic dogs, show only a 40% loss of glucose due to malabsorption (Lotspeich, 1961). In the present study, we injected mice with GTG two hours after PHL administration to avoid a significant loss of GTG in the urine, assuming the reabsorption of GTG in the kidney is affected by PHL. Debona et al. (1968) have demonstrated that continuously high blood levels of GTG are not necessary for lesion formation. In fact the amount of GTG in whole blood begins to decline 30 minutes after administration. Since a high, long-lasting GTG blood concentration is not necessary for VMH lesion formation (Debons et al., 1968), and precautions were taken to avoid excessive renal GTG excretion due to PHL, it is predicted that sufficient amounts of GTG were present in the blood and brain of animals treated with PHL and GTG to cause VMH necrosis. In an effort to demonstrate definitely that PHL did not cause excessive excretion of GTG in the urine, and thus prevent VMH lesion formation, we measured the total amount of gold in a four hour post GTG urine sample from mice treated with and without PHL. Mice injected with water and GTG excreted significantly larger amounts of gold in the urine compared to PHL and GTG treated mice (p<0.001; see Results). This result clearly demonstrates that PHL did not abolish GTG lesion formation in the VMH by increasing the excretion rate of GTG over controls. Furthermore, since no VMH lesions developed in PHL treated, GTG challenged mice, PHL apparently affected the VMH directly, thereby preventing any GTG induced damage from occurring in the area.
The evidence reported in this study indicate the PHL dosage used by Glick and Mayer (1968) was in fact too small to affect food intake, when given IP. The large IP PHL injection used in this investigation inhibited GTG lesion formation in the VMN. Large doses of PHL also inhibited VMN glucose oxidation in vitro. These results substantiate the possible involvement of a glucoreceptor and/or glucose transport mechanism, in relation to VMN function determined by GTG vulnerability. Moreover, our data support the concept of a glucostatic modulation of feeding behavior (Mayer, 1955).
Figure 1. Coronal sections of mouse brain, showing the VHH at the level of the median eminence, 24 hours after a GTG injection. A. Hot water given IP initially, followed by GTG (300 mg/kg). B. PHL (900 mg/kg) given IP initially, followed by GTG (300 mg/kg). Bars represent 0.1 mm. (90X)
Figure 2. The effect of various concentrations of PHL on VMH glucose oxidation in vitro. The bars indicated the mean ± SEM.

* p<0.001
TABLE I. Effect of phlorizin on GTC\textsuperscript{a} lesion formation in the VMH

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>% Incidence of Necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phlorizin (900 mg/kg)</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Water</td>
<td>9</td>
<td>100</td>
</tr>
</tbody>
</table>

\textsuperscript{a} 300 mg/kg.
TABLE II. Effect of GTG Lesions on Glucose Oxidation in Vitro

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Moles x 10^-8 glucose oxidized/gm tissue/hr (X±SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>3.31 ± 0.20</td>
</tr>
<tr>
<td>GTG (300 mg/kg)</td>
<td>8</td>
<td>0.84 ± 0.10*</td>
</tr>
</tbody>
</table>

* Significantly different from control, p<0.001.
CHAPTER 2. GLUCOSE ANALOG EFFECTS ON GLUCOSE OXIDATION IN THE VENTROMEDIAL HYPOTHALAMUS

Introduction

Glucose analogs have been used extensively in the study of glucose transport and glucose-mediated responses in vitro. Glucosamine (GLA), mannosepentulose (MAN), and 2-deoxyglucose (2DG) all block insulin release from pancreatic islets in response to a glucose stimulus (Coore and Randle, 1964a and b; Montague and Taylor, 1968). In addition, pancreatic glucose utilization is impaired by 2DG, MAN, and GLA (Wicks et al., 1955a and b; 1956; Ashcroft et al., 1970; Sols and Crane, 1954; Ishibashi et al., 1981), which suggested a competitive inhibition of glucose binding to pancreatic glucoreceptors and possible competitive inhibition of glucose utilization. In answer to these possibilities, 2DG and MAN inhibit glucose utilization at transport and phosphorylation levels in glucose metabolism (Ullrey et al., 1982; Coore and Randle, 1964a and b). However, GLA can decrease glucose oxidation by inhibiting transport as well as phosphorylation and subsequent glucose metabolism (Ashcroft et al., 1970; Ullrey et al., 1982).

Cold thioglucose (GTG) is a substrate-specific neurotoxin for a small neuronal cell population in the ventromedial hypothalamus (VMH). Destruction of these cells with GTG leads to hyperphagia and eventually
obesity (Debons et al., 1977). However, in pancreatic tissue, GTG causes an enhancement of insulin secretion with or without glucose present in vitro, with no apparent cell toxicity (Caterson and Taylor, 1979; Hahn et al., 1980). This observation suggests that differences may exist between the brain and pancreas with respect to glucose metabolism. To investigate this possibility, we tested the effect of MAN, 2DC, CLA and GTG on VMH glucose oxidation in vitro.

Materials and Methods

Female CFl mice, weighing between 20-25 grams, were housed at 23°C with a 12-hour light:12-hour dark photoperiod, fed Teklad Rat and Mouse Diet and given tap water freely. Animals were decapitated and the VMH was dissected free from the brain at the level of the median eminence as a cube of tissue weighing approximately 3.0 mg. Histological observations and the monitoring of glucose oxidation in normal and GTG-lesioned tissue cubes indicated that 75% of each VMH tissue cube contained neuronal elements vulnerable to GTG (Brown and Viles, 1980; 1982). Two tissue cubes (caps) were placed in culture vessels (25 ml Erlenmeyer flasks with a gas trap) containing 1.47 ml of medium and 7.5 uCi of uniformly labeled [14C] D-glucose. The incubating medium was similar to cerebral spinal fluid, prepared according to Jones et al. (1975). The medium contained 9.6 mM (2.58 mg) glucose, and was supplemented (volume to volume) with 2.6% complete amino acid concentrate, 28.6% rat serum, and 1.3% glutathione (200 mM). The vessels were supplemented with 2.58 mg, 5.16 mg or 7.74 mg (1, 2 or 3
times the quantity of glucose in the medium, respectively) of 2DC, MAN, or GLA, or an equal volume of saline. GTC supplements were 3 or 6 times the amount of glucose in the medium. Millimolar concentrations for the various compounds are given in the figures. Other vessels possessed no tissue but contained 2DC, MAN, GLA, GTC, or saline and served as controls for auto-oxidation of the [14C] D-glucose. The vessels were sealed with rubber septums and gassed with a 95% oxygen:5% carbon dioxide mixture. The VMM caps were allowed to incubate with 2DC, MAN, GLA, GTC, or saline in the medium for 30 minutes at 37°C before the [14C] D-glucose was added. After the addition of [14C] D-glucose, the incubation was continued for 90 minutes. The reaction was stopped and the [14C] CO₂ released by the addition of 9.0 N H₂SO₄ (0.2 ml) to the medium. Hyamine (Sigma) (0.5 ml) was added to the gas trap to absorb the [14C] CO₂ and incubated for an additional 60 minutes. The hyamine was collected from each vessel and counted by liquid scintillation spectrometry with a Beckman 250 liquid scintillation counter. As a test for tissue viability, saline vessels with hypothalamic tissue were supplemented with 15 μl of 2.0 mM dinitriphenol (DNP) (Fisher) in an aqueous solution and processed as described above. Furthermore, as expected, varying the amount of [14C] D-glucose in the incubation system altered glucose oxidation linearly, as a function of labeled glucose activity. Analysis of variance with Dunnett's test was used to analyze for statistical significance between glucose analog treatment and glucose oxidation.
Results

MAN (Figure 1) and GTG (Figure 2) had no effect on glucose oxidation in the VNH. No inhibition of glucose oxidation was observed at any concentration of MAN (1, 2 or 3 times the amount of glucose in the medium) or GTG (3 or 6 times the amount of glucose in the medium). GLA caused a nonsignificant decrease in glucose oxidation at a quantity 3 times that of glucose (Figure 3). The 2X amounts of GLA had little or no effect on CO₂ production from glucose in the VNH. However, GLA present in the medium at a quantity equal to that of glucose caused a significant enhancement of glucose oxidation (Figure 3).

The 2DG did have an inhibitory effect on VNH [¹⁴C] CO₂ production from glucose. Although glucose oxidation was depressed with 2DG levels at 1 or 2 times the amount of glucose in the medium, significant inhibition of glucose oxidation was not observed until the 2DG concentration by weight was 3 times that of glucose (Figure 4). As the 2DG concentration was increased and the glucose concentration remained constant, the inhibition of glucose oxidation in the VNH also increased (Table I). However, the inhibition was not a 1:1 relationship; an amount of 2DG three times greater than glucose was needed in the medium to inhibit glucose oxidation 50%. The \( K_i \) was determined to be \( 11.96 \times 10^{-5} \) M with an inhibition maximum (\( I_{max} \)) of 100%, as determined by a double reciprocal plot (Figure 5). However, a Michaelis-Menten plot for kinetic analysis revealed an atypical curve (Figure 5, inset).
Employing 2DG, MAN, and GLA, three inhibitors of glucose utilization in the pancreas, it was found that quantities of 2DG at least three times greater than glucose were necessary to achieve any significant reduction in glucose oxidation in the VMH. GLA did inhibit glucose utilization at a quantity three times that of glucose present, but this depression was not significant. MAN had no effect. Also, the neurotoxin GTG had no effect on VMH glucose oxidation in vitro. Since 2DG and MAN should block the glycolytic pathway by competitively inhibiting phosphoglucone isomerase (5.3.1.9) (Ashcroft et al., 1970), these sugars would be expected to inhibit glucose oxidation at much lower concentrations. In fact, the apparent $K_i$ of $11.96 \times 10^{-5}$ M for 2DG is much higher than the physiological range and more closely resembles a pharmacological effect. The atypical Michaelis-Menten plot is also suggestive of a non-physiological effect (Figure 5, inset).

MAN has differential effects on the high $K_m$ and low $K_m$ hexokinase in pancreatic islets (Zawalich et al., 1978; Ishibashi et al., 1981). The high $K_m$ hexokinase is blocked by MAN but the low $K_m$ hexokinase is unaffected. This phenomenon may account for the lack of MAN inhibition on VMH glucose oxidation. GLA is a transport and metabolic blocker of glucose utilization. However, no significant inhibitory effect on VMH glucose oxidation was found, although a trend towards a significant decrease in glucose utilization was discerned. No conclusion concerning the metabolic level(s) of GLA inhibition of VMH glucose oxidation can be made from our data. Alternately, since an in vitro incubation system...
was used with an excess of glucose and glucose analogs, the Crabtree effect might be responsible for the apparent inhibition of glucose oxidation, particularly at the high concentrations utilized. Interestingly, GTC had no effect on VMH glucose oxidation. This neurotoxin produces necrosis in the mouse VMH within 24 hours after injection (Debons et al., 1977), albeit no neural damage is seen until 4 hours after GTC injection (personal observation). The short incubation time used in these studies could account for the absence of any GTC effect on glucose oxidation in the VMH. Furthermore, even shorter pancreatic islet incubation times were used in the studies illustrating a GTC potentiation of insulin secretion (Caterson and Taylor, 1979; Hahn et al., 1980). The possibility of damage to the cells of the VMH during dissection and incubation cannot be dismissed. However, the uncoupling of oxidative phosphorylation by DNP produces a threefold increase in glucose oxidation during incubation (Brown and Viles, 1980; 1982), suggesting that potential damage by the experimental procedures was a minimal factor in impairing tissue viability. Moreover, Coore and Randle (1964, a and b) point out that the inhibitors NaN, GLA, and 2DG have no cytotoxicity in the pancreas. This may not be true for VMH tissue, however, 2DG pretreatment can prevent GTC-induced lesion formation in the VMH (Likuski et al., 1967).

None of the glucose analogs tested in these experiments exhibited significant inhibition of glucose oxidation by the VMH at approximate equimolar concentrations. This result suggests that these analogs were not effective in competing with glucose for the glucose utilization
pathways in the VMH of which glucose oxidation is one. Possibly high affinity binding is characteristic of glucoreceptors in the VMH, lessening competitive inhibition by the analogs; this same effect may be operating at the level of the transport or enzyme systems.
Figure 1. The effect of various concentrations of MAN on VHN glucose oxidation in vitro. The bars indicate the mean±SEM. 1X, 2X and 3X represent the amount of MAN in the medium expressed as multiples of the amount of glucose in the medium.
Figure 2. The effect of various concentrations of GTG on VMH glucose oxidation in vitro. The bars indicate the mean±SEM. 3X and 6X represent the amount of GTG in the medium expressed as multiples of the amount of glucose in the medium.
Figure 3. The effect of various concentrations of GLA on VMH glucose oxidation in vitro. The bars indicate the mean±SEM. 1X, 2X and 3X represent the amount of GLA in the medium expressed as multiples of the amount of glucose in the medium. * p<0.01
Figure 4. The effect of various concentrations of 2DG on VMH glucose oxidation in vitro. The bars indicate the mean±SEM. 1X, 2X and 3X represent the amount of 2DG in the medium expressed as multiples of the amount of glucose in the medium. * p<0.005
Figure 5. A double reciprocal plot of the 2DG inhibition kinetic data.

Inset. A Michaelis-Menten plot of the 2DG inhibition kinetic data.
y = 0.1196x + 0.0100
CORRELATION (r) = 0.93
$K_i = 11.96 \text{ mM}$
$I_{\text{max}} = 100\%$
CHAPTER 3. GLUCOSE OXIDATION IN THE VENTROMEDIAL HYPOTHALAMUS
IS NOT AFFECTED BY INSULIN OR OUABAIN BUT
DEPRESSED BY ALLOXAN TREATMENT

Introduction

Insulin enhances the uptake and utilization of glucose in a variety of tissues. A notable exception has been the brain; insulin appears to have no effect on the central nervous system (CNS). Grodsky and Frohman (1966) showed that insulin does not cross the blood-brain barrier. Glucose uptake and utilization in the brain occurs in the absence of insulin (Frohman, 1969), apparently by a diffusion process. However, Frasannan (1972) reported the presence of an insulin effect on glucose uptake in the brain. Moreover, an insulin-sensitive glucoregulator chemoreceptor has been illustrated to exist in the CNS (Szabo and Szabo, 1972; Chen et al., 1975). Havrankova et al. (1978a) have demonstrated insulin receptors are plentiful in the brain. In addition, the hypothalamic nuclei contain the highest levels of insulin compared to other parts of the CNS (Havrankova et al., 1978b).

The ventromedial hypothalamus (VMH) has long been reputed to be involved with the control of food intake and satiety (Debons et al., 1977). Debons et al. (1969; 1970) have demonstrated that insulin has a direct effect on gold thioglucose (GTC) responsiveness in the VMH. The
VMH can be destroyed with a single intraperitoneal injection of CTG, causing hyperphagia and eventually obesity (Marshall et al., 1955). The characteristic lesions induced by CTG are not seen in alloxan-diabetic or anti-insulin serum diabetic animals. Intrahypothalamic insulin injections into the VMH of diabetic animals causes a return of the VMH susceptibility to CTG necrosis. This evidence indicates that the VMH has a sensitivity to insulin, and requires the presence of insulin for normal function. The pathway by which insulin reaches the VMH is unknown, however, there is a strong possibility that this may occur via the cerebral spinal fluid (Margolis and Altszuler, 1967).

Glucose transport can be coupled with the sodium-potassium pump mechanism as a cotransport phenomenon (Stryer, 1975). The brain possesses a heterogeneous, ATP-dependent sodium-potassium transport system (Marks and Seeds, 1979). It is conceivable that glucose transport in the brain and possibly satiety mediation may be coupled with a sodium-potassium pump mechanism. Ouabain, a sodium-potassium pump ATPase inhibitor (Schwartz et al., 1972; Marks and Seeds, 1979), could be used to elucidate the involvement, if any, of the sodium-potassium pump with glucose utilization in the VMH.

To further study the effects of ouabain or insulin on the VMH, an in vitro system for incubating hypothalamic tissue from mice was employed.
Materials and Methods

Female C57 mice, weighing between 20-25 gm, were used in this study. The mice were maintained at 23°C with a 12 hour light:12 hour dark photoperiod, fed Teklad Rat and Mouse Diet and given tap water freely.

Normal animals were utilized for the ouabain investigations, while diabetic animals were used for the insulin experiments. Diabetes mellitus was induced by a single 100 mg/kg intravenous injection of 2.0% aqueous alloxan. On the third day post injection, the animals were tested for glucosuria using Clinistix, as an indicator of the diabetic condition, and used for experimentation.

Animals were decapitated and the VMH was dissected free from the brain at the level of the median eminence as a cube of tissue weighing approximately 3.0 mg. Monitoring glucose oxidation in normal and CTG treated tissue cubes indicated that 75% of each tissue cube contained neuronal elements vulnerable to CTG. Histological examination confirmed this observation. Two tissue cubes (caps) were placed in culture vessels (25 ml Erlenmeyer flasks with a gas trap), containing 1.47 ml of medium and 7.5 uCi of uniformly labeled [14C] D-glucose. The incubating media was artificial cerebral spinal fluid and prepared according to Jones et al. (1975). The vessels were supplemented with 1-2 mUnits or 10 Units of insulin in a small volume, 45 ul of 8.0 mM ouabain or an equal volume of saline. Other vessels possessed no tissue but contained insulin, ouabain or saline, and served as controls for auto-oxidation of the [14C] D-glucose. All vessels were sealed with rubber septums and
gassed for 30 seconds with a 95% oxygen:5% carbon dioxide mixture. The VNH caps were allowed to incubate with saline, ouabain or insulin in the media for 30 minutes at 37°C. After the addition of $[^{14}\text{C}]$ D-glucose, the incubation was continued for 90 minutes. The reaction was stopped and the $[^{14}\text{C}]$ carbon dioxide released by the addition of 9.0 N sulfuric acid (1.2 ml) to the media. Hyamine (0.5 ml) was added to the gas trap to absorb the $[^{14}\text{C}]$ carbon dioxide and incubated for an additional 60 minutes. The hyamine was collected from each vessel and counted by liquid scintillation with a Beckman 250 liquid scintillation counter.

