This is to certify that the

dissertation entitled

Food intake and serum insulin responses to intraventricular infusions of insulin and IGF-I in sheep

presented by

L. A. Foster III

has been accepted towards fulfillment of the requirements for

PhD degree in Agriculture

Roy L. Emery
Major professor

Date 9/5/90

FOOD INTAKE AND SERUM INSULIN RESPONSES TO INTRAVENTRICULAR INFUSIONS OF INSULIN AND IGF-I IN SHEEP

By

L.A. Foster III

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Animal Science

1990

ABSTRACT

FOOD INTAKE AND SERUM INSULIN RESPONSES TO INTRAVENTRICULAR INFUSIONS OF INSULIN AND IGF-I IN SHEEP

By

L.A. Foster

Previous studies reported that intracerebroventricular (ICV) infusion of insulin decreased food intake in rats and baboons. Insulin can bind to insulin like growth factor I (IGF-I) receptors and mimic the response of IGF-I. The objective was to determine the effects of ICV infused insulin or IGF-I on food intake in sheep. In the present study a 6 day ICV infusion of insulin (123ng/kg of body weight/day) but not of IGF-I (123 ng/kg of body weight/day) decreased food intake by 40% ($p<.003$) and body weight ($p<.015$) compared with control sheep. In addition, sheep that received ICV insulin or IGF-I had only half the concentration of insulin in serum as compared with controls. These results support the hypothesis that ICV insulin does not decrease food intake through IGF-I receptors. Nevertheless apparently both insulin and IGF-I in the brain can influence the concentration of insulin in blood.

This is dedicated to my grandma

Mary Kiley Foster

1894-1990

My grandma was 92 (still living by herself and still an excellent cook) when I decided to go back to school after 3 years of practicing veterinary medicine. She thought it was foolish to leave a good paying job, and admixed with presenting me an unceasing array of delicious food and coffee, was in not so uncertain terms telling me exactly that. Such is the psychology of grandmas. Trying to figure out why I was getting another doctorate degree, yet still would not be a "real doctor", she asked what I was going back to school to learn. Wanting to keep it simple, I said that I wanted to find out why sheep or other animals eat. With a look of incredulity, she paused a moment holding her breath, either at a loss for words or so exasperated she could not say them, before stating what to her was only the obvious,

"That don't make any sense,
Everyone knows a sheep's gotta eat to live!"

One day I hope to develope a hypothesis I can state with the conviction my grandma said hers.

ACKNOWLEDGMENTS

I gratefully acknowledge Dr. Della-Ferra for describing the surgical technique and equipment used for the LV cannulation in sheep. Pam Buntz for surgical assistance and George Good, Michigan State's shepherd.

I want to express my gratitude to Dr. Kent Ames for all his help and advice on surgeries and for his willingness to always try just "one more thing".

I would like to thank Jim Liesman for statistical, editorial and technical help. Our discussions not only helped solve my research problems, but also eased East Lansing student-community relation tensions, trade imbalances with Japan, world hunger and put a liberal democratic president in office.

I would like to thank my committee: Dr. Roy Fogwell; Dr. Tom Herdt; and Dr. Dale Romsos, for their helpful suggestions and guidance. Their input was always beneficial.

Finally I would like to thank my major advisor Dr. Emery: it is because of his advice I have decided to make a career in the ingestive behavior area; it is because of his example that I realized the importance of keeping current with the literature.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

An understanding of the basic mechanisms that control long term ingestive behavior would enable huge benefits for human and animal health. One of the difficulties encountered in attempting to find a resolution to this problem is that almost everything can affect food intake, from the animal's surroundings (temperature), food characteristics (caloric density) to changes within the animal (gastric distension). However, very little seems necessary for the regulation of food intake to occur. Food intake will stop during a meal even if the temperature is cold and gastric distension does not occur. Perhaps this is not too surprising if one thinks about the different circumstances where eating is terminated. For example the control mechanisms that are utilized to cause a person to finally stop eating at Thanksgiving dinner would probably not suffice for the continued survival of an antelope grazing in Africa as a lion initiated pursuit. Both of these mechanisms would be for short term control of food intake, the termination of a meal. The ability to control food intake over a period of days and thereby regulate body weight, would have a much larger impact on improving the health of humans and animals.

The Set Point Hypothesis

There is considerable evidence that animals and people regulate their caloric intake and body weight, but the mechanisms are controversial. Adult animals strive to maintain a particular body weight under a variety of imposed conditions. After a period of forced feeding (Cohn and Joseph, 1962; Drewry et al., 1989) or food restriction (Sims et al., 1968) animals adjust voluntary intake to return body weight to what it was before the imposed diet (for review see Hervey, 1988). Observations like these have led to the "set point" hypothesis (Kennedy, 1953; Mayer, 1953,) which states an animal recognizes a certain "weight" as normal. In order to maintain that "weight", animals regulate: the amount of calories consumed; the efficiency of storing those calories; and/or their physical activity (output of calories).

There are two basic questions that make up the set point hypothesis. First, what are the "messenger(s)" that inform the brain as to the body's current "weight" in relation to its "set point"? Second, what aspect of "weight" is monitored, (adipose cell size, amount of liver glycogen or amount of some blood metabolite)? Insulin has been proposed as one of the messengers informing the brain on the amount of adipose tissue (Porte and Woods, 1981; Woods et al., 1985).

The Insulin Hypothesis

One attractive "set point" hypothesis suggests that the amount of fat an animal has determines long term food intake (Woods et al., 1985). This hypothesis suggests that insulin in cerebrospinal fluid (CSF) is the "messenger" to the brain on the amount of fat in the individual. Changes in the concentration of insulin in the CSF will cause opposite changes in food intake. An increased amount of insulin in CSF would cause a decrease in food intake and a decrease in CSF insulin would lead to an increase in food intake.

It has been known for a long time that an acute increase of peripheral insulin will decrease blood glucose and increase food intake (Banting et al., 1922). Therefore it seems rather paradoxical that an increase of insulin in CSF should decrease food intake. In order to explain this conundrum, the hypothesis that 'increases in insulin concentration in CSF decreases food intake' will be dissected into components and examined separately. 1) Body weight is regulated. 2) Average serum insulin concentration varies from day to day in the same direction as changes in amount of adipose tissue. 3) Insulin in CSF is proportional to average serum insulin concentration. 4) Insulin has a physiological role in the CNS and 5) insulin in the CSF can affect the brain so as to decrease food intake.