As a test for tissue viability, saline vessels with hypothalamic tissue were supplemented with 15 ul of 2 mM dinitrophenol in an aqueous solution and processed as described above. Experimental treatments were done in quadruplicate. The t-test was used to test for statistical significance.

Results

Ouabain had no effect on glucose oxidation in the VNH (Figure 1A). This evidence indicates that glucose transport is not dependent on a cotransport mechanism with the sodium-potassium pump. Glucose oxidation in VNH caps from diabetic mice was not enhanced by the presence of insulin (Figure 1B and C). Physiological concentrations (Figure 1B) or pharmacological amounts (Figure 1C) of insulin did not alter glucose oxidation in these diabetic VNH tissue caps. It is noteworthy, however, that untreated, normal tissue caps displayed a much higher glucose oxidation rate than alloxan-diabetic tissue caps ($p<0.001$). No
significant difference existed between the controls in the two insulin experiments (Figure 1B and C). This point will be discussed below. The presence of dinitrophenol, an uncoupler of oxidative phosphorylation, in the incubation media caused glucose oxidation to greatly increase (10.8 x 10^-8 moles glucose oxidized/mg tissue/hour), a process analogous to the Pasteur effect. While not unexpected, the response to dinitrophenol poisoning indicates that tissue viability was not impaired during incubation.

Discussion

Studies by Likuski et al. (1967) have shown glucose may play a major role in determining satiety. Autoradiographic techniques demonstrated GTC and non-necrotizing analogs of GTC become localized in the VMH but only GTC caused a VMH lesion. These results indicated that the effect of GTC is not due to the toxicity of the gold or sulphur moiety, or failure to penetrate the blood-brain barrier (Likuski et al., 1967), but that glucose is of prime importance in the development of GTC-induced pathology and possibly satiety.

Glucose transport and oxidation in the VMH are not coupled with the sodium-potassium pump mechanism. This evidence, however, does not eliminate the possibility that the sodium-potassium pump may be involved with satiety. Intrahypothalamic injections of phlorizin, an inhibitor of glucose transport and oxidation, are known to prevent the hyperphagia and obesity caused by a GTC challenge (Debons et al., 1974a). The phlorizin apparently interferes with the development of the GTC-induced
necrosis in the VMH. Preliminary work in our laboratory indicates that intrahypothalamic infusions of ouabain do not produce the CTC-induced lesions seen in the VMH. This observation supports the idea that the sodium-potassium pump is not involved with CTC necrosis and perhaps satiety.

The action of glucose with possible VMH satiety mechanisms requires insulin (Debons et al., 1969; 1970; 1977). Our data, however, suggest that glucose transport and oxidation do not require an insulin-dependent mechanism. The diabetic condition imposed upon the tissue would be expected to enhance any insulin effects when compared to normal tissue. This result is in agreement with the work of Goodner and Berrie (1977). They demonstrated that normal hypothalamic tissue failed to respond to insulin in vivo and in vitro. Their explanation for this paradox suggested that, (a) insulin may act indirectly on the hypothalamus via neurons from the circumventricular organs (the median eminence apparently does take up insulin), or (b) the permeability of the blood-brain barrier may possibly be altered by insulin, enhancing the rate of glucose transport into the CNS.

Alloxan has been used as an effective diabetogenic agent; a large dose will destroy the beta cells of the pancreas and limit insulin secretion (McEvoy and Hegre, 1977). Prior treatment of the beta cells with D-glucose will protect them from alloxan damage (McDaniel et al., 1976). This result suggests that alloxan competes with glucose for receptors in the cell membrane. Systemic injections of 2-deoxy-D-glucose (2DG) are known to cause hyperphagia (Smith and Epstein, 1969),
and inhibit GTC necrosis in the VMH (Likuski et al., 1967), presumably by competing with glucose transport mechanisms. Recently, intraventricular injections of alloxan were found to abolish the hyperphagic response induced by 2DG, suggesting a competition between alloxan and 2DG for receptor sites in the brain (Woods and McKay, 1978).

Woods and McKay (1978) reported that alloxan treatment eliminated the 2DG hyperphagia response for weeks. The cellular receptors sensitive to 2DG and glucose appeared permanently altered by alloxan. Our data support this finding. VMH tissue caps, previously treated with alloxan by a single intravenous injection, cannot oxidize as much glucose per unit as normal VMH tissue caps similar in weight (comparison of control in Figure 1A with controls in Figure 1B and C). The alloxan-diabetic tissues exhibit a 66% depression in glucose oxidation with respect to normal tissues. This alloxan-induced decrease in glucose oxidation is highly significant (p<0.001).

As early as three days following alloxan treatment, the cell membranes in the VMH are apparently altered (McDaniel et al. 1976). Glucose transport properties and/or receptor mechanisms in VMH membranes seem to be deficient following alloxan treatment. Reduced glucose oxidation rates in alloxan-diabetic VMH caps support this conclusion.
Figure 1. A. The effect of saline (CON) and ouabain (OUA) on the amount of glucose oxidized by normal VMH tissue. B. The effect of saline (CON) and 1-2 mU of insulin (INS) on the amount of glucose oxidized by alloxan-diabetic VMH tissue. C. The effect of saline (CON) and 10 U insulin (INS) on the amount of glucose oxidized by alloxan-diabetic VMH tissue. The bars indicate the mean of four experiments (eight mice) ± S.D. No significant difference was found between the saline control and ouabain treatment or saline controls and insulin treatments.
CHAPTER 4. THE EFFECT OF DIGITOXOSE ON FEEDING BEHAVIOR

Introduction

Mayer (1953) proposed the original glucostat hypothesis of feeding behavior on the basis of gold thioglucose (GTC) vulnerability of the ventromedial hypothalamus (VMH) coupled with subsequent hyperphagia. These data suggested that a mechanism for regulating food intake, which was sensitive to glucose analogs, was located in the VMH. GTC sensitivity in the VMH and subsequent lesion formation can be inhibited by systemic injections of 2-deoxy-D-glucose (2DG) (Likuski et al., 1967). 2DG is a competitive inhibitor of glucose utilization (Wicks et al., 1955b; 1957), which also induces hyperphagia (Likuski et al., 1967). Phlorizin, a competitive inhibitor of glucose transport (Lotspeich, 1961), also will block GTC necrosis in the VMH (Debons et al., 1974a; Brown and Viles, 1982) and induce hyperphagia (Glick and Mayer, 1968). Pharmacological manipulation of VMH glucose utilization may affect feeding behavior. Smith and Epstein (1969) have proposed a glucoprivic mechanism for the control of feeding behavior; brain glucoreceptors stimulate feeding when their glucose utilization is diminished. Since Harrazzi (1976) has demonstrated the presence of glucose-sensitive neurons in the VMH, a glucoreceptor system may exist in the VMH which is important in controlling food intake.
However, specific GTC vulnerability in the VMH has not been widely accepted. Liebelt and Perry (1967) presented evidence that the GTC-induced VMH lesion is nonspecific. They noted that other hypothalamic and extrahypothalamic nuclei can be damaged by GTC, and attributed this general destruction to blood-brain barrier damage. Caffyn (1971; 1972a) examined GTC-induced lesions by electron microscopy and concluded that GTC caused capillary damage, resulting in increased capillary permeability and subsequent downstream ischemia. These conditions produced edema and cellular damage. Levine and Sowinski (1982) found that the triamine, 3,3'-methyleniminobis-(N-methylpropylamine), produced VMH lesions very similar to those of GTC, even though the compounds are structurally unrelated. This evidence suggested that GTC is a drug which causes nonspecific lesions of the VMH via vascular damage.

Recently, Young et al. (1979) have observed that, while regions of the hypothalamus other than the VMH may be affected by GTC, the median eminence is not damaged. They concluded that GTC may have a specificity for certain hypothalamic structures and suggested GTC lesions might result from impairment of glucose uptake and utilization by the VMH. Debona et al. (1979b) have demonstrated that damage to the VMH neuropil in the pericapillary regions occurred prior to observable capillary damage and is restricted to a small area of the VMH. They have suggested that GTC is a specific neurotoxin, damaging target neuronal structures in the VMH before ischemia and edema develop.

Recent studies of VMH glucose oxidation in vitro have revealed that several glucose analogs (Brown and Viles, 1985), including 2DC, and
inhibitors of glucose transport, such as phlorizin (Brown and Viles, 1982), do not inhibit glucose oxidation significantly except at very high concentrations. This result is consistent with the observations of Likuski et al. (1967) that large doses of 2DG are required to block CTG lesion formation in the VMH in vivo. Brown and Viles (1982) have shown that large systemic doses of phlorizin also were required to block CTG lesion formation in the VMH in vivo. However, the high concentrations of these inhibitors necessary to affect VMH glucose oxidation in vitro suggest that these compounds are not very effective inhibitors of glucose oxidation, and possibly not highly competitive glucoreceptor-binding analogs, at least in the VMH.

The sugar digitoxose, 2,3-dideoxy-ribohexose, part of the cardiac glucoside digitoxin, has been shown to inhibit glucose-stimulated insulin release from the pancreatic islets without affecting glucose oxidation in the beta cells. Garcia Hermida and Gomez-Acebo (1975) attributed this phenomenon to the interaction of digitoxose (DIG) with a glucoreceptor in the beta cell membrane, which did not interfere with glucose transport or oxidation. This proposal suggested two pathways for glucose utilization in this system: one affecting stimulus-secretion coupling and the other providing glucose for energy utilization.

In this study, DIG was used as a chemical probe to test several aspects of VMH glucose sensitivity for evidence of differentiated glucose utilization, as proposed for the pancreas. The experiments included: 1) measuring the effects of DIG on feeding behavior as an
indicator of modulation of feeding behavior, 2) the effects of DIG on GTG-induced necrosis as an indication of inhibition of GTG binding in the VMH, and 3) the effects of DIG on VMH glucose oxidation as an indicator of inhibition of glucose utilization.

Materials and Methods

CF1 female mice, weighing 20-25 gm, were used for these experiments. The mice were fed Teklad Rat and Mouse Diet and given tap water freely. A photoperiod of 12 hours light; 12 hours dark and an environmental temperature of 23°C were maintained. A 1000 mg/kg DIG dose was given. If more than one injection was used, the first 4 injections were given at 3-hour intervals. Subsequent administrations were given every 6 hours. GTG was given in a dose of 300 mg/kg. All injections were given intraperitoneally. The Mann-Whitney U and t-test were used to test for significant differences.

Feeding Behavior

Mice were assigned randomly to two groups: normal and obese. Within each group were controls (water injections, n=4) and experimentals (DIG injections, n=4). Since food intake can vary tremendously between the light and dark periods, the effects of DIG were assessed under both light and dark conditions. Under dark conditions, all injections and weighings were done with red light. To enhance feeding, the animals were deprived of food for 18 hours prior to experimentation (Latham and Blundell, 1979). The light and dark experiments were begun 2 hours after the end of the previous 12-hour
photoperiod. The control (water) and DIG injections were given 15 minutes before food (Teklad Rat and Mouse Diet) was made available, and at 1000 hrs for day experiments and 2200 hrs for night experiments. Obese mice were derived from normal mice given a GTG injection. These GTG-treated mice were judged obese if they weighed at least 25% more than their uninjected littermates 6 weeks postinjection. Total food intake and group body weight were monitored every hour for the 6-hour period. To determine whether or not the animal colony was responsive to drug-induced alterations of food intake, mice were selected at random from the stock colony, injected with 2DG (300 mg/kg) at approximately 1000 hrs, and monitored for hyperphagia. All of these 2DG-treated mice (n=8) became hyperphagic. The mice were thus considered responsive to drug-induced changes in feeding behavior.

**DIG and GTG Interaction**

DIG was injected 30 minutes before the GTG injection, between 1000 and 1200 hrs. After the GTG injection, the mice (n=10) were subsequently injected with DIG at the appropriate intervals. A total of 6 DIG injections were given. Control animals (n=10) were given H2O instead of DIG. The mice were decapitated 24 hours after the GTG challenge. The brains were removed from the skull, fixed in Bouin's fluid, dehydrated, and embedded in paraffin. Seven-micron thick sections were made through the VMH at the level of the median eminence and stained with hematoxylin and eosin. The VMH was examined for lesions by light microscopy.
In vitro Glucose Oxidation Studies

Animals were decapitated in the morning and the VMH was dissected free from the brain at the level of the median eminence as a cube of tissue. Two VMH tissue cubes (caps) were placed in a culture vessel (a 25-ml Erlenmeyer flask with a gas trap; n=6) containing 1.47 ml of medium, 7.75 mg DIG in H₂O, and 7.5 uCi of aqueous uniformly labeled [¹⁴C] D-glucose. Control vessels (n=6) contained saline instead of DIG. The incubating medium was similar to cerebral spinal fluid and prepared according to Jones et al. (1975). Other vessels possessed no tissue and served as controls (n=6) for auto-oxidation of the [¹⁴C] D-glucose. All vessels were sealed with rubber septums and gassed for 30 seconds with a 95% oxygen:5% carbon dioxide mixture. The caps were allowed to incubate 15 minutes with DIG in the medium at 37°C. After the addition of [¹⁴C] D-glucose, the incubation was continued for 90 minutes. The reaction was stopped and the [¹⁴C] CO₂ released by the addition of 9.0 N H₂SO₄ (0.2 ml) to the medium. Hyamine (0.5 ml) was added to the gas trap to absorb the [¹⁴C] CO₂ and incubated for an additional 60 minutes. The hyamine was collected from each vessel and [¹⁴C] emissions counted with a Beckman 250 liquid scintillation counter. As a test for tissue viability, saline vessels with hypothalamic tissue were supplemented with 15 ul of 2.0 mM dinitrophenol in an aqueous solution and processed as described above.


Results

Feeding Behavior

The effect of DIG on short-term feeding behavior (a 6-hour interval) was examined under light and dark conditions. DIG was not effective in significantly altering food intake in the dark. However, in the light DIG reduced short-term food intake significantly, p<0.01 (Table I). The effect of DIG on short-term feeding was also tested in mice made obese with GTC. DIG had no effect on short-term feeding in GTC-lesioned, obese mice during the light. Thus, the reduced food intake observed in normal mice in the light was abolished by a GTC challenge.

Normal mice responded typically to changes in the photoperiod; more food was consumed at night than during the day (p<0.01). Also, the obese mice ate more food than the normals (p<0.01).

DIG Inhibition of GTC Necrosis

DIG injections did not alter the vulnerability of the VMH to GTC administration. Mice injected with either DIG and GTC or water and GTC exhibit GTC lesions of similar dimensions (Figure 1). No significant difference existed in lesion size between the two treatments, as determined by planimetry.

In addition, preliminary data indicate that DIG infused into the VMH did not interfere with GTC necrosis. These intrahypothalamic infusions of DIG did not eliminate or alter size of the GTC lesion.
In vitro Glucose Oxidation

DIG enhanced glucose oxidation in the VMH by almost 13% at concentrations 3 times greater than glucose in the incubation medium (Figure 2). However, this difference was not significant. The effect of dinitrophenol, an uncoupler of oxidative phosphorylation, was similar to that observed earlier (Brown and Viles, 1980; 1982). Glucose oxidation increased approximately threefold, indicating that glucose oxidation pathways and regulatory mechanisms were still functional.

Discussion

In this study, it has been demonstrated that the sugar DIG has no effect on GTG-induced lesion formation in the VMH, and in vitro glucose oxidation in VMH tissue. However, DIG injections did decrease daytime food intake significantly without affecting nocturnal feeding behavior. Debons et al. (1968; 1969; 1970) have shown that VMH vulnerability to GTG-induced necrosis is insulin-dependent; diabetic mice are resistant to GTG lesion formation. DIG has been shown to inhibit glucose-stimulated insulin release from the pancreas (Garcia Hermida and Gomez-Acebo, 1975). Thus, if DIG decreased insulin levels sufficiently, GTG lesion formation should be inhibited in DIG-treated mice. However, DIG injections did not prevent the development of a GTG-induced lesion or alter the size of the lesion, nor did an intrahypothalamic infusion of DIG. The possibilities of DIG binding competitively to GTG-sensitive neural tissue or diminishing insulin secretion, and thereby inhibiting GTG lesion formation, is not supported.
VHN caps incubated in vitro with DIG showed no inhibition of glucose oxidation; instead they showed an enhanced (albeit insignificant) glucose oxidation rate, about 13% greater than the controls. Garcia Hermida and Gomez-Acebo (1975) observed a similar enhancement of glucose oxidation in pancreatic islets treated with DIG. This evidence suggested that DIG is not an effective inhibitor of glucose oxidation in the VHN, as has been observed with a number of glucose analogs (Brown and Viles, 1985). Indeed, phlorizin, an inhibitor of glucose transport, showed a significant potentiation of VHN glucose oxidation in vitro, but only at a low concentration (Brown and Viles, 1982).