The first component of this hypothesis is that animals "monitor" and regulate their body weight. As discussed under "The Set Point Hypothesis", adult animals seem to

maintain a certain weight. A classic study by Adolph (1947) demonstrated this principal with a series of experiments. Rats fed diets with increasing amounts of cellulose, kaolin or water (this diluted the caloric density of the diet) ate increasing amounts of the diet to compensate. Changing the flavor of the diet had little or no affect on amount of food consumed. Food directly infused into the stomach caused a compensatory decrease in food ingested for the next three days (rats gavaged with the same amount of water did not have decreased food intakes).

The second component is that changes in the daily average serum insulin concentration directly reflect changes in the amount of adipose tissue. This is supported by the findings that fatter animals and humans have higher serum insulin than thinner animals or humans and that when animals lose or gain weight their basal insulin concentrations change in the same direction (McCann et al., 1989; Owen et al., 1974)

The third component is that the insulin concentration in CSF changes proportionally and in the same direction as serum insulin concentrations averaged over several hours. One aspect of this component is whether peripheral insulin can penetrate into the CSF. Over the last 4 years this once hotly debated issue has been somewhat quelled by a series of experiments done by Porte and Woods' laboratory in Seattle WA. (the same group that proposed the insulin hypothesis). Initially this group reported that IV insulin would increase

the concentration of insulin in CSF of anesthetized dogs (Woods and Porte, 1977). This experiment was criticized because the dogs were anesthetized and anesthesia can affect the blood brain barrier (Ono et al., 1983). In the next experiment, awake human subjects were infused with insulin IV in amounts approximating postprandial concentrations and CSF samples were withdrawn from a lumbar spinal tap. The insulin infusion increased insulin concentrations in CSF (Wallum et al., 1987). In the next experiment, permanent cannulas were implanted into the cisterna magna and jugular vein in rats. While rats were unrestrained and moving, insulin or glucose (to stimulate endogenous release of insulin) was infused IV and samples of CSF withdrawn. Insulin concentration was increased in CSF of rats that received IV infusions of insulin or glucose (Steffens et al., 1988). In their most recent study (Schwartz et al., 1990) they report on the time course for an increase in serum insulin to be reflected in CSF. Significant increases of insulin were not measured in CSF until 90 min after a steady continuous increase in serum insulin had been maintained.

A fourth component is whether insulin normally has a physiological affect on the CNS. Insulin and insulin receptors have been measured throughout the brain and the densest population of receptors were found in areas involved with food intake (Baskin et al., 1983; Havrankova and Roth, 1978; Hill et al., 1986). This would suggest that insulin

normally has some function within the brain. Insulin has been shown to have a postreceptor effect of increasing inositol incorporation within the hippocampus (Figlewicz et al., 1990).

A fifth component is whether an increase of insulin within the CSF decreases food intake and a decrease of insulin within CSF increases food intake. Infusions of insulin into the CSF of rats and baboons has decreased food intake (Ikeda et al., 1986; Woods et al., 1979). Infusions of insulin antibodies into the ventromedial hypothalamus increased food intake (Strubbe and Mein, 1977).

These individual components of the insulin hypothesis have been addressed and experiments done that support them. However, combinations have not been tested, such as: does the concentration of insulin in CSF change with changes in body weight and will infusions of that amount of insulin into CSF affect food intake; do the infusions of insulin into CSF act specifically on insulin receptors to cause the decrease in food intake; will blocking the insulin receptors block the effects of infusions of insulin on food intake?

Mechanism for Insulin's Effects

Neurotransmitters

One mechanism by which insulin could affect food intake is by altering the effects of neurotransmitters. One of the functions of a neurotransmitter is to carry or transfer information from one neuron to another. The 'classical'

neurotransmitters are acetylcholine, 5-HT (serotonin), dopamine (DA), norepinephrine (NE) and epinephrine (Epi). All except for acetylcholine have an extensive array of studies that suggest their involvement with food intake regulation.

5-HT

An infusion of 5-HT into the PVN decreased food intake by decreasing meal size. There were no affects on meal frequency (Shor-Posner et al., 1986). The 5-HT injections caused the largest decrease in fat and carbohydrate ingestion as compared with protein consumption (Shor-Posner et al., 1986). Experiments of this type have contributed to the framework of a hypothesis on how 5-HT is involved in food intake regulation (for review see Wurtman and Wurtman, 1986). Briefly, 5-HT is synthesized from tryptophan. Tryptophan shares a common transport system with other large neutral amino acids to cross the blood brain barrier. A high-carbohydrate meal stimulates insulin secretion; this decreases the amount of large neutral amino acids in blood. More tryptophan crosses the blood brain barrier. Availability of tryptophan is the rate limiting step for 5-HT synthesis in the brain. Therefore 5-HT concentrations within the brain increase and carbohydrate intake is suppressed. A high-protein meal works the opposite, by increasing the amount of large neutral amino acids in plasma, less tryptophan crosses into the brain, less 5-HT is synthesized and food intake, in particular carbohydrate

intake, is increased.

The 5-HT hypothesis complements the insulin hypothesis. Both hypotheses have an increased peripheral insulin component, and in both cases the peripheral increase of insulin leads to a decrease in food intake. A major difference, is the increase in 5-HT is suggested to limit the carbohydrate intake of the next meal (and not effect protein consumption) (Shor-Posner et al., 1986). Whereas central insulin probably takes much longer to have an effect on food intake (see Discussion sect). Still there does not seem to be any proof to suggest that one hypothesis is exclusive of the other.

Norepinephrine

It has been suggested that NE increases carbohydrate consumption by acting on α_2 adrenergic receptors to inhibit satiety neurons within the PVN (for review see Leibowitz, 1988). This hypothesis is based on these findings. 1) Infusions of NE into the PVN stimulates consumption of CHO (Leibowitz et al., 1988). 2) There is an increased release of NE from the PVN with food deprivation (Stanley et al., 1989). 3) There is a decreased release of NE from the PVN after a meal (Stanley et al., 1989). 4) Lesions of the PVN cause hyperphagia (Sakaguchi et al., 1988). 5) Infusion of a specific adrenergic toxin into the PVN decreased CHO intake (Shor-Posner et al., 1986).

An infusion of insulin into the PVN had no effect on NE release within the PVN (Minano et al., 1989). An injection

of insulin into the VMH decreased sympathetic activity and thermogenesis of brown adipose tissue (Amir et al., 1989). A decrease in lipolysis and thermogenesis could fit in with a hypothesis for decreasing food intake; however, these effects of insulin on sympathetic activity are more likely involved with peripheral homeostasis rather than a central control of food intake.

Dopamine

The primary focus on DA has been with Parkinson's and schizophrenia. Much of what is known about schizophrenia has been learned from an amphetamine induced psychosis that has been used as a model for schizophrenia (for review see Chiodo, 1988). Amphetamines cause a large release of DA. A side effect of amphetamines is decreased food intake (Carr and White, 1986). Injections of DA agonists decreased food intake (Rusk and Cooper, 1989). During feeding, endogenous concentrations of DA increased in the same locations in the brain where injections of DA decreased food intake (Hernandez and Hoebel, 1988). DA concentration increased within the medial hypothalamus (includes PVN, DMN, and VMH) during insulin perfusion. Therefore insulin may be affecting food intake via dopaminergic neurons.

Location of Insulin's Action on Food Intake

The primary emphasis of research on the neuroanatomical location of food intake regulation has focused on the hypothalamus. More than 20 years ago it was demonstrated that lesions of the VMH caused hyperphagia and lesions of

the LH made animals anorexic (Hoebel and Teitelbaum, 1962). Other lesions in the hypothalamus such as the PVN (Kirchgessner and Sclafani, 1988), increase intake; the dorsomedial hypothalamic nuclei (DMN), decrease intake (Bernardis et al., 1987). Lesions in higher structures of the brain, such as the amygdala (Fonberg, 1989) caused active food rejection in dogs.

Of perhaps greater relevance than inducing a lesion and examining whether a behavior is changed or lost, is inducing a lesion and examining which behaviors stay the same. For example, if a lesion affected motor activity so that an animal could not walk, the intake of that animal would drop to zero. Even though, the animal would eat normally if food was within reach.

Chronic decerebrate rats have an intact spinal cord and brainstem (no hypothalamic, thalamic or cortical functions). Chronic decerebrate rats and intact rats ingest or refuse the same solutions at the same concentrations (Grill and Norgren, 1978a). Chronic decerebrate rats and intact rats will stop drinking a solution after they have consumed the same amount and are sated (Grill and Norgren, 1978b). This demonstrates that integration between sensory information from visceral structures (information on satiation), taste (type of solution in oral cavity) and motor output (rejection of a substance) must occur someplace caudal to the hypothalamus.

There has been some type of interaction reported for

most structures within the brain and insulin. Injection of a physiological concentration of insulin into the jugular vein decreased the activity of taste neurons within the brainstem (Giza and Scott, 1987). These authors suggested that the decreased sensitivity of taste neurons to glucose, after an IV insulin injection, could be a mechanism by which insulin reduces feeding (Giza and Scott, 1987). An alternative interpretation, is that the changes in neuronal activity to insulin, contribute to the increased food intake that occurs after a peripheral injection of insulin that induces hypoglycemia. The decreased activity within the taste neuron to glucose, might cause the animal to prefer a higher concentrated glucose solution. If a certain activity within the neuron was related to a preferred amount of sweetness; then after the insulin injection, a higher glucose concentration would be necessary to obtain that same activity. In support of this, it has been reported that in sodium-deprived rats (sodium deprived rats display an avid sodium appetite (Fregley et al., 1965)), the activity of taste neurons to sodium decreases (Jacobs et al., 1988). In either case, the neuronal response was to exogenous insulin. The insulin concentration within blood will vary considerably in response to meals. Mechanisms that are directly responsive to plasma insulin concentration would probably be involved with short term food intake regulation. It has not been reported if similar changes in neuronal activity occur from an increased concentration of insulin in

CSF. If this occurred, then altered taste might be a mechanism where insulin affects long term food intake.

Infusions of insulin into the LH increased the activity of glucose sensitive neurons (Oomura and Kita, 1981). Most other factors, such as glucose, free fatty acids, amino acids or serotonin that might indicate that a meal had or was occurring, decreased the activity of glucose sensitive neurons (reviewed by Oomura, 1988). In this instance the effect of insulin is opposite to the expected result. Specific infusions of insulin into the LH did not have any affect on food intake (McGowan et al., 1990). This would suggest that insulin is not affecting food intake via the LH.

Infusions of glucose into the VMH increase the activity of glucose responsive neurons. Infusions of insulin tend to inhibit activity, but infusions of glucose and insulin cause a greater stimulation than glucose infusions alone (Oomura and Kita, 1981). The same factors that decreased the activity of glucose sensitive neurons in the LH increase the activity of glucose responsive neurons in the VMH (reviewed by Oomura, 1988). Infusions of insulin specifically into the VMH decrease food intake (McGowan et al., 1990). Lesions of the VMH cause obesity and hyper-insulinemia (King et al., 1988). Stimulation of the VMH decreases food intake (Hoebel and Teitelbaum, 1962). The evidence strongly supports the inclusion of the VMH within the neuroanatomical pathway by which insulin affects food intake.

Insulin's Effects on Meal Patterns

An experiment has not been reported that was designed to specifically examine what alterations occur in meal patterns to account for the decreased intake in response to insulin infusions. When reported, authors describe a general decrease in intake with no exclusive effect limited to meal size, duration or intermeal interval. In an experiment with baboons ICV insulin reduced feeding comparably at every interval. The authors did not state what the length of the interval was, only that they were examining meal patterns (Woods et al., 1980). Insulin infused ICV in rats decreased both day and night food intake, but the authors stated that since rats normally eat at night, there was only a significant decrease of food intake during the night (Brief and Davis, 1984). Injections of insulin antibodies into the VMH only increased intake during the night time (Strubbe and Mein, 1977). Which again is not surprising since rats do not normally eat much during the day. Infusions of small amounts of insulin directly into the medial hypothalamus, that did not affect total food intake, increased daytime food intake (McGowan et al., 1990). The authors suggested that this could have been a compensatory increase in food intake due to insulin's effects of decreasing food intake at night. Although the evidence is far from conclusive, it seems that insulin decreases meal size, frequency and duration during times when an animal normally eats.