DIG significantly reduced food intake in mice during the light photoperiod, but had no effect on feeding in the dark. It might be expected that an effect would be more noticeable with darkness, since mice consume the majority of their food at night. However, this photoperiod-dependent difference in feeding behavior could be explained by a hypothalamic circadian rhythm. The suprachiasmatic nucleus in the hypothalamus, an area associated with the control of diurnal variations, selectively bound 2DG during the day but not at night (Schwartz and Gainer, 1977). In addition, Larue-Achagiotis and LeMagnen (1979) have shown that a 2DG challenge inhibited food intake at night but stimulated feeding during the day. The daytime results from their study (Larue-Achagiotis and LeMagnan, 1979) are incongruent with our data. This inconsistency may be linked to the ability of 2DG to be a glucoprivic stimulus, while DIG is not. Ritter et al. (1981) have demonstrated that
the glucoreceptors responsible for eliciting hyperphagia, under conditions of glucoprivation, are located in the hindbrain and not in the hypothalamus. A glucoprivic stimulus resulting from the administration of 2DG or 5-thioglucose (5TG) is known to produce an increase in food intake (Ritter et al., 1981). These two compounds also inhibit GTG lesion formation, presumably by binding to VMH glucoreceptors (reference for 2DG-Likuski et al., 1967; 5TG-Brown, 1983). Since DIG has an opposite effect on feeding behavior compared with these glucoprivic compounds and DIG has no effect on GTG-induced necrosis in the VMH, apparently DIG cannot be described as a substance capable of inducing glucoprivation or binding to hypothalamic glucoreceptors. DIG probably does not affect daytime feeding behavior by a central mechanism.

Garcia Hermida and Gomez-Acebo (1975) demonstrated that DIG affected glucose-stimulated insulin secretion, presumably by binding to beta cell membrane glucoreceptors. It has been well-documented that peripheral hepatic glucoreceptors play a role in the regulation of feeding behavior (Novin et al., 1973; Russek, 1970). The exact mechanism is unclear, but these peripheral glucoreceptors may produce feeding and/or satiety signals, which are relayed to the brain via vagal afferents (Niijima, 1969). Phlorizin, when infused intraventricularly into the brain, produced hyperphagia, apparently by binding to central glucoreceptors (Glick and Mayer, 1968). An infusion of phlorizin into the liver decreased the vagal afferent discharge rate, probably by affecting hepatic glucoreceptors (Niijima, 1969). Furthermore,
infusions of 2DG into the hepatic portal system stimulated feeding (Novin et al., 1973; Russek, 1970). Since DIG is known to affect insulin secretion via glucoreceptors, it is conceivable that DIG may affect other peripheral glucoreceptors, such as those found in the liver. Thus, our observation that DIG inhibited daytime feeding behavior may be due to a diurnal alteration of vagal afferent discharge mediated by hepatic glucoreceptors. Further investigations are needed to explore this possibility.
Figure 1. Cross sections of mouse brain, depicting the VMH at the level of the median eminence, 24 hrs after a CTC challenge.

A. Water given IP initially followed by CTC (300 mg/kg) IP.
B. DIG (1000 mg/kg) injected IP followed by 300 mg/kg CTC parenterally. Bar equals 1.0 mm
Figure 2. The effect of DIG on glucose oxidation in the VMH in vitro. The bars indicate the mean ± SEM. No significant difference is apparent.
Moles x 10^-4 glucose oxidized/gm tissue/hour

<table>
<thead>
<tr>
<th></th>
<th>SALINE CONTROL</th>
<th>DIG (35mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n = 6</td>
<td></td>
<td>n = 6</td>
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The bar chart shows the comparison of glucose oxidation between saline control and DIG (35mM) conditions.
## TABLE I. The effect of digitoxose (DIG) on food intake in food-deprived mice in light or dark

<table>
<thead>
<tr>
<th></th>
<th>Normal mice food intake (gm/gm body weight, mean ± SEM)</th>
<th>GTG obese mice food intake (gm/gm body weight, mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Water (Control, n=4) Dig (n=4)</td>
<td>Water (Control, n=4) DIG (n=4)</td>
</tr>
<tr>
<td>Light</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Range)</td>
<td>0.008 ± 0.005 * 0.085-0.092</td>
<td>0.073 ± 0.007 * 0.064-0.077</td>
</tr>
<tr>
<td></td>
<td>0.132 ± 0.048 * 0.080-0.176</td>
<td>0.134 ± 0.050 * 0.088-0.188</td>
</tr>
<tr>
<td>Dark</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Range)</td>
<td>0.108 ± 0.012 * 0.096-0.119</td>
<td>0.111 ± 0.012 * 0.103-0.125</td>
</tr>
</tbody>
</table>

* A significant difference between treatments, p<0.01.
SECTION II.

NEUROTRANSMITTER MANIPULATIONS
CHAPTER 5. TRYPTOPHAN EFFECTS ON GTC-INDUCED
VENTROMEDIAL HYPOTHALAMIC NECROSIS

Introduction

Cold thioglucoao (CTC) has been used for over 30 years as a means of inducing obesity in experimental animals. This obesity syndrome is believed to be caused by CTC-induced necrosis of the ventromedial hypothalamus (VMH), but the mechanism of CTC action in the hypothalamus has remained an enigma. Recently, however, serotonin (5-
hydroxytryptamine, 5HT) has been implicated in mediating CTC lesion formation in the VMH.

Debons et al. (1978; 1979a) have presented evidence suggesting that the development of VMH lesions induced by CTC is mediated by 5HT release. Drugs that depleted 5HT stores or antagonized 5HT action prevented CTC-induced necrosis in the VMH. Although 5HT abnormally increases capillary permeability (Sparrow and Wilhelm, 1957), CTC-induced necrosis of the VMH can occur without structural capillary damage (Debons et al., 1977; 1979b). The VMH has a high level of 5HT (Brownstein et al., 1976), and CTC apparently damages serotonergic neurons causing a release of 5HT. This 5HT release, in turn, damages the adjacent capillaries as a consequence of the neuropil destruction induced by CTC. These damaged neuronal components are possibly those involved with the reputed satiety function of the VMH (Blundell, 1977).
Caffyn (1972a and b) also observed hypothalamic edema associated with the GTG injection. Prevention of 5HT mediated edema inhibited GTG lesion formation, even though VMH levels of 5HT are high (Debons et al., 1979a). These data presented by Debons et al. (1977; 1978; 1979a; 1979b) support the ideas that (1) GTG-induced necrosis is not produced by vascular damage as proposed by Caffyn (1971; 1972a), but by a toxic flood of 5HT released from damaged neuronal cells and (2) GTG can serve as a useful probe for hypothalamic function. It follows that the precursor of 5HT, tryptophan (TRP), could influence GTG necrosis in the VMH.

TRP, an essential amino acid, is normally supplied to animals through the diet. A high fat diet (Curzan and Knott, 1974; Hyypa et al., 1977), insulin injections (Fernstrom et al., 1974; Mackenzie and Trulson, 1978), fasting (Fernstrom et al., 1974; Curzan and Knott, 1974), and malnutrition (Miller et al., 1977) are all known to increase TRP levels in the brain. Since the rate limiting enzyme in the 5HT synthetic pathway, tryptophan hydroxylase, is not saturated with substrate under normal conditions (Growdon, 1979), an increase in brain TRP would be expected to increase brain 5HT levels (Warsh et al., 1979). Thus, each of the above diets or physiological nutritional states would increase 5HT levels in the central nervous system. Low levels of TRP in an otherwise normal diet should cause decreases in brain 5HT content. Sahakian et al. (1979) have shown this condition to be true.

The evidence for the involvement of 5HT in the control of feeding behavior is strong (Blundell, 1977; Blundell and Latham, 1979). The
precursor of 5HT, TRP, suppressed food intake when injected in rats or mice (Blundell, 1977; Latham and Blundell, 1979). Presumably this effect is brought about by the metabolism of TRP to 5HT (Latham and Blundell, 1979). It is conceivable that factors controlling 5HT metabolism may influence the responsiveness of the VMH to GTC. The production of 5HT is dependent on the presence of the precursor TRP. As mentioned earlier, since TRP is an essential amino acid and must be supplied through the diet, the amount of TRP ingested may influence the metabolism of TRP and 5HT.

These physiological and nutritional states, reflecting increased blood and brain TRP levels, may be related to GTC hypothalamic vulnerability. The availability of TRP for the production of 5HT in the central nervous system could be a primary factor in GTC lesion formation. Recently, Young et al. (1979) have shown that mice maintained on a high fat diet, versus normal chow or high carbohydrate diet, have significantly larger GTC-induced VMH lesions. They postulated that the high fat diet resulted in peripheral glucose intolerance causing elevated blood levels of GTC or glucose. An alternative explanation for the increased sensitivity of the VMH to GTC in mice fed a high fat diet is plausible. The increased level of nonesterified fatty acids in the blood from the high fat intake resulting in an increase in 5HT brain content (Fernstrom et al., 1974; Pardridge, 1977), could result in more extensive damage to the VMH due to a GTC challenge, since 5HT is implicated as mediating the GTC-induced VMH pathology (Debons et al., 1978; 1979a).
If high levels of TRP and 5HT can increase VMH vulnerability to GTG, then low TRP levels may depress GTG necrosis in the VMH. Brain TRP levels are low in diabetics (Mackenzie and Trulson, 1978). The resulting decreased brain 5HT content may depress the susceptibility of the VMH to GTG to such a low level, that necrosis is inhibited in diabetics. Debons and co-workers (1968) discovered that a GTG injection in diabetic mice caused no necrosis of the VMH, suggesting that VMH GTG sensitivity required the presence of insulin. Injections of insulin increase brain TRP content (Mackenzie and Trulson, 1978). The insulin administration reported by Debons et al. (1969; 1970), to reverse GTG insensitivity of the VMH in diabetics may be due to increased 5HT synthesis, mediated by an increased brain TRP concentration.

Since TRP (Latham and Blundell, 1979) and probably 5HT (Blundell, 1977; Blundell and Latham, 1979) levels are correlated with food intake, manipulations of TRP in the daily diet could possibly be a means of controlling hypothalamic GTG sensitivity in normal or diabetic mice.

**Materials and Methods**

**General Description**

Female CF1 mice, weighing between 20-25 gm, were used in this study. The mice were maintained at 23°C with a 12 hour light:12 hour dark photoperiod, fed Teklad Rat and Mouse Diet and given tap water freely.
Normal Mice

Experiments were either short term (24 hours or less) or long term (more than 24 hours). TRP in an aqueous suspension was given in two doses, 150 or 400 mg/kg intraperitoneally (IP); GTC was injected IP in a dose of 300 mg/kg. TRP administration (3 infusions) varied from 3 hour to 8 hour intervals, during the short term studies. Long term experiments had 1 TRP injection per day. Controls were given water and GTC.

Mice were decapitated 24 hours after GTC injection. The brains were removed from the skull, fixed in Bouin's fluid, dehydrated, embedded in paraffin, sectioned at 7 μm, stained with hematoxylin and eosin, and examined by light microscopy. The size of the lesion was determined by the method described by Young et al. (1979). Briefly, the cross sectional area of the lesion at its largest point was measured using a planimeter and an overhead slide projector at a magnification of 46 times. The t-test was used to determine significant differences.

For rapid lesion detection and empirical comparisons between experimental treatments and controls, brains fixed in Bouin's fluid were separated at the level of the median eminence and the presence or absence of a lesion determined with a dissecting microscope.

Diabetic Animals

Diabetes mellitus was induced by an intravenous injection of alloxan (150 mg/kg). The diabetic condition was confirmed 3 days post injection using Clinistix as an indicator of glucose in the urine. An aqueous suspension of TRP was given IP (200 mg/kg) or IP and orally (400
Results

Normal Animals

All normal TRP treated animals (IP or oral) and controls in the short term experiments challenged with GIG, exhibited lesions of similar dimensions upon light microscopy inspection. The dose of TRP (400 mg/kg IP) and interval between the 3 TRP injections appeared, empirically, to have no effect on GIG lesion formation in the VMH. Planimetry of a short term experiment with TRP given orally confirmed this observation (Table I and Figure 1). No significant difference in lesion size existed between TRP-treated and control animals.

Long term experiments produced varied results. The experiment using oral TRP injections (150 mg/kg) for 7 days did not produce any size differences in the VMH lesion between TRP-treated and control animals (Table II). The long term study lasting 8 days produced a significant reduction in lesion size between experimentals and controls. These mice, receiving the TRP (150 mg/kg IP each day), displayed much smaller VMH lesions induced by GIG than the controls (Figure 2 and Table III).

Alloxan Diabetic Mice

Diabetes mellitus prevents GIG necrosis in the VMH via insulin deficiency. Animals given TRP (200 mg/kg/day IP), during the development of the diabetic condition (3 days), and challenged with GIG did not exhibit GIG necrosis in the VMH (Table IV). Two series of TRP
(400 mg/kg, IP only or IP and oral) injections administered at a 7 hour interval to 3 day alloxan diabetics also caused no change in the expected response to GTG, which was given 4 hours after the first set of TRP injections (Table V). No GTG lesions were observed in the VMH. TRP was unable to reverse the inhibition of GTG lesion formation in the VMH due to the absence of insulin.

Discussion

Debons et al. (1979a) have shown that 5HT is a potential mediator of GTG necrosis in the mouse VMH. Changes in the 5HT content in the hypothalamus would be expected to alter the responsiveness of the VMH to GTG. TRP loading increases the content of TRP and 5HT in the brain (Wurtman and Fernstrom, 1974; Crowdon and Wurtman, 1979), thereby increasing the probability of a larger VMH lesion due to a GTG challenge. Supplements of TRP, however, did not cause an increase in lesion size following a GTG challenge, but rather exhibited no effects on lesion size and in one experiment caused a reduction in lesion size.

The rate of 5HT turnover may have an effect on VMH sensitivity to GTG. When the 5HT content of the brain is increased, the turnover rate of 5HT is accelerated (Gessa and Tagliamonte, 1974). Chronic injections of TRP increase TRP hydroxylase activity in the brain, however, TRP levels are not higher compared with controls (Diez et al., 1976). A rapid turnover rate of 5HT would, in effect, cause the cellular pools of 5HT to remain constant or perhaps decrease in the presence of high TRP levels. Thus, levels of brain 5HT in animals receiving TRP
supplementation could be similar to the controls. Mackenzie and Trulson (1978) have shown this phenomenon to be true. Manipulations that caused changes in brain TRP concentrations produced no alterations in 5HT metabolism. GTC-induced VMH lesions of similar or smaller size might, therefore, be expected in both the controls and the animals receiving TRP.

In no instance did TRP administration produce an increase in the size of VMH lesions due to GTC. However, in the 8 day long term study, TRP caused a significant decrease in the size of GTC-induced VMH lesions. TRP loading over an 8 day period may accelerate the turnover of 5HT in the brain. As implied by Diez et al. (1976), this increased 5HT turnover rate may have resulted in a relative decrease in 5HT content in the VMH. The 5HT reduction, but not depletion, could account for the smaller lesions in the 8 day TRP injected mice. Indeed, Eccleston and Nicolaou (1978) have shown that rats given TRP displayed an increased 5HT turnover rate. Oral TRP in either short or long term experiments showed no effect on GTC lesion formation in the VMH. The reason for this difference is unclear, however, the intragastric loads of TRP may not have been completely absorbed, thereby decreasing any systemic TRP effect.

Diabetes prevents the occurrence of GTC-induced pathology in the mouse VMH (Debons et al., 1968; 1969; 1970). Diabetes also causes a decrease in brain TRP concentrations. Increasing brain TRP levels may produce a VMH sensitivity to GTC. Our results demonstrate that TRP treatment does not alter VMH vulnerability to GTC in diabetic mice.
However, insulin administration will cause a return of CTG lesion formation in diabetics (Debons et al., 1969; 1970). Insulin injections also produce an increase in brain TRP levels without a change in 5HT metabolism (Nackensie and Trulson, 1978). Since even TRP levels appear to have no effect on 5HT concentrations at least with respect to CTG lesion formation in diabetics, sensitization of the diabetic VMH to CTG with insulin is the foundation for CTG lesion formation in this model. 5HT and TRP do not appear to contribute significantly to the alteration of the VMH sensitivity in diabetic mice.

Debons et al. (1979a) have shown that 5HT acts as a mediator for CTG lesion formation. Since TRP is the nutritional precursor for this neurotransmitter, we investigated whether or not TRP loading had any effect on CTG lesion formation. Our results suggest that TRP and 5HT play a role in CTG-induced necrosis in the mouse VMH but that this role may be overshadowed by hormonal influences.
Figure 1. The effect of oral TRP on a short term basis on GTG lesion formation. A. Control—a cross-section of the VMH at the level of the median eminence from a mouse given water orally and challenged with GTG. B. A cross-section of the VMH at the median eminence level from a mouse treated with TRP and subsequently given GTG. No significant difference exists between the water and TRP treatments.
Figure 2. The effect of long term TRP (150 mg/kg/day for 8 days) treatment on GTG lesion formation. A. Control—a cross-section of the VMH at the median eminence level from a mouse given water orally and challenged with GTG. B. A cross-section of the VMH at the level of the median eminence from a mouse treated with TRP and challenged with GTG. The TRP treated mice displayed significantly smaller GTG-induced lesions compared with the controls, p<0.001.
**TABLE I. The effect of tryptophan (TRP) given orally on short term basis on CTC lesion formation**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Lesion size (mean ± SD) (cm²; x46)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
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<td>5.3 ± 2.0</td>
</tr>
<tr>
<td>TRP</td>
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<td>7.4 ± 4.5</td>
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TABLE II. The effect of oral tryptophan (TRP) for 7 days on GTG-induced necrosis in the hypothalamus

<table>
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</thead>
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<tr>
<td>TRP</td>
<td>7</td>
<td>6.0 ± 2.0</td>
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</table>
TABLE III. The effect of tryptophan (TRP) given intraperitoneally for 8 days on CTC necrosis in the VMH

<table>
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</thead>
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<td>TRP</td>
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</table>

* Significantly different from the water controls, p<0.001.
TABLE IV. The effect of tryptophan (TRP) treatment during the development of diabetes on GTG lesion formation

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<tr>
<td>TRP</td>
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TABLE V. The effect of tryptophan (TRP) treatment on CTC lesion formation in diabetic mice

<table>
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<th>Treatment</th>
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<tr>
<td>TRP</td>
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<td>0</td>
</tr>
<tr>
<td><strong>400 mg/kg TRP—oral and IP</strong></td>
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<tr>
<td>Control</td>
<td>7</td>
<td>0</td>
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<tr>
<td>TRP</td>
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CHAPTER 6. THE EFFECT OF ALPHA-METHYLPARATYROSINE AND TYROSINE LOADING ON GOLD THIOGLUCOSE LESION FORMATION IN THE VENTROMEDIAL HYPOTHALAMUS

Introduction

Catecholamines (CA) are known to have profound effects on feeding behavior. Grossman (1960) was the first to show that intraventricular administration of norepinephrine (NE) stimulated hyperphagic activity. Injections of dopamine (DA) had no effect (Leibowitz, 1973). Destruction of dopaminergic fiber systems (Rowland et al., 1979) caused hypophagia in rats made hyperphagic by ventromedial hypothalamic (VMH) electrocoagulation. Cuts made in the ventral noradrenergic bundle produced hyperphagia (Gold, 1973; Grossman and Grossman, 1977). Treatment of rats with drugs known to deplete central NE abolished eating (Leibowitz, 1975). On the other hand, opposite effects also were found with NE depletors. Leibowitz (1975) suggested that alpha-adrenergic receptors stimulated feeding, while beta-adrenergic and dopaminergic receptors inhibited food intake. Recent work by Leibowitz and Rossakis (1979) supports this multi-receptor idea. Injections of L-dopa into the perifornical lateral hypothalamus (PLH) suppressed feeding. Blockage of the dopaminergic and beta-adrenergic receptors or inhibition of dopa decarboxylase reversed the suppression. L-dopa
appeared to decrease food intake, in part, by increasing CA synthesis in specific neurons located in the PLH.

It is well-known that destruction of the VMH by gold thioglucone (CTG) causes an obesity syndrome (Debons et al., 1977). Lorden et al. (1979) have demonstrated that CTG lesions produce significant decreases in hypothalamic NE and DA. Since levels of DA and NE are high in the hypothalamus (Palkovits et al., 1974), these losses in NE and DA may be involved with the hyperphagia observed in CTG treated animals. However, Lorden and co-workers (1979) could find no correlation between body weight gain and reduction in hypothalamic NE or DA concentrations.

Recently serotonin (5HT) has been implicated as a mediator of CTG-induced necrosis in the VMH (Debons et al., 1979a). The CTG lesion may result from a toxic flood of 5HT released by damaged cells during the initial stages of lesion development (Debons et al., 1979a). However, the fact that 5HT appears to participate in CTG induced necrosis, does not rule out the idea that CA may still be associated with increased feeding caused by VMH lesions. 5HT seems to be involved with the effects of NE and DA (Blundell, 1979), suggesting the existence of a 5HT-CA axis. Increases in 5HT brain content depressed DA dependent circling behavior in mice (Wilson and Pycock, 1976). Injections of 5HT into the nucleus accumbens increased the levels of DA metabolites in the area, suggesting that DA turnover was enhanced (Pycock et al., 1978). In vitro studies have indicated that 5HT terminals will take up DA but apparently not NE (Berger, 1978). In addition, 5HT uptake has been demonstrated in NE and DA neurons (Lichtensteiger et al., 1967).
Interactions between 5HT and CA neurons may contribute to the hyperphagia and obesity resulting from GTC administration (Coscina et al., 1978).

To further investigate the involvement of CA in the GTC induced obesity syndrome, two experiments were done. The effect of tyrosine (TYR) administration on GTC lesion formation in the mouse VMH was assessed. TYR is the precursor for CA synthesis. In addition, alpha-methylparatyrosine (AMPT) was given to mice to deplete the brain of CA stores and the effect of this treatment on GTC-induced necrosis in the VMH investigated.

Materials and Methods

General

Female CF1 mice, weighing between 20-25 gm were used in this study. The mice were maintained at 23°C with a 12 hour light:12 hour dark photoperiod, fed Teklad Rat and Mouse Diet and given tap water freely.

Following the various experimental methods described below, the mice were decapitated 24 hours after the GTC injection. The brains were removed from the skull, fixed in Bouin's fluid, dehydrated, embedded in paraffin, sectioned at 7 μm, stained with hematoxylin and eosin, and examined by light microscopy. The size of the lesion was determined by the method described by Young et al. (1979). Briefly, the cross sectional area of the lesion at its largest point was measured using a planimeter and overhead slide projector at a magnification of 46 times.
The t-test was used to test for significance between TYR or AMPT treated animals and their controls.

**AMPT Treatment**

Aqueous AMPT, an inhibitor of TYR hydroxylase and a CA depletor, was given intraperitoneally (IP) in doses of 250 or 350 mg/kg and followed by GTG (400 mg/kg IP) at 2 to 8 hours after AMPT. A combination dose of AMPT also was given—350 mg/kg with 175 mg/kg administered 23 hours later. GTG was given within one hour after the second AMPT injection. Controls received water and GTG.

**TYR Loading**

Mice were injected with TYR (200 or 400 mg/kg) IP and/or orally with a tube as an aqueous suspension. The long term experiments involved injections of 200 mg/kg TYR IP and orally (a total of 400 mg/kg) for 6 days, once a day. GTG (300 mg/kg IP) was given on the fifth day.

One short term investigation involving the oral administration of 400 mg/kg TYR, 4 times over a 24 hour period at 4 to 9 hour intervals was done. GTG (300 mg/kg IP) was injected 3 hours after the first TYR injection.

All controls for the TYR loading experiments received the appropriate amounts of water and GTG.
Results

Mice given TYR for 6 days orally and IP and injected with GTG developed lesions very similar in appearance to controls (Figure 1). No significant difference in lesion size existed between the TYR-treated and controls (see Table I). In addition, TYR given orally only to mice as described above, did not alter GTG lesion size in the VMH significantly (Table II).

AMPT planimetry data from all levels were pooled and analyzed. No significant differences in lesion size existed among dose levels. The lesions formed in the mice given AMPT were significantly larger compared to the controls (p<0.001) (Table III and Figure 2).

Discussion

GTG causes a distinct VMH lesion and obesity syndrome very similar to the obesity phenomenon induced by electrolytic destruction of the VMH. Since Rowland et al. (1979) have demonstrated that the electrolytic VMH obesity syndrome can be reversed by destruction of DA neurons, perhaps alteration in brain CA levels are involved with GTG lesion formation in the VMH.

Oral and/or IP TYR administration had no significant effect on GTG necrosis in the VMH. This result implies that increases in CA concentrations by TYR loading (Fernstrom and Wurtman, 1974) do not have an effect on GTG lesion formation. The lack of a TYR loading effect is consistent with the evidence reported by Lorden et al. (1979). Although central CA levels in the hypothalamus decrease as a result of
GTG necrosis, these lower CA stores could not be correlated with the direct effects of GTG, i.e. hyperphagia and obesity, using body weight as a monitor.

Although our data indicate that potential dietary manipulations of CA had no effect on GTG necrosis in the VMH, depletion of central CA concentrations with AMPT enhanced GTG lesion formation. Animals injected with AMPT developed significantly larger GTG lesions in the VMH compared with controls. This finding suggests that CA may be involved with the development of GTG-induced necrosis. Since the VMH damage apparently is enhanced by the absence of CA, perhaps CA feedback has been compromised. However, Debons et al. (1979a) have shown that GTG necrosis is dependent on the presence of serotonin (5HT) and/or tryptophan hydroxylase activity in the VMH. On the other hand, interactions between CA and 5HT have been suggested. Biochemical (Eccleston and Nicolaou, 1978; Pycock et al., 1978) and behavioral (Sahakian et al., 1979) evidence support the idea of a 5HT-CA axis. Moreover, Berger (1978) demonstrated that cerebral 5HT terminals can take up DA on a competitive basis with 5HT. In the absence of DA, due to AMPT treatment, these 5HT terminals might take up more 5HT than under normal conditions. Such an increase in 5HT in VMH neurons may account for the larger GTG lesions exhibited in AMPT treated animals. In the presence of high CA levels (TYR pretreatment), the amount of 5HT taken up by the neurons would be decreased. However, if CA turnover is increased due to the TYR stimulation of CA synthesis, the level of 5HT
in the terminals may remain relatively constant. Thus, no significant change in GTC lesion formation would be observed in TYR treated or control animals.
Figure 1. The effect of TYR loading (6 days orally and IP) on GTG lesion formation. A. Control. A cross-section of the mouse VMH at the level of the median eminence. This animal was treated with water and challenged with GTG. B. A cross-section of the VMH at the median eminence level from a mouse treated with TYR and challenged with GTG. No significant difference exists between the two treatments (see Table I).
Figure 2. The effect of acute AMPT treatment on GTC lesion formation.

A. Control. A cross-section of the mouse VMH at the level of the median eminence. This animal was treated with water and challenged with GTC.

B. A cross-section of the VMH at the median eminence level from a mouse treated with AMPT and challenged with GTC. The AMPT treated mice displayed significantly larger GTC-induced lesions compared with controls, p<0.001
TABLE I. The effect of oral and IP tyrosine (TYR) on GTG-induced necrosis

<table>
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<th>Treatment</th>
<th>n</th>
<th>Lesion size (mean ± SD) (cm² ± SD)</th>
</tr>
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<tbody>
<tr>
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<td>6.2 ± 2.5</td>
</tr>
<tr>
<td>TYR</td>
<td>9</td>
<td>8.6 ± 5.1</td>
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TABLE II. The effect of short term, oral tyrosine (TYR) on GTG lesion formation

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<td>5.3 ± 2.8</td>
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<tr>
<td>TYR</td>
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<td>4.0 ± 2.5</td>
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<tr>
<td>Treatment</td>
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<td>Lesion size (mean ± SD) (cm² x46)</td>
</tr>
<tr>
<td>-----------</td>
<td>----</td>
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</tr>
<tr>
<td>Control</td>
<td>11</td>
<td>5.9 ± 1.9</td>
</tr>
<tr>
<td>AMPT</td>
<td>15</td>
<td>11.5 ± 3.9*</td>
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* Significantly different from the control, p<0.001.
SECTION III.

HORMONAL MANIPULATIONS
CHAPTER 7. THE EFFECT OF ESTRADIOL BENZOATE ON GOLD THIOGLUCOSE NECROSIS IN THE VENTROMEDIAL HYPOTHALAMUS

Introduction

Estrogen (EST) can influence several aspects of carbohydrate metabolism including gluconeogenesis, glycogen synthesis, and blood glucose levels (Wade and Gray, 1979). Glucose uptake in muscle (McKerns et al., 1958) and adipose tissue (Gilmour and McKerns, 1966) are enhanced by EST. This cellular uptake of glucose may be due to the estrogenic stimulation of insulin secretion from the islets (Costrini and Kalkhoff, 1971; Wade and Gray, 1979). Moreover, tissues apparently are supersensitized to insulin by EST (King and Cox, 1973), consequently increasing glucose metabolism (Wade and Gray, 1979).

In addition to affecting carbohydrate metabolism, EST can alter food intake and body weight (Fishman, 1976; Wade and Gray, 1979). As EST levels increase during the ovulatory cycle, food intake and body weight decrease (Fishman, 1976). Ovariectomized (OVX) animals do not exhibit these decreases. Replacement therapy for OVX animals with EST or EST analogs produces a reduction in food intake and weight (Fishman, 1976; Wade and Gray, 1979). It is noteworthy that EST apparently decreases body weight only by altering carbohydrate intake (Wurtman and Baum, 1980).
The exact mechanism of EST action on food intake is unknown, but may involve the ventromedial hypothalamus (VMH). Implants of EST directly into the VMH suppress food intake (Jankowiak and Stern, 1974). When surrounding nuclei received EST implants, no changes in feeding behavior were observed. This result was expected since the VMH has a high affinity for EST, while adjacent areas do not (Jankowiak and Stern, 1974).

The VMH also is affected by the drug gold thioglucose (GTC). A single intraperitoneal injection of GTC causes a specific lesion in the VMH, followed by a chronic hyperphagia and obesity (Marshall et al., 1955). Debons et al. (1979b) have demonstrated that GTC is highly specific for neural tissue in the VMH and that GTC lesion formation is dependent on the presence of insulin in the VMH (Debons et al., 1968; 1969; 1970). An intrahypothalamic injection of insulin caused a return of GTC sensitivity in the VMH of diabetic animals. Although insulin apparently does not affect glucose oxidation in the brain in vivo or in vitro (Goodner and Berrie, 1977; Brown and Viles, 1980), this hormone is present in the brain (Havrankova et al., 1978b). Recently, GTC-induced VMH necrosis has been shown to be sensitive to EST. Young et al. (1978) demonstrated that EST administration increased significantly the size of GTC lesions. These authors suggested that glucose uptake and/or storage may be enhanced in the VMH by EST.

Since EST can facilitate glucose uptake and enhance insulin effects in various tissues (Wade and Gray, 1979), treatment of diabetic animals with EST might cause a reversal of VMH insensitivity to GTC necrosis.
In order to examine the possible effects of EST on VMH glucose oxidation in a more quantitative fashion, in vitro incubations using \(^{14}\text{C}\) glucose were performed.

**Materials and Methods**

C57 female mice, weighing 20-25 gm were used in this study. The animals were maintained at 23°C on a 12 hour light:12 hour dark photoperiod, fed Teklad Rat and Mouse Diet, and given tap water freely. Mice were made diabetic by a single intravenous injection of alloxan, a 300 mg/kg dose in a 3% aqueous solution. Mice were judged diabetic and used for experimentation if glucosuria was evident using a Clinistix, three days after alloxan administration. A pharmacological dose of estradiol benzoate (EB), an EST analog, in oil (10 µg per mouse per day) or oil alone was injected subcutaneously for 4 days, beginning on the day of the alloxan injection. Once diabetes was established on the third day after alloxan administration, the mice were challenged with a 300 mg/kg CTC dose. The animals were decapitated 24 hours later. The brains were removed from the skull and fixed in Bouin’s fluid for 48 hours. The brains were dehydrated, embedded in paraffin, sectioned at 7 µm, mounted on glass slides, stained with hematoxylin and eosin, and the VMH examined for CTC-induced pathology by light microscopy.

The procedure for the in vitro VMH glucose oxidation studies has been described previously (Brown and Viles, 1980; 1982). Briefly, culture vessels with VMH tissue in medium were supplemented with 1 or 3 µg of EB in 15 µl of ethanol, resulting in 0.67 and 2.0 mM of EB.
respectively in the culture medium. Control vessels received ethanol only. Tissue incubated with dinitrophenol served as an indicator for tissue viability, as described earlier (Brown and Viles, 1980; 1982). The t-test was used for determining statistical significance.

Results

Diabetic controls given oil alone and GTG did not display any lesion formation (Figure 1). Diabetic mice given EB in oil and challenged with GTG did not develop lesions in the VMH (Figure 1). EB, therefore, did not reverse the inhibition of GTG-induced lesion formation caused by an absence of insulin in diabetic mice. EB (1 ug or 3 ug in ethanol) had no significant effect on glucose oxidation in VMH caps compared to controls supplemented with ethanol alone (Figure 2). Since ethanol may have an acute affect on glucose oxidation, the controls with ethanol were compared with a control group supplemented with saline instead of ethanol. Ethanol depressed glucose oxidation significantly (p<0.001) (Figure 2). The studies of Brown and Viles (1980; 1982) confirmed that the hypothalamic tissue was viable during experimentation. Empirically, these dinitrophenol results were confirmed in this study.

Discussion

Diabetic mice do not develop GTG lesions because lesion formation is insulin-dependent (Debons et al., 1968; 1969; 1970). Since EST is known to supersensitize tissue to insulin (King and Cox, 1973) and
enhance GTG lesion formation in the VNH (Young et al., 1978), we postulated that EST might cause a return of GTG sensitivity in the VNH of diabetic mice, much in the same manner that adrenalectomy reverses GTG insensitivity in diabetics (Debons et al., 1974; 1977). However, EB supplements given to diabetic mice had no effect on GTG lesion formation. Apparently, EST is not effective in reversing the diabetic block to GTG lesion development in the VNH. Although EST is known to have an influence on carbohydrate metabolism (Wade and Gray, 1979), this effect is probably not significant compared to aberrations in carbohydrate metabolism associated with diabetes.

Glucose oxidation in vitro was measured to determine if EST does affect VNH glucose metabolism. EB had no apparent effect on glucose oxidation in the VNH. This result suggests that EST does not have a significant effect on VNH glucose transport and utilization. However, ethanol was the solvent for EB and may have interfered with glucose oxidation. Alcohol administration can alter glucose transport and metabolism in the small intestine, liver, heart, and brain (Roe, 1979). Specifically, Towell and Erwin (1982) have shown that ethanol treatment in the isolated perfused mouse brain at 37°C decreased the glucose utilization rate in the hypothalamus significantly. Our ethanol controls confirmed this finding; these vessels exhibited over a 62% decrease in glucose oxidation as measured by $^{14}$C CO$_2$ evolution. Thus, any EB effects in this in vitro system may have been masked by the alcohol solvent.
Figure 1. The effect of estradiol benzoate (EB) treatment on GTG lesion formation in the VMH of alloxan-diabetic mice. A. Control. Diabetic mice given oil alone and GTG. No lesion is apparent in the VMH. B. A diabetic mouse given EB in oil and challenged with GTG. No necrosis of the VMH can be discerned.
Figure 2. The effect of ethanol or ethanol and estradiol benzoate (EB) on glucose oxidation in the mouse VMH. Saline controls oxidized significantly more glucose compared with each condition containing ethanol, p<0.001
Moles x 10^-8 glucose oxidized/mg tissue/hour

- Saline Control
- Ethanol Only
- 1.0 μg (0.67 mM) EB
- 3.0 μg (2.0 mM) EB

* denotes significance at the p<0.05 level.
CHAPTER 8. STRESS INHIBITS GOLD THIOGLUCOSE-INDUCED
HYPOTHALAMIC DAMAGE

Introduction

Gold thioglucose (GTC) is known to induce hyperphagia coupled with
destruction of the ventromedial hypothalamus (VMH) (Debons et al.,
1977). This sensitivity of the VMH to GTC is altered by the presence or
absence of various hormones. For instance, insulin was necessary for
development of GTC necrosis in the VMH, since diabetic animals are
insensitive to a GTC challenge (Debons et al., 1968; 1969; 1970).
Glucocorticoid administration abolished GTC necrosis in the VMH (Debons
et al., 1977; Brown, 1982a). In addition, mice that were insensitive to
a GTC challenge due to diabetes mellitus became vulnerable to the drug
after adrenalectomy (ADX) (Debons et al., 1974b). Injections of
estradiol benzoate super-sensitized the VMH to GTC-induced damage (Young
et al., 1978).