Insulin's Effects at the Cellular Level

Insulin has been reported to have no affect on slices from the hypothalamus or cortical gray matter for glucose uptake, oxidation or lipogenesis in brain (Goodner and Berrie, 1977). In cell cultures derived from the whole brains of 1 day old rats, insulin inhibited NE uptake more than maprotiline, an antidepressant and specific NE uptake inhibitor (Boyd et al., 1986). In cells taken from the hippocampus, insulin stimulated inositol incorporation into inositol phosphate and inositol lipids (Figlewicz et al., 1990).

As stated in a review by Recio-Pinto and Ishii (1988) there are many complications in trying to discern the specific functions of insulin on brain neurons. Since there are more than 10 times as many glial cells as neurons in the brain, many preparations will be contaminated with endothelial cells, fibroblasts or glial cells which may contain their own insulin receptors. But it should not be ruled out that insulin may be having major affects on neurons via glial cells. Hexokinase, a rate limiting enzyme in glucose metabolism, is absent in some neurons; yet glucose is almost the sole source of energy in the brain (Katoh-Semba et al., 1988). It has been suggested that some neurons depend on glucose metabolites such as pyruvate supplied by glial cells (Katoh-Semba et al., 1988). The other major point Recio-Pinto and Ishii (1988) made is that insulin and IGF's are survival factors and therefore results

from dose response curves need to be interpreted with caution, since cells in the control and low dose cultures may just be dying. Another problem with relating these in vitro experiments to ingestive behavior is that insulin is involved with other functions and behaviors in the brain. There has been a lot of work determining insulin's involvement with neuron growth or differentiation (for review see Recio-Pinto and Ishii, 1988). Intraperitoneal insulin injections changed the electrical activity of 27% of neurons in the olfactory bulb and 21% of the neurons in the amygdala to odors (Cain, D.P., 1975). Sleep deficits in streptozotocin treated rats were normalized by ICV insulin (Danguir, 1984). Therefore many intracellular changes in some brain areas, caused by insulin, are probably not involved with food intake.

Another important aspect involved in interpreting the results of acute experiments, is that insulin's affects on food intake are chronic. This is a point that has generally been overlooked when the results from acute experiments are used to explain how insulin affects food intake (Myers et al., 1986; Oomura and Kita, 1981). Electrophysiological and microdialysis measurements need to be made over several days of insulin infusion in order to make more confident suggestions on changes caused by insulin that affect food intake.

Insulin Hypothesis Via IGF-I Receptor ?

There is a possibility that all of the pharmacological manipulations of insulin that have affected food intake have caused a response via the IGF-I receptor. High concentrations of insulin can bind to the IGF-I receptor and elicit a similar response to IGF-I (Rechler and Nissley, 1986). There are IGF-I receptors throughout the brain and there are more IGF-I receptors in the choroid plexuses of all ventricles than insulin receptors (Davidson et al., 1990; Shemer et al., 1987). The choroid plexuses are located in the ventricles and are responsible for the synthesis of most of the CSF (Wright, 1982). Peptide infusions into the ventricles would first contact the choroid plexus.

Insulin and IGF-I are structurally similar (Rinderknecht and Humbel, 1978). Receptors for IGF-I and insulin: both contain α and β subunits (McElduff et al., 1988); both receptors can bind either peptide; both receptors can be down regulated by either peptide; the β-subunit of both receptors have intrinsic tyrosine-specific kinase activity that is activated by autophosphorylation (Kasuga et al., 1982; Rechler and Nissley, 1986; Sasaki, et al., 1985). Central insulin and IGF-I receptors appear to maintain these homologies, except insulin receptors do not appear to be down regulated by increased amounts of central insulin (Havrankova et al., 1981; Manin et al., 1988) and both types of receptors are smaller in the brain than

peripherally (McElduff et al., 1988). It is unknown if IGF-I receptors in the brain are down regulated in the presence of increased IGF-I concentrations. In an earlier study a large bolus of IGF-I did not affect food intake in rats (Lauterio et al., 1987). However, insulin does not affect food intake when bolused ICV either (Lauterio et al., 1987; Tsujii and Bray, 1990). The question "are the affects of chronic insulin infusions on food intake via IGF-I receptors?" needs further study.

Insulin Hypothesis In Sheep

In sheep the liver has a net output of glucose in both fed and fasted states (Brockman and Laarveld, 1986). Monogastric species, when fed a typical high carbohydrate diet, only have a net output of glucose in the fasted state. Short chain fatty acids are the primary energy source absorbed from the gut in sheep (Bergman et al., 1966). The concentration of serum insulin is 50% lower than in monogastrics and serum insulin varies less in response to meals, compared to monogastrics (Hove and Blom, 1973). These features make the sheep a unique model to test the hypothesis that increasing concentrations of insulin in CSF lead to decreased food intake.

OBJECTIVE

The objective was to determine the effects of ICV infused insulin or IGF-I on food intake in sheep.

MATERIALS AND METHODS

Animals and Housing

Adult castrated male Dorset crossbred sheep (40-60 kg) were kept in individual metabolism cages. The building was continuously lighted and maintained at a temperature of 18±8°C. Animals were fed a pelleted diet (89% DM, 66.4% total digestible nutrients (TDN), 12% crude protein, 18% crude fiber, 2% fat; Countrymark; Columbus, OH) and water ad libitum. Animals were given new food once daily at which time refusals were weighed.

Infusion Solutions

Artificial CSF (A-CSF) was made according to methods reported earlier (Cserr, 1965; Pappenheimer et al., 1962,) except 100 mg/liter bovine serum albumin was added and the solutions were sterilized by filtering rather than autoclaving.

TABLE 1

Artificial Cerebrospinal Fluid Composition

Constituent	mEq/l	Compounds Used	Amount of Compound mg/l
Na	150.0	NaCl	7310.
K	3.0	KCl	224.
Ca	2.3	$NaHCO_3$	2100.
Mg	1.6	Na_2HPO_4	23.
Cl	135.0		
HCO_3	25.0	$CaCl_2$	128.
PO_4	0.5	$MgCl_2 \cdot 6H_2O$	163.
		Albumin	100.

Porcine insulin was generously donated by Eli Lilly (Indianapolis, IN.) and IGF-I was generously donated by Monsanto (St. Louis, MO.). Each powdered peptide was reconstituted with 0.01 N HCl to make stock solutions with concentrations of 1 mg/ml. Each stock solution was diluted with A-CSF and infused at a dose of 5.6 ug, in 280 ul per day, which amounts to 123 ng/kg of body weight/day for both peptides or 3.4 mU/kg/day for insulin. This insulin dose is greater than the dose used in baboons (0.1 mU/kg/day) (Woods et al., 1979) and less than the dose used in rats (6 mU/kg/day) (Ikeda et al., 1986). The same ng dose of IGF-I was chosen so there would not be a difference in amount of protein added. Since molecular weights are similar (~6000 for insulin and ~7000 for IGF-I) molar infusions were also similar.

Experimental Design

Sheep were adapted to diet, housing and handling for at least 10 days prior to beginning the baseline period. Four trials were completed; the first starting in October and the last ending in March. Trials 1 and 2 used eight sheep each, four infused with A-CSF and four infused with insulin in A-CSF. Trials 3 and 4 were similar to trials 1 and 2 except nine sheep were used in each trial, three were infused with A-CSF (Controls), three with insulin in A-CSF (INS) and three with IGF-I in A-CSF (IGF-I).

Surgery

Ventricular cannulas were permanently implanted under isoflurane inhalation anesthesia. A 1.5 cm long, 16-gauge guide cannula was cemented to the skull with dental acrylic (Columbus Dental; St. Louis, MO) 0.5 cm lateral to the midline and 0.5 cm below the bregma suture line. Stainless steel screws were placed in the skull to anchor the acrylic. Polyethylene tubing (PE) was filled with sterile artificial CSF, and attached to a 20 gauge, short beveled needle. The tubing was held higher than the sheep's head while the 20 gauge needle was lowered through the guide cannula into the brain. The level of A-CSF dropped in the tubing when the needle entered the ventricle. The tubing was then held below the sheep's head and CSF was siphoned out of the ventricle. A visibly steady drip of CSF out of the tubing was required before the needle was secured to the guide cannula. An osmotic pump (Alzet, Palo Alto, CA.) was inserted subcutaneously in the dorsal cervical area and connected to the 20 gauge needle via PE-60 tubing filled with the appropriate test solution.

The criteria used to verify the patency of the system included: 1. observing a consistent drip of CSF out of the PE tubing at time of surgery; 2. infusing Bromophenol blue dye (Sigma; St. Louis, MO) into the PE tubing just prior to euthanasia and finding blue stained ventricles at necropsy; 3. a two fold increase of insulin and IGF-I in the CSF of insulin and IGF-I-infused sheep, respectively, as compared

with controls. Of the sheep infused with either insulin or IGF-I, there was only one sheep that had dye stained ventricles yet did not have elevated insulin in the CSF sample. This sheep was not included in the analysis.

Blood and CSF Sampling Procedure

After 6 days of continuous ICV infusion, animals were weighed and a 5 ml CSF sample was obtained aseptically from a spinal tap into the cisterna magna of anesthetized sheep. For trials 3 and 4, blood was collected from the jugular vein and another CSF sample was taken approximately 4 hr later. Blood and CSF samples were stored in siliconized, sterile serum tubes on ice until centrifugation. The supernatant was divided into 2 ml aliquots and frozen until chemical determinations.

Assays

Insulin was measured by radioimmunoassay utilizing commercial kits; ImmoPhase (Corning, Medfield, MA.) for trials 1 and 2; and Micromedic (ICN, Costa Mesa, CA) for trials 3 and 4. IGF-I was determined using a heterologous radioimmunoassay (Della-Ferra et al., 1986).

Analysis of Data

For the analysis of food intake, the baseline period consisted of 6 days prior to surgery. Food intake for days 3 through 6 of the infusion period were averaged for analysis after determining no treatment*DAY interaction in a split plot analysis removing sheep within treatment sum of squares from the residual. The first model compared INS and

A-CSF using data from all four trials with fixed factors of treatment, trial, and treatment*trial interaction. The second model compared IGF-I, INS and A-CSF using data from trials 3 and 4. There was no significant trial effect for the second model, therefore trial was not included in the analysis. Average food intake of the baseline period was used as a covariate for both models.

For the analysis of body weight, the difference in body weight from the end of the baseline period to the end of the infusion period for each treatment were analyzed in a model with fixed factors of treatment, trial, and treatment*trial interaction.

For the analysis of serum and CSF values of insulin and IGF-I, the same model as body weight was used. Due to the large range of values for insulin in CSF, there was heterogeneous variance. Natural logarithms of the CSF insulin values had homogeneous variance. Therefore the statistical analysis was done on the natural log transformation, but actual values are reported.

Criteria for Animals Included in Analysis

Two sheep died during this experiment; one was hydrocephalic and the other never recovered from anesthesia. Ten sheep were removed from the experiment due to failure of fulfilling criteria for patency of the system. Three sheep were removed from trial one, two from trial two, three from trial three and four from trial four, leaving a total of 10 of 14 controls, 9 of 14 INS and 3 of 6 IGF-I infused sheep.

RESULTS

Intake and Body Weight

Insulin infused sheep ate 40% less ($p<.003$) and lost more weight ($p<.015$) than controls (FIGURES 1 and 2). There were no differences in intake ($p<.4$) or body weight ($p<.5$) between IGF-I infused sheep and controls (FIGURE 2). There was no difference in food intake for A-CSF infused sheep between baseline period and the infusion period (2.4 ±0.1 kg/day; vs. 2.1 ±0.1 kg/day; $p<.08$) (FIGURE 1).

Serum values

Sheep infused ICV with INS or IGF-I had less than half the serum insulin concentration measured in sheep infused with A-CSF ($p<.01$; Table 2). There were no differences in serum IGF-I caused by treatments.

CSF values

By design, animals infused with INS or IGF-I had to have two times the concentration of the infused peptide as compared with controls. The range of values for insulin infused sheep varied from 11 to 1100 uU/ml, with an average of 255 uU/ml.

Cannula Placement

At the time of surgery the cannulas were angled slightly rostral and medial. This caused most of the cannula tips to be in the medial ventral portion of the lateral ventricle (LV) where the LV join together to form the third ventricle. At necropsy all cannula tips were

TABLE 2

Serum and cerebrospinal fluid (CSF) concentrations of insulin (INS) or IGF-I

Treatment Group	TRIALS 1 AND 2			TRIALS 3 AND 4					
		CSF INS		Serum			CSF		
	n	(uU/ml)	Range	n	INS (uU/ml)	IGF-I (ng/ml)	IGF-I (ng/ml)	INS (uU/ml)	Range
A-CSF	5	3	1-6	5	60 ±6.4*	101 ±14	4 ±2.7	8	1-16
INS	5	255¶	12-492	4	28 ±6.7	101 ±18	4 ±3.0	323¶	74-1120
IGF-I				3	11 ±9.0	90 ±24	16 ±2.9§	4	2-16

Plasma and CSF samples were withdrawn following a 6 day continuous infusion of artificial CSF (A-CSF), INS or IGF-I. The data represent means ±SEM, except for values of INS in CSF, where the range is given instead.