Caffyn (1971;1972a and b) and Liebelt and Perry (1967) have
suggested that GTC is a nonspecific hypothalamic vascular toxin.
 Allegedly capillary breakdown, edema and ischemia, caused by GTC
administration, resulted in VMH lesion development (Caffyn, 1971; 1972a
and b). However, this evidence has been questioned recently (Young et
al., 1979; Debons et al., 1979b). Debons and colleagues (1979b) have
shown, by ultrastructural studies, that GTG necrosis is very specific. The initial pathology is limited to an area between the arcuate and VMH nuclei. Debons et al. (1979b) concluded GTG necrosis is specific for neural components in the VMH.

Furthermore, the neurotransmitter serotonin (5HT) has been suggested to be the mediator of GTG necrosis in the VMH (Debons et al., 1979a). Depletion of 5HT stores with para-chloro-phenylalanine, reserpine or antagonism of 5HT action with cyproheptadine, methylsergide or chlorpromazine, all prevented GTG lesion formation (Debons et al., 1979a; Briese and Murzi, 1981). However, 24 hours after drug treatment, when 5HT levels are still low, mice challenged with GTG develop typical VMH lesions. Apparently this phenomenon is caused by a return of tryptophan (TRP) hydroxylase activity for the production of 5HT.

Stress can lead to alterations in systemic (Azmitia and McEwen, 1974) and brain (Kakihana and Moore, 1978) glucocorticoid concentrations and 5HT metabolism (Yuwiler, 1979). Moreover, these alterations in glucocorticoid and 5HT activity by stress may not be independent effects of the stress. Fasting, cold exposure, restraint, ether anesthesia, alcohol administration, frustration, formalin injections, novelty, trauma and electric foot shock all increase glucocorticoid concentrations in the plasma and brain (Boulouard, 1963; Kakihana and Moore, 1978; Yuwiler, 1979). Electric foot shock, ether anesthesia and cold exposure also increase TRP hydroxylase activity (Azmitia and McEwen, 1974). Glucocorticoid administration alone, to normal experimental animals, will increase TRP hydroxylase activity (Azmitia
and McEwen, 1974), the rate limiting enzyme in 5HT synthesis, while ADX blocks the stress-induced increase in TRP hydroxylase activity (Sze et al., 1976). Other investigators (Palkovits et al., 1976; Sze et al., 1976) have shown that stress does not alter TRP hydroxylase activity but lowers 5HT concentrations by causing an increased turnover rate of 5HT. In addition, recent reports support the idea that increased 5HT levels parallel increased glucocorticoid concentrations in the body (McEwen, 1980). These studies indicate that stress can alter 5HT metabolism.

As reviewed above, stress can alter glucocorticoid levels and 5HT synthesis, compounds which are known to play a role in CTC lesion formation. In this study, we investigated the effect of two stress syndromes, cold exposure and food deprivation (starvation), on CTC-induced necrosis in the mouse VMH. In addition, the effect of TRP supplements on CTC lesion development during the starvation syndrome was tested.

Materials and Methods

CF1 female mice, weighing between 20–25 gm were used in this study. The animals were housed at 23°C with a 12 hour light:12 hour dark photoperiod, fed Teklad Rat and Mouse Diet and given tap water freely.

Unless stated otherwise, CTC was given intraperitoneally (IP) in a 300 mg/kg dose. Cold stress consisted of a 29 hour exposure at 4°C in a cold room. CTC was administered 5 hours after the beginning of the cold exposure period. Mice deprived of food were fasted 72 hours before CTC was injected. Controls for the cold stress or fasted experiments were
maintained at 23°C and fed lab chow ad lib, respectively.

Bilateral ADX's were done on mice under metofane anesthesia. The animals were allowed to recover 1-3 days before being subjected to the stress of food deprivation and challenged with CTG. All ADX mice were given 0.9% saline instead of tap water to compensate for the loss of the mineralcorticoids.

In separate experiments TRP was given to food deprived animals. In the first study, mice were starved for 3 days. On the third day, TRP (400 mg/kg) was administered IP two times, once two hours before CTG (200 mg/kg IP) and again 5 hours after CTG. The brains from these animals were examined for lesions but not processed for photographic purposes. In another study, TRP (100 mg/kg IP) was injected into mice 2 or 3 times a day at 4 to 8 hour intervals over 4 days of starvation. On the third day following removal of the food supply, the mice were challenged with CTG (300 mg/kg). The VNH of these animals was examined for neural damage but not photographed.

Animals were decapitated 24 hours post CTG injection. The brains were removed from the skull and the VNH was dissected free as a cube of tissue and fixed overnight in 10% formalin. These tissues were embedded in 8% agar and sectioned at 50u using a Smith and Farquhar tissue sectioner. CTG-induced pathology of the VNH was rapidly determined in these sections using the Penfield modification of the del Rio Hortega silver carbonate reaction for oligodendroglia (Humason, 1972) and light microscopy. For photographic purposes, brains from the food deprived, cold exposed mice and controls challenged with CTG were fixed in Bouin's
fluid, dehydrated, embedded in paraffin, sectioned at 7μm, stained with hematoxylin and eosin, and photographed using a light microscope.

Results

CTC necrosis was inhibited in animals subjected to food deprivation (Figure 1) or cold exposure (Figure 2). The VMH of these stressed animals challenged with CTC was indistinguishable from normal untreated mice. Control animals, fed and maintained at 23°C, developed typical VMH lesions when challenged with CTC. Inhibition of CTC lesion formation in stressed animals was always complete and consistent (Table I).

Preliminary data from ADX mice that were starved showed no lesion formation when given CTC. The VMH from these animals was indistinguishable from the starved mice given CTC. Apparently removal of removal of glucocorticoids had no effect on VMH insensitivity to CTC during starvation.

TRP administration had no effect on lesion formation during food deprivation. Animals given the two 400 mg/kg TRP injections (IP) at a 7 hour interval after 3 days of starvation and challenged with 200 mg/kg CTC 2 hours after the first TRP injection, exhibited no CTC-induced necrosis in the VMH (Table II). When TRP (100 mg/kg IP) was given 2 or 3 times a day at 4 to 8 hour intervals for 4 days during starvation, and the animals then challenged with CTC (300 mg/kg IP) on the third day, similar results were received (Table III). No VMH necrosis could be detected.
Discussion

Since injections of cortisone or hydrocortisone are known to cause inhibition of CTC lesion development in the VMH (Debons et al., 1977; Brown, 1982a), it is tenable that environmental and physiological conditions which elevate glucocorticoid concentrations in the plasma may also alter the VMH response to CTC. Stress elevates glucocorticoid levels in the blood and brain (Kakihana and Moore, 1978), and might insensitize the VMH to a CTC challenge. Caffyn (1972b) has suggested that CTC necrosis in the VMH is the result of a vascular inflammatory response. This work suggested that glucocorticoids, as well as other anti-inflammatory drugs such as aspirin, could abolish CTC-induced pathology by preventing the inflammatory response in the VMH. However, Debons et al. (1974b) have shown that cortisone, in amounts insufficient to prevent CTC necrosis in normal animals, can abolish CTC lesion formation in diabetic-ADX (Long-Lukens) animals. Moreover, ultrastructural evidence has been reported indicating that the primary site of CTC action is neural and not vascular (Debons et al., 1979b).

Glucocorticoid injections increase TRP hydroxylase activity in the brain (Azmitia and McEwen, 1974), while ADX inhibits the acceleration of TRP metabolism observed with glucocorticoid administration or stress (Sze et al., 1976). Since CTC lesion development appears to be dependent on the VMH 5HT and TRP hydroxylase activity (Debons et al., 1979a), it might be expected that glucocorticoid injections would enhance CTC-induced damage in the VMH. However, it is well known that glucocorticoid treatment prevents CTC lesion formation (Debons et al.,
1977; Brown, 1982a). Palkovits et al. (1976) and Sze et al. (1976) have demonstrated decreased concentrations of brain 5HT, under stress conditions. They postulated that an increase in 5HT turnover rate yielded decreased 5HT pools. These lowered 5HT stores, due to the high turnover rate of 5HT, could explain the desensitizing effect glucocorticoids and stress have on the mouse VMH when challenged with CTG.

Our data show that CTG-induced necrosis in the VMH is eliminated by the imposition of stress. No CTG lesions were detected in mice subjected to cold exposure or food deprivation. This result is consistent with reports made in the literature (Debons et al., 1977; Brown, 1982a). As described above, CTG lesion formation is dependent on the activity of TRP hydroxylase and probably 5HT content in the VMH. Although stress increases glucocorticoid levels, thereby increasing TRP hydroxylase activity, the tremendous turnover rate of 5HT that results from the glucocorticoid stimulation may actually decrease the pool size of 5HT in the VMH (Eccleston and Nicolaou, 1978). Thus, CTG lesion formation would be eliminated by stress. Another possibility is that glucocorticoids may bind to cells in the VMH and block CTG necrosis directly (Kakihana and Moore, 1978; Brown, 1982a).

Diet is known to affect the levels of neurotransmitters in the brain (Wurtman and Fernstrom, 1974). Since 5HT is implicated as a mediator of CTG lesion formation, TRP was injected into starved mice. It was anticipated that if 5HT is a major factor in the development of CTG necrosis in the VMH, administration of TRP would increase the TRP
hydroxylase activity and 5HT level in the VMH and perhaps reverse the inhibition of GTG lesion formation during stress. As the results indicate, TRP treatment during starvation did not sensitize the VMH to a GTG challenge. This evidence suggests that 5HT concentrations and TRP hydroxylase activity, although important in the mediation of GTG-induced damage in the VMH, may be of secondary importance to the glucocorticoid status in the animal. Apparently increases in glucocorticoid levels in the blood and brain will counteract any effect a 5HT metabolism change will have on GTG lesion formation.

During starvation, removal of the adrenal gland will cause a significant decrease in the blood glucocorticoid levels and perhaps sensitize the VMH to GTG during starvation. However, our preliminary data indicate that ADX and food deprived mice do not develop VMH lesions, when challenged with GTG. Two alternatives may have caused this result in the ADX-starved mice. During starvation, ketone bodies are the main energy source for the brain. Young et al. (1979) have suggested that GTG vulnerability of the VMH is associated with glucose utilization in the area. With little glucose available to the hypothalamus, GTG lesion formation may be eliminated. Thus, the VMH of stressed and ADX mice, utilizing ketone bodies mainly for energy, would not be susceptible to GTG necrosis. On the other hand, the ADX may have been incomplete. In this case, the mice would not develop a glucocorticoid deficiency. In addition, auxillary adrenal-like bodies are known to exist in mice (Soffer, 1956). This tissue may have compensated for the glucocorticoid loss due to ADX.
Another aspect of GTG lesion formation, that must be considered during periods of food deprivation, is the insulin requirement. It has been demonstrated that during starvation, insulin blood levels are significantly reduced (Dubuc et al., 1982). Interestingly, insulin deficient mice do not develop VMH lesions when challenged with GTG (Debons et al., 1968; 1969; 1970). This lowered insulin concentration in the body may contribute to inhibition of GTG lesion formation. Although this possibility is tenable, it is probably unlikely. Starvation does not abolish insulin secretion. Adrenalectomy will reverse GTG insensitivity in diabetic mice even though insulin levels are undetectable. The glucocorticoids apparently play a major role in GTG lesion formation (Debons et al., 1982; Brown, 1982a).

Recently, using abdominal irritation, induced with carrageenan in mice, GTG lesion formation was inhibited during this stress (Brown, 1982b). Controls displayed typical bilateral lesions in the VMH from GTG administration. Immediately before the GTG injection, a tail vein blood sample was taken and the corticosterone concentration measured by radioimmunoassay, in both control and experimental animals. Systemic corticosterone levels were significantly elevated over controls. This evidence suggests that increases in glucocorticoid levels following stress inhibit GTG lesion formation.

In conclusion, this report indicates that stress, due to food deprivation or cold exposure, will prevent GTG lesion formation in the mouse VMH, probably by elevating systemic glucocorticoid levels. TRP loading had no effect on sensitizing the VMH to GTG in starved mice.
Apparently, the glucocorticoid status of the animal is of utmost importance for GTC lesion development, while 5HT changes during starvation apparently are of little consequence.
Figure 1. Cross-sections of the VMH at the level of the median eminence from mice challenged with GTC. A. A mouse deprived of food for 3 days and then given GTC. B. Control—a mouse fed ad lib and administered GTC.
Figure 2. Cross-sections of the VMH at the median eminence level from mice challenged with GTG. A. A mouse maintained at 4°C for 29 hours. GTG was given 5 hours after the cold exposure began. B. Control. A mouse maintained at room temperature (23°C) and given GTG.
TABLE I. The effect of fasting and cold exposure on CTG-induced necrosis in the VMH

<table>
<thead>
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<th>Stress Treatment</th>
<th>n</th>
<th>VNH necrosis (% incidence)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fasted</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control + GTG</td>
<td>12</td>
<td>100</td>
</tr>
<tr>
<td>Fasted + GTG</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td><strong>Cold exposure</strong></td>
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<td></td>
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<tr>
<td>Control + GTG</td>
<td>12</td>
<td>100</td>
</tr>
<tr>
<td>Cold + GTG</td>
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<td>0</td>
</tr>
</tbody>
</table>
TABLE II. The effect of tryptophan (TRP) given 3 days after starvation on CTG lesion formation

<table>
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<tr>
<th>Treatment</th>
<th>n</th>
<th>Incidence of Necrosis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starved + H₂O + CTG</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Starved + TRP + CTG</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Fed + H₂O + CTG</td>
<td>7</td>
<td>100</td>
</tr>
</tbody>
</table>
TABLE III. The effect of tryptophan (TRP) given to mice during starvation on CTG lesion formation

<table>
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<th>Treatment</th>
<th>n</th>
<th>Incidence of Necrosis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starved with ( H_2O ); CTG</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Starved with TRP; CTG</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Fed with ( H_2O ); CTG</td>
<td>6</td>
<td>100</td>
</tr>
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</table>
SECTION IV.

ULTRASTRUCTURE OF GIG LESION FORMATION
CHAPTER 9. AN ULTRASTRUCTURAL STUDY OF GOLD THIOGLUCOSE
LESION FORMATION IN THE VENTROMEDIAL HYPOTHALAMUS.
I. SUBNECROTIC GOLD THIOGLUCOSE DOSE

Introduction

A single intraperitoneal injection of gold thioglucose (CTG) in mice causes hyperphagia and eventual obesity (Brecher and Waxler, 1949). This obesity syndrome induced by CTG has been attributed to the destruction of the ventromedial hypothalamus (VHH) by CTG (Marshall et al., 1955).

The idea that gold thioglucose (CTG) is a vascular toxin to capillaries in the ventromedial hypothalamus (VHH) has enjoyed widespread acceptance. Liebelt and Perry (1967) reasoned that since extrahypothalamic structures appeared damaged by CTG, the destruction was non-specific. Caffyn (1971) added ultrastructural evidence to this postulate. The capillary endothelium appeared disrupted in the early development of CTG-induced lesions in the VHH. Furthermore, Levine and Sowinski (1982; 1983; also Nochlin and Levine, 1982) have reported the triamine, 3,3'-methylimino-bis-(N-methylpropylamine) (MIMPA), produced nearly identical hypothalamic lesions as those lesions induced in the VHH by a CTG challenge. These authors surmized that since MIMPA and CTG lesions cause similar VHH destruction, these very different compounds
induce VMH lesions by a common non-specific action, namely breakdown of
the hypothalamic microvasculature.

However, Young et al. (1979) have noted that the median eminence,
an area with a weak blood-brain barrier, is not vulnerable to GTG
necrosis, suggesting GTG is not a general vascular or neural toxin.
Interestingly, NIMPA injections also do not damage the median eminence
(Nochlin and Levine, 1982). Debons and his colleagues (1977; 1979b)
have demonstrated, using a subnecrotic or microlesion dose of GTG (100
mg/kg), that the capillary structure in the VMH was not destroyed by
GTG. Instead the perivascular neuropil was clearly damaged.

The ultrastructural studies done by Caffyn (1971; 1972a and b) and
Debons et al. (1977; 1979b) present conflicting interpretations with
respect to GTG action in the VMH. Additional electron microscopic
examination could reduce the apparent divergence in these two studies.

In this paper, we have examined sequentially the ultrastructural
development of GTG microlesions (VMH necrosis not visible at the light
microscope level) in the mouse VMH.

Materials and Methods

Female C57 mice, weighing 20–25 g, were used in this study. The
mice were maintained at 23°C, on a 12 hour light:12 hour dark
photoperiod, fed Teklad Rat and Mouse Diet, and given tap water freely.