* Serum insulin concentrations were 50% lower in sheep infused with INS or IGF-I as compared to sheep infused with A-CSF ($p<.01$).

§ Sheep infused with IGF-1 had higher concentrations of IGF-I in CSF than sheep infused with INS or A-CSF ($p<.02$).

¶ Sheep infused with INS had higher concentrations of INS in CSF than sheep infused with IGF-I or A-CSF ($p<.001$).

FIGURE 1. Mean food intakes by day within treatments for sheep receiving intracerebroventricular (ICV) infusions of: artificial CSF (CONTROL) or insulin for trials 1-4 (upper panel); CONTROL, insulin, or IGF-I for trials 3 and 4 (lower panel).

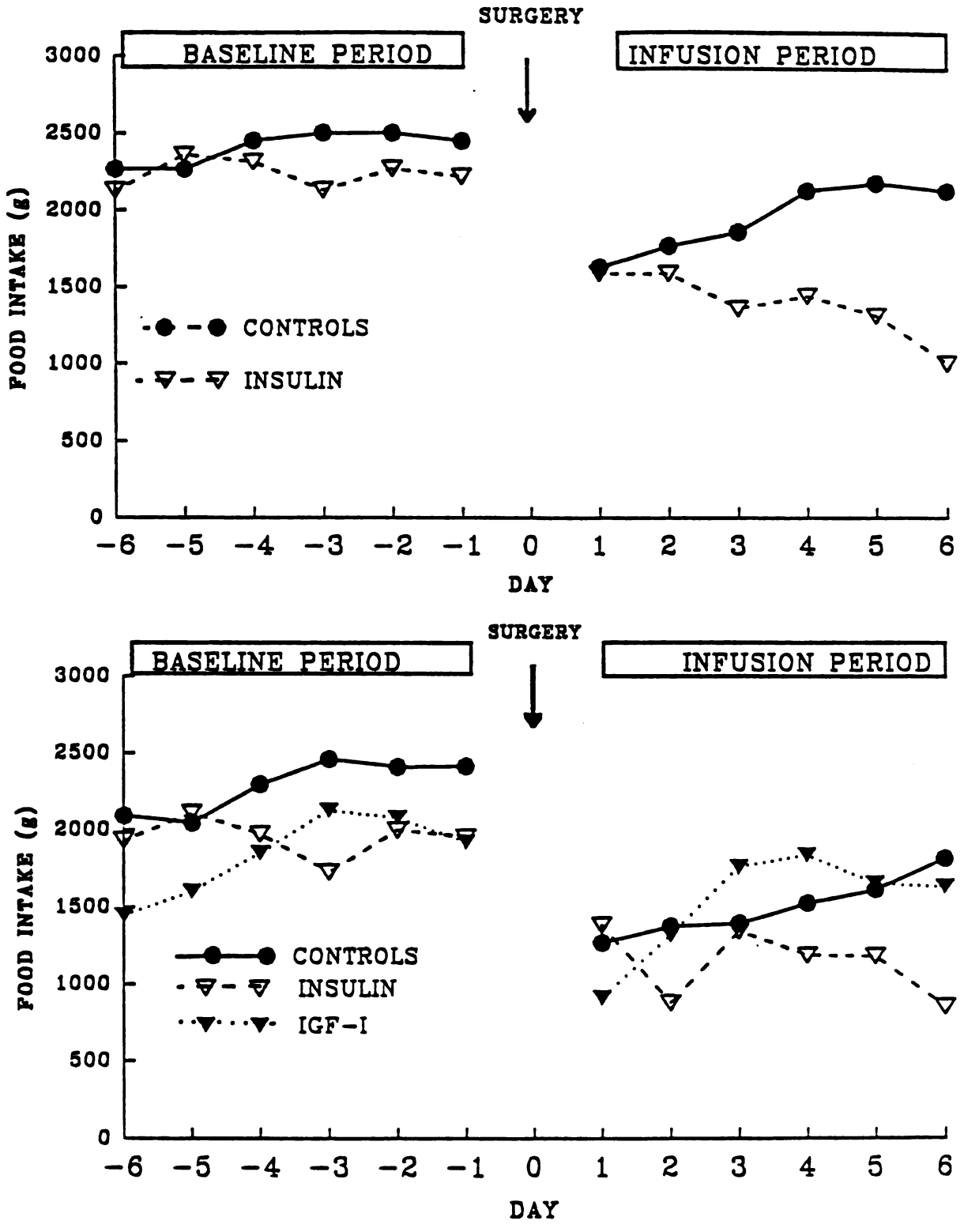
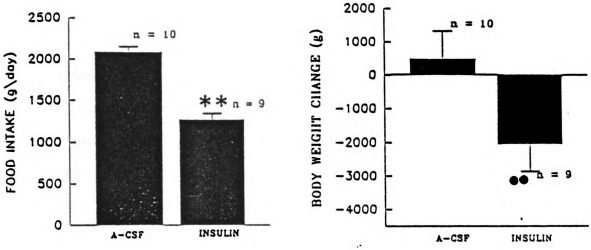

SURGERY
BASELINE PERIOD
INFUSION PERIOD
FOOD INTAKE (g)
3000
2500
2000
1500
1000
500
0
CONTROLS
INSULIN
-6 -5 -4 -3 -2 -1 0 1 2 3 4 5 6
DAY
SURGERY
BASELINE PERIOD
INFUSION PERIOD
FOOD INTAKE (g)
CONTROLS
INSULIN
IGF-I
DAY

FIGURE 1

FIGURE 2. Mean food intakes for days 3-6 of infusion (left panels) and body weight differences (right panels) for sheep recieving intracerebroventricular infusions of A-CSF or insulin for trials 1 through 4 (top panels) or infusions of A-CSF, insulin, or IGF-I for trials 3 and 4 (bottom panels). **$p<.003$ insulin infused sheep ate less than controls. *$p<.02$ insulin infused sheep ate less than A-CSF and IGF-I infused sheep. ●●$p<.015$ insulin infused sheep lost more weight than controls. ●$p<.03$ insulin infused sheep lost more weight than A-CSF and IGF-I infused sheep.