Groups of 5 mice each were administered 100 mg/kg GTG
intraperitoneally (IP) and sacrificed 4, 8, or 24 hours after injection.
Controls (n=5) received water IP. All mice were perfused with 5%
gluteraldehyde in 0.1 M phosphate buffer (Sorenson), adjusted to pH 7.4, for 3 minutes at a flow rate of 1 ml/15 seconds. Following perfusion, the brain was removed from the skull and the VNH removed as a cube of tissue. This cube was placed in 5% gluteraldehyde overnight. Following washes in 0.2 M phosphate buffer with 4% sucrose, the tissue cube was embedded in 8% agar and sectioned coronally at 100 um, using a Smith and Farquhar tissue sectioner. The section containing the cell poor area, described by Debons et al. (1979b) as the origin of CTC-induced pathology, was located with a dissecting microscope, utilizing the median eminence as a marker. This section was processed for electron microscopy. The section was post fixed for 1 hour with 1% osmium tetroxide in a vernal-acetate buffer with 1.5% potassium ferricyanide to enhance contrast (Langford and Coggeshall, 1979). Following washes in vernal-acetate buffer with 4% sucrose, the tissue was stained en bloc with uranyl acetate (Hayat, 1975), in vernal-acetate buffer for 2 hours. After additional washes in vernal-acetate buffer with sucrose, and dehydration in ethanol to acetone, the section was allowed to stand in a mixture of acetone and Spurr's epoxy overnight under vacuum. The section was then placed in Spurr's medium overnight and under vacuum. Curing of the block was accomplished over the next 24 hours in a 60°C oven. Silver sections (90 nm thick) were obtained by using a Sugg diamond knife and LKB Huxley ultramicrotome. The sections were picked up on clean 200 mesh grids, stained with 1% lead citrate (Venable and Coggeshall, 1965), and examined with a Hitachi H-11E electron microscope, operated at 50 KV with a 50 um objective aperture.
Results

**Normal VMH tissue**

Tissue located in the cell poor area between the arcuate and VMH nuclei, corresponding to the site of origin of the GTG lesion, was examined. The region was characterized by a well developed microvasculature, densely packed cell processes of several sizes, myelinated axons, many synapses and a few cell bodies (Figure 1). The capillaries exhibited characteristic structure with the lumen surrounded by endothelial cells and associated basement lamina. Distal to and surrounding the basement lamina were flattened cell processes similar to astrocytic end feet. Surrounding this complex was an array of cell processes of several sizes and types, tightly packed together with no extracellular spaces. The small processes were organized in discrete bundles interspersed among large processes. The large processes contained microtubules, mitochondria, cisternae of the endoplasmic reticulum, and plasmalemmal surface membranes. The cell bodies were large, nucleated and contained an array of mitochondria, cisternae of the endoplasmic reticulum, and golgi complexes. A large number of synapses were observed with characteristic synaptic vesicles and synaptic junctions. These were most frequently located between apposed large processes. Myelinated axons were scattered throughout the mass of cell processes.
100 mg/kg CTC

4 hours post injection  At four hours after injection with 100 mg/kg CTC, the cell poor area, which is the site of origin of the lesion, exhibited minimal structural changes from the normal, control tissue samples (Figure 2). Patches of cytoplasm of some of the large processes was electron lucid. Accumulation of membranous vacuoles and an absence of microtubules were characteristically observed in the large lucid processes. The mitochondria, however, were not swollen and showed no structural changes when compared to the normal, control condition. Synapses joining the large, lucid processes were not disrupted. Capillaries and myelinated fibers appeared normal.

8 hours post injection  At 8 hours after injection with a microlesion dose of CTC, distinctive changes were observed in the region of lesion origin (Figure 3). The microvasculature appeared identical to the normal, control tissue. However, in the pericapillary areas cellular processes adjacent to the basement lamina were electron lucid. Cytoplasmic organelles except mitochondria were absent and a few membranous vacuoles were distributed throughout the membrane bound lucid area. The plasmalemmal surface membranes bounding these areas exhibited no structural discontinuities, although there was some apparent swelling. Other areas in the adjacent neuropil also were observed to be electron lucid. These areas were located adjacent to nearby large processes and the other surrounding cellular components. These apparent spaces, however, were bounded by plasmalemmal membranes and contained recognizable mitochondria as well as membranous vacuoles.
Large processes in the pericapillary area occasionally exhibited loss of electron density and the presence of membranous vacuoles. In addition, some mitochondrial profiles exhibited swelling or distention. These processes always contained microtubules. Interestingly, profiles of the electron lucid large processes were usually found adjacent to the other electron lucid processes in the neuropil. Synapses observed in the area occasionally exhibited some swelling, however, the synaptic junctions remained intact.

24 hours post injection At 24 hours after injection with a microlesion dose of GTG, pronounced structural changes were observed in the cell poor region where the lesion originated (Figures 4A and B). These changes involved the cellular elements surrounding the microvasculature. The capillary endothelium at 24 hours was identical to the capillary endothelium in the normal, control tissue. There were no structural discontinuities in the endothelial cells and desmosomes and tight junctions were not separated or disrupted. Immediately adjacent to the capillary basement lamina, electron dense, membrane bound spaces were observed. These spaces contained membrane bound vacuoles, punctate electron dense particles, and occasional mitochondria. Areas of the neuropil also contained profiles similar to the pericapillary spaces. These electron lucid areas were bounded by plasmalemmal membranes with membranous vacuoles and electron dense particles, and occasional mitochondria. Areas of the neuropil also contained profiles similar to the pericapillary spaces. These electron lucid areas were bounded by plasmalemmal membranes with membranous
vacuoles and electron dense particles in the lumen of the spaces. The
electron lucid spaces were interdigitated among the large and small
processes in the neuropil. Many of the large processes were also
electron lucid and contained membranous vacuoles. The synaptic
junctions, however, remained intact. In some cases, the cell bodies
contained swollen vacuolated structures, extremely dark cytoplasm and
occasional darkly stained inclusion bodies distributed through extremely
electron dense cytoplasm. Myelinated axons, on the other hand, were
identical to those seen in normal, control tissue.

Discussion

GTC has been proposed to cause VMH lesions and subsequent changes
in feeding in behavior by acting as a vascular toxin, which alters
capillary permeability, leading to ischemia and subsequent tissue
destruction in the lesion area (Caffyn, 1971; 1972a and b; Liebelt and
Perry, 1967; Levine and Sowinski, 1982; 1983; Nochlin and Levine, 1982).
Using a necrotic dose (macrolesion) of GTC, Caffyn (1971) reported
that the microvasculature of the VMH was damaged by GTC. She described
capillary breakdown and edema, which destroyed the adjacent neuropil.
Early during GTC lesion development, Caffyn (1971) described swollen
processes around the capillary in the neuropil. Presumably these
structures were dendrites or astrocytic end feet. Longer periods after
the GTC challenge, neuronal destruction developed with extensive
distention of a large area. Debons et al. (1977; 1977b), on the other
hand, have described the pericapillary changes in the VMH during
microlesion formation following a subnecrotic dose of GTG. These authors reported fragmentation and dissolution of the neuropil in the area surrounding the capillaries. No vascular destruction was detected.

In this study tissue from the cell poor region between the arcuate nucleus and the VMH was examined 4 hours after injection with a microlesion dose of GTG. This tissue was essentially identical to tissue from the small area in normal, control animals. Eight hours after injection with GTG, pronounced changes were observed in the pericapillary areas corresponding to astrocytic end feet and adjacent large processes. The striking loss of electron density in the cytoplasm and accumulation of membranous vacuoles suggests degeneration of the cytoplasm and some of the membranous organelles. This apparent cytoplasmic degeneration is even more pronounced in the pericapillary areas and adjacent large processes in the 24 hours post injection samples. In addition, at 24 hours degenerative changes were seen in some cell bodies and synaptic terminals.

Significantly, our findings do not indicate any observable damage to the capillaries in the cell poor region between the arcuate and VMH, 24 hours after injection with a microlesion dose of GTG. Rather pericapillary elements, either astrocytic end feet or dendrites, exhibited loss of membranous organelles and loss of electron density, similar to the cytoplasmic dissolution that has been reported by Debons et al. (1977; 1979b). Some large cellular processes in the surrounding neuropil also exhibited similar changes, suggesting that axonal processes may also be damaged. Occasionally, cell bodies adjacent to
damaged processes exhibited an increased electron density, which may be indicative of damage.

Caffyn (1971) has suggested that hypothalamic damage produced by GTC could serve as another experimental model for cerebral edema studies. Whereas it is difficult to estimate changes in capillary permeability by observation, structural evidence of edema that might result is more easily determined. Techniques used to include cerebral edema, such as trauma to the brain, diptheria toxin injection, cerebral compression, sinus obstruction or jugular ligation, all produced edema with minimal vascular distortion (Wechsler 1967). After development of edema, the affected area became necrotic, involving damage to both neuronal and glial cells. The neuronal degeneration following experimentally induced edema reported by Wechsler (1967) strongly resembled the neuronal damage reported in this study. A search for structural manifestation of edema during microlesion development revealed no extracellular spaces that could be attributed to edematous swelling, rather the damaged processes noted above were distended or swollen. Despite loss of cytoplasmic density and cytoplasmic membranous organelles, the plasmalemmal surface membranes were intact with no structural discontinuities. There was no evidence of fragmentation of cells or accumulations of cellular debris in the microlesion area. The capillaries and surrounding neuropil had not been disrupted and areas of the VNH nearby the GTC-induced microlesion appeared normal.

Definitive VNH damage was not readily identifiable until 8 hours after the 100 mg/kg GTC injection. At 8 hours and later, pericapillary
changes were evident. Large fibers showed distention of the cytoplasm and loss of cytoplasmic density. GTG-induced changes involving large processes also were evident in areas not directly associated with capillaries, however, smaller fibers in the neuropil appeared undamaged. In all instances, the capillary endothelium was normal.

At 24 hours post GTG injection, more extensive damage was evident. Large perivascular and non-perivascular processes displayed almost complete cytoplasmic dissolution with little debris. Degenerating cell bodies were present and synaptic junctions and swollen synaptic boutons were associated with these cells.

The large neuronal processes appeared to be the main VMH component affected by GTG administration in subnecrotic doses. Small processes in the neuropil, as well as myelinated processes, were apparently undamaged. The damaged processes might be dendrites. However, Wechsler (1967) determined that the initial swelling in other model systems involving induction of edema occurred in astrocytic processes. These swollen processes were nearly identical to those we found. However, close examination of the large processes in our micrographs revealed the present of microtubules and tubular smooth endoplasmic reticulum, indicative of dendrites (Rhodin, 1974). In addition, it is evident that synapses were formed with these large processes. Moreover, the large degenerating cell bodies seen 24 hours after administration of a microlesion dose of GTG are neurons. Definitive synaptic complexes were visible on these electron dense cells. It is conceivable that degeneration of these neurons began at the dendritic level. Based on
this information, the VMH structures affected initially by a subnecrotic
dose of GTC, are neuronal, as opposed to glial.

Our findings that neural rather than vascular components in the VMH
are affected by GTC are consistent with the reports made by Debons et al.
(1977; 1979b). The hypotheses of Liebelt and Perry (1967), Caffyn
(1971) and Levine and Sowinski (1982; 1983; also see Noclin and Levin,
1983) need to be reexamined. GTC may not be vascular toxin, inducing
edema and ischemia in the hypothalamus and eventually neural necrosis.
The evidence presented here indicates that GTC is a specific nervous
tissue poison for the VMH. With such a high specificity for the
hypothalamus, GTC could be used as an experimental, pharmacological
probe for hypothalamic function (Caffyn 1971; Debons et al., 1979b).
Figure 1. A section from the VMH of an untreated mouse, illustrating normal and intact perivascular neuropil. Uranyl acetate and lead citrate stains. Magnification 7400x.
Figure 2. A VMH section from a mouse treated with 100 mg/kg CTC and sacrificed 4 hours post injection. Little change is observed in this tissue compared with normal VMH. Uranyl acetate and lead citrate stains. Magnification 7400x
Figure 3. A section from the VMH of a mouse challenged with 100 mg/kg GTG and sacrificed 8 hours later. Pericapillary damage and loss of cytoplasm density in large processes is evident. Uranyl acetate and lead citrate stains. Magnification 7400x.
Figure 4A. Section of the VMH from a mouse given 100 mg/kg GTC and sacrificed 24 hours post injection. Pericapillary distention of the neuropil and definite loss of large process density is observed. Uranyl acetate and lead citrate stains.

Magnification 7400x
Figure 4B. Section of the VMH from a mouse given 100 mg/kg CTG and sacrificed 24 hours post injection. A degenerating neuron and dendrites are depicted. Arrows indicate areas of synaptic complexes on dendritic processes and the dying neuron. Uranyl acetate and lead citrate stains.

Magnification 7400x
Introduction
A single intraperitoneal injection of gold thioglucose (GTC) in mice causes hyperphagia and eventually obesity (Brecher and Waxler, 1949). This obesity syndrome induced by GTC has been attributed to the destruction of the ventromedial hypothalamus (VMH) by GTC (Marshall et al., 1955). Little is known about the development of the GTC lesion in the VMH, and the data available in the literature conflict. Liebelt and Perry (1967) proposed that since extrahypothalamic structures appeared damaged by GTC, the destruction was non-specific. Caffyn (1971; 1972 a and b) reported that GTC lesion formation in the VMH is non-specific and due to vascular damage (1982; 1983). However, Young et al. (1979) have noted that the median eminence, an area with a weak blood-brain barrier, is not vulnerable to GTC necrosis, suggesting GTC is not a general vascular toxin. Debons and his colleagues (1977; 1979b) have proposed that GTC is not a vascular toxin. Using subnecrotic doses of GTC, pericapillary neural damage was observed in a small area of the VMH by electron microscopy. The neuropil showed signs of a breakdown in cellular integrity, while the capillaries appeared normal and undamaged.
Since earlier work by Caffyn (1971; 1972 a and b) involved the induction of lesions with large doses of GTC, we have examined the effects of two necrotizing doses of GTC on the VMH over time, using electron microscopy, in an effort to characterize the onset and development of VMH lesions caused by large doses of GTC. Necrotizing doses of GTC produce discrete VMH lesions, macrolesions, easily visible with the light microscope. Doses of 300 and 800 mg/kg were used to bracket the range of drug concentration that produces a VMH macrolesion.

Materials and Methods

Female C57 mice, weighing between 20-25 gm, were used in this study. The mice were maintained at 23°C on a 12 hour light:12 hour dark photoperiod, fed Teklad Rat and Mouse Diet and given tap water freely. The mice were administered 300 or 800 mg/kg GTC intraperitoneally (IP) and sacrificed 2, 4, 6, or 12 hours after injection. The VMH from each mouse was prepared for ultrastructural observation as detailed in the preceding chapter. The cell poor region lying between the arcuate and VMH nuclei, corresponding to the area of lesion onset and preliminary pericapillary damage (Debons et al., 1977; 1979b), was examined by electron microscopy.

Results

Normal VMH Tissue

The neuropil was tightly packed with large and small processes, myelinated fibers, and synaptic figures (Figure 1). Large processes contained numerous mitochondria, microtubules, and endoplasmic reticulum.
profiles. Synaptic terminals, with many vesicles, formed synaptic complexes with the large and small processes in the area. Capillaries exhibited characteristic morphology. The lumen was surrounded by endothelial cells which, in turn, rested on a basal lamina. Adjacent to the capillary basal lamina were flattened cellular processes corresponding to astrocytic endfeet. Cell bodies were large and nucleated, contained mitochondria, profiles of endoplasmic reticulum and membranous vacuoles.

300 mg/kg CTC

6 hours post injection Large membrane bound, electron lucid spaces were adjacent to the capillary basal lamina (Figure 2). Scattered in the neuropil were similar electron lucid and membrane bound profiles. Some of these electron lucid spaces also contained membranous vacuoles. Cell bodies were large and nucleated, with many mitochondria, profiles of endoplasmic reticulum, and golgi complexes. Myelinated fibers were normal. Capillary morphology was intact; the endothelial cells and basal lamina resembled those found in normal tissue. Synaptic profiles were not distorted and many of these profiles were adjacent to electron lucid, membrane bound structures.

12 hours post injection Pericapillary destruction was apparent (Figure 3). Large electron lucid, membrane bound areas were present adjacent to the capillary basal lamina. However, the capillary endothelium and basal lamina were intact and not disrupted. Cell bodies contained mitochondria, endoplasmic reticulum and many golgi complexes. Occasionally, electron lucid membranous vacuoles were seen in the cell
body cytoplasm. Synaptic figures and myelinated fibers were normal. A generalized distention of the neuropil was apparent; electron lucid areas with no membrane boundaries, indicative of tissue edema, were scattered throughout the neuropil in areas adjacent to the capillaries.

**800 mg/kg CTC**

*2 hours post injection* The neuropil was densely packed with large and small processes, synaptic profiles, and myelinated fibers (Figure 4). No damage to these elements was apparent. Large processes contained microtubules, mitochondria and profiles of endoplasmic reticulum. On occasion, electron lucid membranous vacuoles were evident in these large processes. Cell bodies exhibited normal morphology with abundant mitochondria and profiles of endoplasmic reticulum. Synaptic figures were not disrupted. Capillary endothelial cells and the basal lamina were intact.

*4 hours post injection* The microvascular endothelial cells and basal lamina were intact (Figure 5). However, processes adjacent to the capillary basal lamina were electron lucid and contained membranous vacuoles. These electron lucid processes also were found in the neuropil not associated with a capillary. Synaptic figures were normal and frequently occurred adjacent to these electron lucid processes. Large processes contained microtubules, mitochondria, endoplasmic reticulum profiles, and occasionally electron lucid membranous vacuoles. Cell bodies were normal with mitochondria, endoplasmic reticulum and membranous vacuoles. Small processes and myelinated fibers exhibited no damage. Generally the tissue morphology was similar to the 6 hours post
injection 300 mg/kg GTG tissue.