TRIALS 1 – 4



TRIALS 3 AND 4

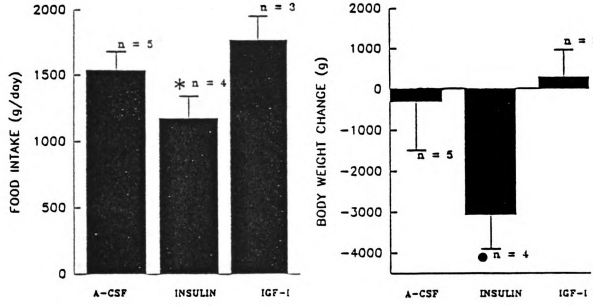


FIGURE 2

the lateral ventricles for the INS and A-CSF infused sheep. The tips of cannulas for the IGF-I infused sheep were actually in the third ventricle.

DISCUSSION

When insulin is infused ICV, food intake is often (Brief and Davis, 1984; Ikeda et al., 1986; Woods et al., 1979) but not always (Brief and Davis, 1984; Lauterio et al., 1987; Manin et al., 1988) decreased. A generalization that may explain these conflicting studies is: ICV insulin reduces food intake only when chronically infused at a relatively large and steady dose. That insulin does not have a short term affect on food intake is supported by the experiments in which insulin bolused into rat ventricles had no short term affect on food intake (Lauterio et al., 1987; Plata-Salaman and Oomura, 1986; Tsujii and Bray, 1990). Insulin was infused into baboons for three days before a significant decrease in food intake occurred (Woods et al., 1979). In this experiment no significant decreases in food intake were measured until after two days of infusion.

This necessity for a chronic ICV infusion of insulin in order to negatively affect food intake might be due to the distance from the infusion site to the active site, or the changes initiated by insulin might take time. That higher doses of insulin decrease the time lag between the start of an ICV infusion and a significant decrease in food intake (Brief and Davis, 1984), support the idea that insulin must

diffuse to the active site. However, ICV infusions of insulin can affect pancreatic function almost immediately (Woods and Porte, 1975), which suggests that at least some active sites are available to insulin in CSF. The idea that insulin might cause slow changes, also fits in well with the proposal that insulin is a component of the "set point" hypothesis. Animals normally take at least a day to compensate for sudden changes made in the energy density of their food or in their energy expenditure (Adolph, 1947; Edholm et al., 1955).

It also appears that a large dose of insulin, relative to endogenous CSF insulin concentrations, must be infused into the CSF to decrease food intake. The endogenous insulin concentration in CSF of baboons is 3 uU/ml (Woods et al., 1981), 150 -1500 uU/day of insulin were required to decrease food intake when infused into the ventricles of baboons (Woods et al., 1979). In rats an ICV infusion of 5 mU/day of insulin did not alter food intake whereas 7.5 and 10 mU/day caused a dose dependent decrease in food intake (Brief and Davis, 1984). The normal concentration of insulin in CSF of rats is 6 uU/ml (Manin et al., 1988). In this experiment the endogenous concentration of insulin was increased in CSF 10 to 100 times.

There is no question that the doses infused for both INS and IGF-I were pharmacological; however since the active site is unknown, the concentration of insulin at the active

site might be in the physiological range. Duffy and Pardridge (1987) have identified an insulin transporter between blood and brain parenchyma, suggesting that insulin goes from blood to brain to CSF. This would mean that the infusion site was "downstream" of the active site and it might be expected that much higher doses would be required.

Alternative Explanations

The decreased food intake with insulin in this trial might have been due to a non-specific protein effect. This is not likely since the same dose of IGF-I was also infused, plus the large amount of albumin (.1mg/ml) that was included in the A-CSF should have masked any nonspecific protein effect that insulin might have had. In earlier reports glucagon (Woods et al., 1979) or denatured insulin (Plata-Salaman et al., 1986) were infused ICV with no effect of food intake.

Insulin infused ICV might cross the blood brain barrier and have an effect peripherally. This is not likely since the amount of insulin infused into the CSF was very minor compared to peripheral insulin concentrations. If the amount of insulin infused ICV (2.3uU/kg/min) for 15 min (two times the half life of insulin in blood) (Grizard et al., 1988) was given in a single dose into the blood, the insulin concentration in blood would increase by approximately 0.7 uU/ml or less than 2%. In sheep, intravenous jugular infusions ranging from 20 to 2000 uU/kg/min for 24 hr had no

effect on food intake (Deetz et al., 1980).

High concentrations of insulin bind to IGF-I receptors and elicit a response similar to IGF-I (Rechler and Nissley, 1986). It is possible that large doses of insulin (relative to endogenous concentrations of insulin in CSF) need to be infused into the CSF to decrease food intake because insulin is acting via IGF-I receptors. These results would suggest that this was not the case since there was a trend for IGF-I to increase, not decrease, food intake (FIGURE 2).

It is possible that IGF-I did not cause an effect since it was infused into the third ventricle; however, insulin decreases food intake when infused into the third ventricle (Brief and Davis, 1984; Ikeda et al., 1986). There is mixing of CSF between the ventricles (Wright, 1982); insulin infused into the right lateral ventricle will spread into the third ventricle and on to the left lateral ventricle (Manin et al., 1990). This would suggest that IGF-I infused into the third ventricle would diffuse into the lateral ventricles.

Insulin and a Fat Diet

Arase et al. (1988) reported that ICV insulin decreased intake in rats fed a high-carbohydrate diet, but not in rats fed a high-fat diet. Sheep can be said to be on a high-fat diet, based on what is absorbed from the gut into the blood, but sheep primarily absorb short chain fatty acids rather than long chain fatty acids (Bergman et al., 1966). These

results suggest that long chain fatty acids -not short chain fatty acids- prevent the effects of ICV insulin on food intake in high fat diets.

Rats need to be tested with a high-fat diet of short chain fatty acids to determine if they respond similar to sheep. This would have interesting implications into the mechanism of insulin's actions on food intake. Short chain fatty acids are absorbed directly across the gut into the blood stream whereas long chain fatty acids are absorbed into the lymph; long chain fatty acids require carnitine acyl transferase to cross the mitochrondrial membrane to be oxidized whereas short chain fatty acids do not (McGarry and Foster, 1980).