6 hours post injection A large amount of disruption was observed throughout the neuropil (Figure 6). Few large processes appeared normal; most were electron lucid and membrane bound. Occasionally membranous vacuoles were found within these electron lucid processes. In addition, extensive distention of the extracellular space was observed, and these distended areas contained membranous electron lucid vacuoles. Small processes, synaptic profiles, and a few larger processes were not distorted. Cell bodies exhibited no damage and contained mitochondria, endoplasmic reticulum, and golgi complexes. Capillaries displayed an intact endothelial cell structure and basal lamina.

12 hours post injection A generalized destruction of the VMH was observed (Figures 7A-D). Membrane bound, electron dense figures contained large and small, electron dense and lucid membranous vacuoles and membrane profiles resembling endoplasmic reticulum. Synaptic complexes were formed with these electron dense figures. These synaptic profiles were disrupted but contained clustered synaptic vesicles. On occasion, the synaptic vesicles were distended and mitochondria were swollen and distended. Cell bodies were no longer intact and pycnotic nuclei were present. A few membrane bound, electron lucid processes were observed. Large accumulation of membranous debris was observed in the area. A large amount of extracellular, electron lucid space was observed. Capillaries demonstrated an initial breakdown of the endothelium and basal lamina, as well as stasis of blood in the lumen.
Occasional myelinated axons were swollen and no normal structures, cellular or subcellular, could be identified.

Discussion

Debons and collaborators (1977; 1979b) have proposed that GTG is not a vascular toxin as suggested by Liebelt and Perry (1967), Caffyn (1971), Nochlin and Levine (1982) and Levine and Sowinski (1982; 1983). The macrolesion doses of GTG used in this study produced a striking pathology with a rapid onset. The initial phase involved loss of electron density of cellular processes in the pericapillary spaces, extending out into the neuropil. Separation of the cellular elements of the neuropil by large spaces, corresponding to edematous swelling, followed this initial phase. Finally, destruction and dissolution of neuropil elements appeared with an accumulation of cellular debris in the area.

The VMH pathology resulting from large dose GTG administration, resembled models of experimental brain edema (Wechsler 1967). The primary response was intrafiber distention in large VMH processes, followed later by interfiber separation. The onset of necrotic degeneration corresponded with the establishment of large spaces in the tissue as the elements of the neuropil separated. The degenerative phase was characterized by disrupted synapses and dissolution of the tissue elements of the neuropil with the accumulation of large amounts of cellular debris in the extracellular spaces. The small fibers of the neuropil were destroyed, capillaries appeared occluded, swollen, and
distorted myelinated fibers were apparent, and mitochondria were grossly enlarged and occasionally disrupted. Eventually the macrolesion dose of GTG led to complete degeneration of the cellular components in the area of the lesion.

The general response of the VMH to subnecrotic and necrotic doses of GTG appeared similar. Intrafiber destruction and distention of these large processes, dendrites, resulted following the GTG injection. Subsequent interfiber separations in the neuropil appeared. Necrosis of the VMH began when degenerating, electron dense cells were observed. VMH necrosis was minimal and apparently temporary with microlesion doses of GTG. On the other hand, macrolesion amounts of GTG caused extensive distention and destruction of the entire lesion area. Furthermore, the rate of lesion formation appeared to be directly related to GTG dose. It is noteworthy that none of the cerebral edema models (Wechsler 1967) or GTG-induced VMH necrosis produced large numbers of lysosomes or phagocyte infiltrations for cellular digestion of debris. Only distention and cellular destruction were seen with extensive necrosis.

Using a necrotic dose of GTG to produce a VMH macrolesion eventually caused mitochondrial distortion; the mitochondria swelled, became spherical in shape, and electron lucid. Subnecrotic doses of GTG, used to produce a VMH microlesion, did not induce noticeable alterations in mitochondrial structure or appearance. The observed abnormal change in mitochondrial morphology, and possibly function, following a necrotic dose GTG challenge, may be related to the appearance of permanent hypothalamic damage, since the microlesion is
only temporary with normal morphology apparent within 5 days following injection with subnecrotic doses of GTG (Debons et al., 1979b). Normal functional mitochondria may be required for recovery from GTG-induced lesions.

Debons et al. (1979b) have shown that GTG lesion formation is correlated with VMH tryptophan hydroxylase activity and to a certain extent VMH serotonin (5HT) content. However, VMH edema must be present before GTG-induced necrosis will occur, regardless of the tryptophan hydroxylase activity or 5HT levels in the area. These investigators postulated that GTG acts directly on VMH neural components, eventually causing massive VMH edema due to 5HT release. Since the hypothalamus is rich in 5HT (Brownstein et al., 1976) and 5HT is a potent edema-producing agent in mice (Bulle, 1957), this idea is attractive. Our observations are consistent with this hypothesis. Dendritic swelling was observed before a generalized destruction of the VMH began. This initiation of GTG lesion formation was probably a direct effect of GTG on the dendrite leading to interfiber swelling and structural damage. Synaptic profiles began to degenerate, possibly releasing 5HT. The 5HT liberation may have produced abnormal capillary permeability, which caused additional edema and destruction, as well as microvascular damage.

Edema and destruction in the VMH was much more evident and extensive with a necrotizing dose of GTG than a subnecrotic dose. In addition, mitochondria and myelinated fibers were damaged following administration of a macrolesion dose of GTG, an effect not seen in the
microlesion system. Our present studies, the preceding paper and the work of Debons et al. (1977; 1979 a and b) suggest that GTG is a substrate-specific neurotoxin; vascular damage occurs secondary to neural destruction.
Figure 1. A section from the VMH of an untreated mouse illustrating normal and intact neuropil and vasculature. Uranyl acetate and lead citrate stains. Magnification 7350x
Figure 2. A VMH section from a mouse treated with 300 mg/kg GTG IP and sacrificed 6 hours after injection. Pericapillary distention of the neuropil is evident. Uranyl acetate and lead citrate stains. Magnification 7350x
Figure 3. A section from the VMH of a mouse treated with 300 mg/kg GTG IP and sacrificed 12 hours after GTG administration. Extensive intercellular neuropil distention is seen while the capillary appears undamaged. Uranyl acetate and lead citrate stains. Magnification 7350x
Figure 4. A VMN section from a mouse treated with 800 mg/kg CTG IP and sacrificed 2 hours after injection. No vascular or neural damage is apparent. Uranyl acetate and lead citrate stains. Magnification 7350x
Figure 5. A section of the VMH from a mouse treated with 800 mg/kg GTG IP and sacrificed 4 hours after injection. An intact capillary is visible with perivascular distention of the neuropil. Uranyl acetate and lead citrate stains.

Magnification 7350x
Figure 6. A VMH section from a mouse treated with 800 mg/kg CTG IP and sacrificed 6 hours after injection. Extensive interfiber distention is illustrated with undamaged capillaries. Uranyl acetate and lead citrate stains. Magnification 4800x
Figure 7A. A profile of a degenerating neuron. A section of the VMH from a mouse treated with 800 mg/kg GTG IP and sacrificed 12 hours after GTG administration. Small arrows indicate disrupted or swollen synaptic figures associated with the neuron. The large arrow points to a swollen mitochondrion. Uranyl acetate and lead citrate stains. Magnification 16700x.
Figure 7B. Extensive disruption of the VMH is seen from a mouse given 800 mg/kg GTC IP and sacrificed 12 hours after injection. The arrow points to a deformed mitochondrion. Note the outline of a cell body nucleus in the upper half of the plate. Uranyl acetate and lead citrate stains. Magnification 6750x.
Figure 7C. Myelinated fiber distention from the VMH of a mouse treated with 800 mg/kg GTG IP and sacrificed 12 hours after injection. Arrows indicate swollen mitochondria. Uranyl acetate and lead citrate stains. Magnification 6750x
Figure 7D. A damaged capillary in the VMH of a mouse treated with 800 mg/kg CTC IP and sacrificed 12 hours after injection. Complete neuronal destruction is seen surrounding the capillary. Uranyl acetate and lead citrate stains. Magnification 9700x
GENERAL CONCLUSIONS

The investigations reported in the preceding chapters comprise an effort to understand the function of the putative satiety center in the ventromedial hypothalamus (VMH). One of the early theories of how the VMH controlled feeding behavior centered on a glucose currency. Proponents of the glucostatic hypothesis reasoned that this brain region possessed cells that monitored blood levels of glucose. As vascular glucose concentrations in the VMH increased to an appropriate threshold, satiety resulted, at least in part, by an alteration of VMH neuronal activity.

Gold thioglucose (GTG) has been a useful pharmacological probe in the study of this VMH satiety center. It has been known for over 30 years that a systemic GTG injection produces a focused bilateral lesion in the mouse VMH. This VMH lesion causes a sustained hyperphagia and eventually leads to obesity. Early researchers suggested that GTG destroyed the neuronal integrity of the satiety center, thus abolishing the animal's ability to limit food intake. It is hypothesized that GTG produces hypothalamic damage by the binding of its glucose moiety to the VMH chemoreceptors which normally initiate satiation, however, the exact mechanism is not understood.

Glucose can have a dual role in the biochemical management of body function by binding with functional or nutritional receptors. It is not
unreasonable to expect that, in tissues that possess dual receptors for
glucose, glucose oxidation may be regulated differently from other
tissues. We have found that glucose utilization in the VMH is not
dependent on insulin. Furthermore, we discovered that VMH glucose
oxidation is affected minimally or not at all by a variety of glucose
analogs or by interference with the Na-K pump. A plausible explanation
for the differences in VMH responses to these procedures from responses
that were observed with different tissues is that the VMH glucoreceptors
differ from those of other cells. An analogous situation is seen with
pancreatic islets. Experimental evidence indicates that the pancreas
possesses two classes of glucose receptors; one concerned with glucose
metabolism and the other with hormone secretion. Digitoxose inhibits
the functional but not the nutritional glucoreceptors in pancreatic
islets. However, we have observed that this sugar does not alter either
receptor in the VMH to a great extent, since both glucose oxidation and
the ability of GTG to induce VMH lesion formation are unaltered by
digitoxose. Digitoxose does cause a reduction of food intake during day
light hours, but this effect may be a peripheral phenomenon instead of a
central one. On the other hand, alloxan, a diabetogenic inhibitor of
glucoreceptors, depressed VMH glucose oxidation and also depressed
feeding in response to a stimulus. A similar situation exists after
phlorizin treatment. We demonstrated that phlorizin depressed VMH
glucose oxidation and inhibited GTG-induced lesion formation in the VMH.
These data indicate that both alloxan and phlorizin affected the
functional as well as the nutritional glucoreceptors in the VMH.
Hormones are able to modulate a variety of physiological and behavioral systems, and feeding behavior is not an exception. Insulin, estrogen and glucocorticoids, substances that influence carbohydrate metabolism, can produce changes in feeding behavior and alterations of the VMH in response to a GTC challenge. Hyperinsulinemia causes a hyperphagia in normal animals, presumably a consequence of the lowered blood glucose levels. We have shown that insulin does not influence VMH glucose oxidation; however, other evidence indicates that insulin has a direct effect on the functional VMH glucoreceptors, thereby influencing GTC-induced necrosis in the VMH. Mice rendered diabetic by alloxan treatment or anti-insulin serum are resistant to GTC lesion formation. Intrahypothalamic administration of insulin in these diabetic animals will cause a return of GTC sensitivity in the VMH.

Estrogen is known to produce a depressive effect upon food intake, presumably by a direct action of the hormone on cells in the VMH. In addition, estrogen treatment of ovariectomized mice significantly increases the size of GTC-induced lesions in the VMH. Since estrogen enhances the uptake of glucose in muscle or liver, we postulated that estrogen may also increase glucose oxidation in the VMH. The data generated from this experiment are inconclusive, but estrogen clearly has an effect on functional VMH glucoreceptors, as shown by its influence on GTC lesion formation.

Since glucocorticoids influence carbohydrate metabolism and their release is indirectly controlled by hypothalamic releasing factors, it is tenable that this steroid may influence feeding behavior. Although
the administration of glucocorticoids has produced contradictory
findings concerning food intake, removal of the adrenal glands reduced
food intake. In mice made diabetic by treatment with alloxan,
adrenalectomy ameliorates the diabetes and causes sensitization of the
VMH to GTC. Conversely, glucocorticoid homeostasis may also change the
responsiveness of the VMH to a GTC challenge. Mice were subjected to
one of two types of stress, food deprivation or cold exposure. These
conditions are known to increase systemic levels of glucocorticoids.
Either stressor completely inhibited GTC lesion formation in the VMH.
The elevated levels of glucocorticoids under these conditions may have
afforded direct protection of the VMH, or may have altered VMH
sensitivity to GTC by inducing cellular metabolic changes.

Elucidation of the mechanism of GTC lesion formation in the VMH
could provide an essential clue to understanding the functional
glucoreceptors, which appear to be involved with satiation. Early
investigators reported that GTC damage manifested itself by a breakdown
of the VMH microcirculation. They believed that neuronal destruction
was secondary to the ischemia induced by GTC. However, more recent
evidence has demonstrated that neuronal degeneration is the primary
effect of a GTC challenge. We have confirmed the latter finding with
ultrastructural observations of the VMH after both necrotic doses of GTC
and doses so low that only electron microscopic evidence of degeneration
was detectable. Therefore, it appears that GTC is a substrate-specific
neurotoxin in the mouse VMH, albeit other brain cells possessing
functional glucoreceptors may also be vulnerable to GTC. Moreover, we
determined that the first portions of the neuron affected by GTG are dendrites and dendritic trunks. Apparently, GTG either acts directly on the dendrites or produces an elevation in the concentration of some substance which damages the dendrites.

Serotonin (5HT) is present in very large concentrations in the VMH. A current hypothesis of GTG lesion formation is that this drug causes a massive efflux of 5HT from terminals, thereby producing damage to adjacent dendrites. We reasoned that if this were the case, loading an animal with tryptophan should increase available 5HT in the VMH and thereby increase the size of GTG-induced lesions. Unexpectedly, this idea was not supported by our data; tryptophan pretreatment did not alter lesion size in mice. Only if tryptophan pretreatment was prolonged for several days did a GTG injection alter lesion area, and in this instance, the lesion size was reduced, not increased. Tryptophan treatment can increase 5HT stores in the brain and the 5HT turnover rate. However, other studies indicate that brain 5HT levels are unchanged following tryptophan supplementation, but that tryptophan hydroxylase (an enzyme required for 5HT synthesis) activity and 5HT turnover are increased. If this is the case, any decrease in tryptophan hydroxylase activity prior to or without a change in the 5HT turnover rate would result in lower levels of central 5HT. Such lower 5HT levels in the VMH would reduce the size of a 5HT mediated GTG lesion.

Catecholaminergic neurons are numerous in the hypothalamus. Since central catecholamines are involved with feeding behavior, we addressed the question of what effect catecholamines may have on GTG lesion
formation in the VMH by loading mice with tyrosine to enhance the synthesis of catecholamines. However, following a GTG challenge in these animals, no significant difference existed in lesion size between controls and loaded mice. Therefore, we tried the converse experiment, pretreatment with alpha-methylparatyrosine, a catecholamine synthesis inhibitor. This regimen produced significantly larger lesion following GTG administration, which indicates that absence of catecholamines sensitizes the VMH to GTG. Catecholaminergic terminals possess a 5HT uptake mechanism, which may render depleted catecholaminergic neurons in the area more susceptible to 5HT uptake. Assuming that this enhanced 5HT influx into these catecholamine depleted neurons is toxic, when the area is flooded with 5HT, the development of large GTG lesions would be expected and in agreement with our result.

In summary, the following scheme for GTG-induced lesion formation is proposed. GTG concentrates in the VMH when given intraperitoneally. This drug, in an as yet unknown mechanism, is believed to cause release of 5HT from specific VMH neurons. This flood of 5HT is toxic, initially producing dendritic damage and edema. Subsequent neuronal breakdown and degeneration leads to secondary destruction of other cellular elements, and eventually (approximately 24 hours after a GTG injection) the entire VMH is obliterated. With time, the edema decreases and area scars appear. Alterations in insulin, glucocorticoid, estrogen or neurotransmitter homeostasis can change VMH responsiveness to a GTG challenge. Future investigative efforts should focus on clarifying the mechanism of GTG action in the VMH, and characterizing the glucose/CTG
receptor, i.e., the functional glucoreceptor. Although the VMH is involved with feeding behavior, the idea that this area is the exclusive satiety center is now questioned. A neurocircuitry system may be a more appropriate concept. If a direct link could be established between feeding and GTG neuronal damage, the role of a VMH satiety center as an essential element of such a circuit would be confirmed.
APPENDIX.

RELATED INVESTIGATIONS
CHAPTER 1. INHIBITION OF GOLD THIOGLUCOSE LESIONS BY INTRAHYPOTHALAMIC SALINE INJECTIONS

Introduction

Stereotaxic placement of chemicals into the brain has been widely employed in the past. This technique is extremely useful in assessing the effects of brain implants on the central nervous system (CNS) and bodily functions (Singh and Avery, 1975). One area of investigation that has utilized the stereotaxic implantation technique is the study of CNS control of food intake.

Gold thioglucone (GTC), when injected in mice intraperitoneally (IP), will cause necrosis and subsequent lesions in the ventromedial hypothalamus (VMH), producing hyperphagia and eventual obesity (Brecher and Waxler, 1949; Debons et al., 1974a). It has been demonstrated in mice, that a unilateral intrahypothalamic (IH) injection of phlorizin, a glucose transport inhibitor, into the VMH at the level of the median eminence abolished the GTC-induced VMH necrosis, on the same side of the brain receiving the injection (Debons et al., 1974a). IH saline injections, used as controls, had no effect on the GTC-induced pathology.

The contention that the extent of GTC-induced necrosis in the VMH could be used as a measure of VMH glucose metabolism has been
successfully employed (Young et al., 1979). Utilizing this technique, we tried to determine whether or not ouabain, a Na-K pump ATPase inhibitor, injected IH into the VMH had any effect on GTG necrosis in the area. The ouabain experiments were inconclusive, however, the control saline infusions produced unexpected results. Specifically, the saline control experiments appeared to inhibit GTG lesion formation.

Materials and Methods

C57 female mice, weighing 20-24 gm, were maintained at 23°C on Teklad Rat and Mouse Diet and tap water, with a 12 hour light:12 hour dark photoperiod. The procedure for stereotaxic infusions into the VMH of mice has been previously described (Debons et al., 1970). Ether was used as the anesthetic. Isotonic saline was infused (2 ul total volume) unilaterally with a Hamilton microliter syringe into the VMH at the level of the median eminence. Following the surgery, the mice were injected IP with 800 mg/kg CTG and sacrificed 24 hours later. The brains were removed from the skull, fixed in Bouin's fluid, embedded in paraffin, sectioned at 7 um, stained with hematoxylin and eosin, and the VMH examined by light microscopy. Lesions were examined in mice receiving saline infusions and GTG and mice receiving only GTG. The cross sectional area of the lesion at its largest point was measured (Young et al., 1979) using a planimeter and overhead slide projector at a magnification of 46 times. The paired t-test was used to determine significant differences in GTG lesion size between saline infused and untreated mice challenged with CTG.
Results

In all instances, with proper placement of the chemical, saline IH infusions caused a partial or complete inhibition of GTG necrosis in the VMH (Figure 1). This result suggests an altered VMH glucose transport and metabolism (Young et al., 1979). Of the seven animals used, three demonstrated a partial inhibition of GTG-induced necrosis, while four exhibited complete inhibition of GTG-induced pathology on the side of the brain receiving the intracranial infusion.

The GTG lesions were generally confined to the VMH and arcuate nuclei. The cross sectional area of the lesions in saline infused and untreated mice given GTG was measured at its largest point (Young et al., 1979) (see Table 1). The GTG lesion area in saline infused mice was significantly smaller than the lesion area in non-infused mice ($p<0.001$). These data indicate that saline infusions can inhibit GTG necrosis and subsequent lesion formation.

Discussion

These results are in contrast to illustrations of IH saline injections published earlier (Debons et al., 1974a). These photomicrographs show no apparent inhibition of the GTG lesion by saline. However, it is noteworthy that, in the animals receiving IH saline (depicted in Debons et al., 1974a), measurements of the lesioned area, on either side of the third ventricle, indicated that the side receiving the saline infusion had a smaller lesion than its untreated, contralateral counterpart.
These conflicting data question the technique of stereotaxic IH infusions into the brain. Perhaps glucose transport and metabolism is readily altered by an acute intracranial disturbance. The inhibition of GTG-induced lesions in the VMH by IH saline injections make this idea plausible. It is not entirely clear what aspect of the IH infusion is causing the inhibition of GTG necrosis. The saline infusion itself, the actual mechanical insertion of the syringe needle, or the pressure of injection are all possible mechanisms by which inhibition could be produced.

Much excellent work has been done with stereotaxic implantation and manipulation (Singh and Avery, 1975), thus the technique should not be abandoned. However, experimental design utilizing stereotaxic IH infusions should be scrutinized and the results interpreted with caution.
Figure 1. Cross sections of the mouse brain showing the VMH at the level of the median eminence, 24 hours after various injections. A. A mouse given GTG IP. Characteristic lesions appear on either side of the third ventricle in the VMH. B. A mouse given an IH infusion of saline on the right side of the brain (arrow indicates needle track) with subsequent injection of GTG IP. The right side shows partial inhibition of the GTG necrosis, while the left side has a full size lesion comparable to Figure 1A. C. A mouse given an IH of saline on the right side of the brain (arrow indicates needle track) with a subsequent injection of GTG IP. The right side shows complete inhibition of the GTG necrosis, while the left side has a full size lesion comparable to Figure 1A. (x42)
<table>
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<th>Treatment</th>
<th>n</th>
<th>Cross sectional area of lesion (cm² x 46)(mean ± SEM)</th>
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<tr>
<td>Saline infusion + GTC</td>
<td>7</td>
<td>1.40 ± 0.71*</td>
</tr>
<tr>
<td>GTC only</td>
<td>7</td>
<td>7.86 ± 1.55</td>
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</tbody>
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* Significantly different from non-infused mice challenged with GTC, p<0.001.
CHAPTER 2. RESISTANCE OF THE GERBIL HYPOTHALAMUS TO GOLD THIOLUCOSE LESION FORMATION

Introduction

Brecher and Waxler (1949) discovered that a single intraperitoneal (IP) injection of gold thioglucoce (GTG) would cause hyperphagia and subsequent obesity in mice. Histological examination of the brain revealed that the ventromedial hypothalamus (VMH) was destroyed by GTG (Marshall et al., 1955; Mayer and Marshall, 1956). This GTG-induced bilateral lesion of the VMH is easily visible with the naked eye at the level of the median eminence. Two-deoxy-D-glucose (2DG) (Likuski et al., 1967) and phlorizin (Brown and Viles, 1982; Debons et al., 1974a) inhibitors of glucose utilization, prevent GTG lesion formation in the VMH of mice. Furthermore, insulin deficiency (Debons et al., 1968; 1969; 1970) and elevated systemic glucocorticoid levels (Debons et al., 1979b) abolish GTG necrosis in the VMH. This evidence suggested that GTG vulnerability could be used as an indicator of VMH glucose metabolism (Blaustein et al., 1976; Likuski et al., 1967; Young et al., 1979).

Much controversy has appeared in the literature concerning the specificity of GTG-induced lesion formation. Some experimental evidence has suggested that GTG affects brain areas that possess a weakness in
the blood-brain barrier (Caffyn, 1971; Liebelt and Perry, 1967).

However, using subnecrotic doses of GTG, Debona and co-workers (1979b) demonstrated by electron microscopy that GTG attacked neuronal components prior to any vascular damage. Collapse of the capillary endothelium was secondary. In addition, this initial GTG destruction of neuropil was confined to a small area between the VMH and arcuate nuclei. GTG appeared to exhibit an anatomic specificity for a small area of hypothalamic neuropil and a functional specificity for receptors affected by changes in glucose metabolism (Debons et al., 1977; 1979b). Thus, GTG could be used effectively as a pharmacological probe for hypothalamic function (Debons et al., 1979b). Moreover, we have confirmed Debons' observations (unpublished data).

Glucoprivic stimuli (2DG injections) not only eliminate GTG necrosis, they also cause hyperphagia (Likuski et al., 1967). This hyperphagic response has been found in mice (Likuski et al., 1967), rats (Smith and Epstein, 1969), goats (Houpt, 1974), sheep (Houpt, 1974), rabbits (Houpt and Hance, 1971), dogs (Russek, 1971), cats (Rowland, 1981), monkeys (Smith and Epstein, 1969), and humans (Laslo et al., 1961). Hamsters and gerbils, however, do not respond to 2DG administration with hyperphagia (Ritter and Balch, 1978; Rowland, 1978); even a preferred sunflower seed diet is not overeaten by hamsters administered 2DG (Sclafani and Eisenstadt, 1980).

This lack of hyperphagia, seen in gerbils and hamsters, due to 2DG injections suggested that a reputed area for satiety, the VMH, may be different functionally and/or structurally compared with other mammals.
Using GTG as a probe, we investigated this possibility in gerbils. In addition, manipulations of systemic insulin and glucocorticoid concentrations were done by injection and adrenalectomy (ADX) respectively, to enhance any necrosis produced by GTG in the gerbil VMH.

Materials and Methods

Female Mongolian gerbils (Meriones unguiculatus), weighing 45-64 gm were used in this study. They were given Teklad Rat and Mouse Diet with sunflower seed and tap water or 0.9% saline if bilaterally ADX. A temperature of 23°C and a 12 hour light:12 hour dark photoperiod were maintained in the animal care facility.

Six experimental groups were established. The first group, control gerbils, received a water injection IP (n=5). Group 2 (n=10), group 3 (n=4), and group 4 (n=4) received 800, 1600 and 3600 mg/kg GTG IP respectively. The fifth group received insulin (5 units Iletin II, IP,) 90 minutes prior to GTG (n=12). Blood glucose was assayed by the glucose oxidase method. The sixth group was ADX, under ether anesthesia, and injected with GTG 3 days after the surgery (n=8). Gerbils were decapitated 24 or 48 hours after the GTG injection. The brains were removed from the skull, fixed in Bouin's fluid, embedded in paraffin, sectioned at 7 um, stained with hematoxylin and eosin, and the VMH examined by light microscopy. Blood glucose statistical differences were determined using the t-test.
Results

Figure 1 depicts the VMH from a control gerbil injected with water 24 hours before decapitation. No damage of the neuropil could be discerned. Gerbils challenged with GTG at the extraordinarily large dose of 3600 mg/kg showed no evidence of VMH necrosis or lesions (Figure 2). Furthermore, no gerbils injected with GTG alone, at any dose mentioned above, developed necrosis of the VMH. The controls (Figure 1) were indistinguishable from the gerbils injected with the high GTG dose (Figure 2). Animals sacrificed 48 hours after GTG administration produced identical results.

Since alloxan diabetes is known to desensitize the VMH to GTG challenges in mice (Debons et al., 1968; 1969; 1970) insulin was administered to gerbils to enhance any possible GTG response in the VMH. These gerbils did not show any signs of necrosis or lesion formation in the VMH (Figure 3). The insulin was biologically active; blood glucose levels were drastically reduced, p<0.001 (Table I).

An IP injection of hydrocortisone renders the mouse VMH insensitive to GTG necrosis (Debons et al., 1979b). Since gerbils may develop spontaneous hyperadrenocorticism (Vincent et al., 1979), ADX were done to eliminate systemic glucocorticoids. ADX gerbils challenged with GTG showed no sign of VMH pathology (Figure 4).

For comparison, a cross section of the VMH from a mouse challenged with GTG (500 mg/kg) is depicted in Figure 5.
Discussion

Our results clearly indicate that the gerbil VMH reacts differently from the mouse VMH to GTG. GTG administration, in a dose 12 times that necessary to produce necrosis in mice, does not damage the gerbil VMH. In addition, hormonal manipulations that normally would sensitize the mouse VMH to GTG have no effect in the gerbil.

The mouse and gerbil also differ in their response to another glucose analog, 2DG. 2DG administration stimulates feeding in the mouse (Likuski et al., 1967), but has no effect in the gerbil (Rowland, 1978). Interestingly, glucoprivic stimuli from 2DG (Likuski et al., 1967) or 5-thioglucone (5TG) (Brown, 1983) injections block GTG necrosis in the mouse VMH. These data imply that the mouse VMH neurons sensitive to GTG may be the same population of neurons sensitive to glucoprivation. Indeed, the idea of a GTG-lesioned glucostat system in mice has been postulated (Luby et al., 1981; Marrazzi and Holliday, 1981). However, Ritter et al. (1981) have demonstrated convincingly in rats that the glucoreceptors modulating glucoprivic feeding in response to 5TG are located in the hindbrain. As Ritter et al. (1981) have pointed out, this finding does not disprove the existence of hypothalamic glucoreceptors. The facts that GTG causes lesions in the VMH and 2DG and 5TG interfere with GTG lesion formation, support the idea that the mouse hypothalamus possesses a population of glucoreceptive neurons. Although there is no evidence that glucoreceptive cells are present in the gerbil hypothalamus, if we assume they exist, the absence of any response in the gerbil VMH to a massive GTG challenge implies that
gerbil hypothalamic gluoreceptors differ substantially from mouse hypothalamic gluoreceptors.

An explanation for the gerbil's lack of response to GTG or 2DG administration may lie in their biological history. The gerbil may have had significant changes occur in central feeding control mechanisms over evolutionary time. Since this animal is adapted to a desert environment, potential food sources may be limited, making continual feeding advantageous. Feedback loops to brain regions from peripheral receptors involved with controlling food intake or central areas mediating satiety may have been altered by natural selection, accounting for gerbil insensitivity to GTG lesion formation and 2DG-induced hyperphagia. These ideas are consistent with those proposed by Brown and Farrar (1980) for frogs. They found that frogs also are resistant to GTG necrosis in the VMH and speculated that, (1) frogs lack a substrate-specific modulation of feeding behavior, or (2) changes have occurred in hypothalamic food intake control mechanisms through time.
Figure 1. A cross section of the VMH from a gerbil at the level of the median eminence, given water and decapitated 24 hours later. No hypothalamic damage is observable. The bar represents 0.1 mm (105x)

Figure 2. A cross section of the VMH from a gerbil at the median eminence level, challenged with CTG (3600 mg/kg) and sacrificed 24 hours after the injection. No abnormalities are discernible in the VMH. The bar represents 0.1 mm (105x)
Figure 3. A cross section from the VMH of a gerbil at the level of the median eminence, treated with insulin and GTC (800 mg/kg) and sacrificed 24 hours after the GTC injection. No necrosis or lesion in the area is apparent. The bar represents 0.1 mm (105x).

Figure 4. A cross section of the VMH at the median eminence level, from an ADX gerbil challenged with GTC (800 mg/kg) and sacrificed 24 hours later. No damage to the VMH is seen. The bar represents 0.1 mm (105x).
Figure 5. A cross section of the VMH at the level of the median eminence from a mouse challenged with 500 mg/kg CTG and sacrificed 24 hours later. The typical bilateral CTG-induced VMH lesion is seen on either side of the third ventricle.

The bar represents 0.1 mm (105x)
TABLE I. Blood sugar levels in gerbils given insulin and GTG

<table>
<thead>
<tr>
<th>Condition</th>
<th>n</th>
<th>mg % glucose (mean ± SEM)</th>
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<tbody>
<tr>
<td>Before insulin injection</td>
<td>12</td>
<td>149.0 ± 11.2</td>
</tr>
<tr>
<td>90 minutes after insulin administration and before GTG injection</td>
<td>11</td>
<td>54.8 ± 6.9*</td>
</tr>
<tr>
<td>24 hours after GTG administration</td>
<td>4</td>
<td>45.5 ± 11.1</td>
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</tbody>
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* Significantly different from the other two conditions, p<0.001.
CHAPTER 3. ABSENCE OF LESIONS IN THE HYPOTHALAMUS OF FROGS TREATED WITH GOLD THIOGLUCOSE

Introduction

Gold thioglucofo (GTG) has been known for almost three decades to cause obesity. It selectively destroys neurons in the brain and thus has become a pharmacological tool for studying central nervous control of food intake in mammals.

Brecher and Waxler (1949) were the first to show that a single intraperitoneal injection of GTG will cause hyperphagia and eventual obesity in mice. Histological studies have demonstrated that GTG selectively destroys neuronal cells in the ventromedial hypothalamus (VNH) (Marshall et al., 1955). This area of the brain seems to be responsible for controlling food intake and has been called the satiety center. As further substantiation of the involvement of the VNH in satiety, electrolytic destruction (Hetherington and Ranson, 1940), cauterization (Stevenson, 1969) and knife cuts (Albert and Storlien, 1969) in this area can lead to hyperphagia and obesity.

The frog, Rana pipiens, has been used extensively in physiological investigations but very little is known about the control of food intake in this species or in other lower vertebrates. We therefore chose to investigate whether or not a GTG sensitive satiety center exists in Rana pipiens.
Materials and Methods

Adult *Rana pipiens* weighing 20-30 gm were obtained from Turtox Biological Supply. The animals were housed in moist aquaria at 23°C under a 12 hour light:12 hour dark routine. Frogs were force-fed liver when not in use, but were fasted 3-5 days before experimentation. Animals were acclimatized to laboratory conditions for at least 2 weeks before use. Experiments were conducted in late October.

Six frogs were injected intraperitoneally with a single dose of 800 or 1000 mg/kg aqueous CTC. Six control frogs were injected with a similar volume of saline. Animals were killed after 48 hours and their brains were fixed in Bouin’s solution for 48 hours. The brains were embedded in paraffin and the entire diencephalon serially sectioned at 7 μm. Sections were stained with hematoxylin and eosin and surveyed by light microscopy for pathological changes that might have been induced by CTC.

Results

CTC failed to cause either necrosis or lesions in the frog hypothalamus. All brain sections appeared normal and CTC-treated brains were indistinguishable from those of the controls.

Discussion

The absence of lesions after CTC treatment of these frogs suggests that the hypothalamus is either less responsive to CTC than that of mice and rats, in which this dose is considered to be pharmacological (Debons
et al., 1977), or is not responsive at all. Either alternative indicates considerable change in hypothalamic control of food intake through evolutionary time and suggests some possibilities concerning satiety in this species.

First, frogs may not possess a satiety center for controlling food intake. Perhaps they have only a single center, more analogous to the mammalian hunger or feeding center, in the lateral hypothalamus. In this case feeding could take place continually when food is available and perhaps only stop when peripheral receptors, such as stretch receptors in the gut or chemoreceptors in the liver, inhibit feeding by nervous input to the feeding center. Peripheral input to the hypothalamic centers from receptors such as hepatic satiety receptors is being investigated in mammals (Russek, 1970; 1971).

Second, frogs might have a satiety center that is seasonally functional, being active only in warmer months when food is most available. Frogs seasonally store lipids and carbohydrates (Farrar, 1972; Mizell, 1965), but the possible role of hyperphagia in this storage has not been investigated.

Lastly, it is possible that Rana pipiens has a satiety center insensitive to GTG. Likuski et al. (1967) have shown that the glucose moiety of GTG is necessary for VHM necrosis in mice. However, it is not clear to what extent amphibian metabolism is based on a glucose currency (Copeland and deRoos, 1979), and thus to what extent food intake control would be based on changes in glucose concentration. Adult frogs primarily eat insects, which are generally not high in carbohydrates.
Blood glucose levels are characteristically lower in amphibians than in other vertebrates at comparable temperature and metabolic rate (Umminger, 1977). If there is an amphibian satiety center it could be more sensitive to fatty acids or amino acids than to glucose. Further investigation of food intake control in amphibians and other lower vertebrates is desirable.
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