Serum Values

There was a large and significant decrease in serum insulin concentration for sheep infused with either INS or IGF-I as compared with A-CSF ($p<.01$). Decreased intake might explain the reduced serum insulin in the INS infused sheep. Animals with decreased food intake will have decreased serum insulin (de Boer et al., 1985; Heitmann et al., 1986). However the trend was for IGF-I infused sheep to eat more than controls, suggesting that IGF-I either decreased pancreatic insulin secretion or increased the rate of insulin turnover.

A subcutaneous infusion of IGF-I in humans decreased insulin secretion, based on C-peptide concentration (Guler

et al., 1989). Plasma insulin concentrations were not affected which suggests that insulin turnover was decreased also. During the subcutaneous IGF-I infusion, serum IGF-I concentrations were increased four times higher than normal. In this experiment there were no differences in plasma IGF-I. It is not likely that the IGF-I infused in sheep was acting peripherally. It is possible the high concentration of IGF-I obtained in humans might have crossed the blood brain barrier and affected insulin secretion. This experiment supports the proposal that IGF-I lowers serum insulin via a central mechanism.

Pancreatic insulin secretion is altered by stimulating the hypothalamus (de Jong et al., 1977). Therefore, neural connections exist to the pancreas that might be activated by IGF-I. Electrical stimulation of the hypothalamus can alter enzyme kinetics in the liver (Shimazu and Ogasawara, 1975). Therefore, it is possible that IGF-I might affect insulin turnover via the CNS. An alternative explanation is that IGF-I induces the release of another peptide or hormone that is then responsible for the peripheral changes in serum insulin concentrations. ICV infusion of IGF can decrease pituitary release of GH by increasing hypothalamic somatostatin concentrations (Berelowitz et al., 1981; Tannenbaum et al., 1983).

A significant decrease in serum insulin due to an ICV infusion of insulin or IGF-I has not been reported

previously. Most studies have measured a nonsignificant decrease in serum insulin in response to an ICV infusion of insulin (Arase et al., 1988; Manin et al., 1988; Steffens et al., 1985; Woods et al., 1979); except in an experiment with anesthetized dogs, an ICV infusion of insulin caused an increase in serum concentration of insulin (Woods and Porte, 1975). Possible reasons for this discrepancy might be due to factors such as: the dogs had been fasted prior to insulin infusion; the dogs were anesthetized; or the experiment only had a 3 hr duration.

CSF Concentrations

Reasons for the huge variation in insulin concentration within the CSF, even though the same amount of insulin was being infused, is unknown, but has been found by other workers (Buonomo, personal communication). In the last two trials, two CSF samples were obtained per sheep approximately 4 hr apart. There were large differences in insulin concentrations within, as well as among sheep (data not presented). Within the sheep infused with insulin, the concentration of insulin in CSF was not correlated with food intake. The production and outflow of CSF is very responsive to hydrostatic pressure (Pappenheimer et al., 1962; Wright, 1982), so perhaps the variation was due to the way the sheep had been holding his head just prior to sampling.

SUMMARY AND CONCLUSIONS

I think that insulin has a specific effect on food intake within the central nervous system (CNS). Infusion of insulin into the ventricle of the brain decreased food intake. Infusion of insulin antibody into ventromedial hypothalamus caused an increase in food intake (Strubbe and Mein, 1977). The dose of insulin I infused was too small to cross the blood brain barrier and have an effect peripherally. Insulin binding sites have been identified within the brain in areas associated with food intake (Havrankova and Roth, 1978; van Houten and Posner, 1981; Wilcox et al., 1989). Insulin has been shown to have a postreceptor effect of increasing inositol incorporation within the hippocampus (Figlewicz, et al., 1990). In conclusion, ICV infusion of insulin significantly decreased food intake and body weight as compared to IGF-I or A-CSF infused sheep. A surprising, albeit unique, finding was that IGF-I and INS infused sheep had significantly lower serum insulin concentrations than A-CSF infused sheep. Further experiments will be necessary to elucidate the mechanism(s) by which this occurred.

FUTURE DIRECTIONS

One of the most exciting results found, was that central administration of IGF-I and insulin decreased plasma insulin. This experiment needs to be repeated with more frequent withdrawal of blood samples. The minimal analysis

on the blood samples would include insulin, glucose, and C-peptide concentrations. With this information, the time course for the decrease in plasma insulin could be charted. A decreasing amount of C peptide would support the hypothesis that there was a decreased secretion of insulin (versus an increased turnover of insulin).

Another important relationship to determine is the change in serum insulin over time as compared to the change in food intake. If serum insulin changed before the change in food intake, this would suggest that the decreased peripheral insulin was not due to decreased intake.

If C-peptide concentration decreased, then it would be worthwhile to sever the vagal branches to the pancreas and repeat the infusion experiment with insulin and IGF-I. If the decrease in serum insulin was eliminated, this would suggest that the peptides were decreasing insulin secretion via a neural mechanism. If C-peptide was not decreased, than a selective vagotomy to the liver might be indicated to determine if the decrease in serum insulin was due to a change in insulin turnover within the liver that was neurally mediated. If a hepatic vagotomy had no effect, than measuring other hormones or peptides, such as somatostatin, might be indicated.

Three directions that could be pursued from the insulin and food intake part of this research would include: 1) a "classic" approach that has typified previous research

investigating the role of a peptide with food intake regulation. 2) An approach that would examine the "basic" mechanisms of insulin in the brain, with molecular biology techniques. 3) An approach that would work on elucidating the "central hardware" for food intake regulation.

Classic Approach

Typically after an ICV infusion of a peptide has been shown to affect food intake, micro-infusions are done in representative sites throughout the brain. In the most sensitive areas, several different amounts of that peptide are infused to determine: a dose response curve, affects on macronutrient selection and affects on meal patterns. The most sensitive area can be lesioned to determine if this will attenuate the response to an ICV infusion. Then various combinations of manipulations such as: lesions, pharmacological agonists or antagonists, and diets are tested.

Molecular Biology Approach

Since: insulin receptors are found throughout the brain; insulin is involved with different behaviors and functions in the brain; and the location of insulin's affects on food intake are unknown; it is likely that work on post receptor effects of insulin would lead to other fields of research than food intake. However, a mechanism can be postulated on how insulin could affect long term food intake at the cellular level. Since insulin is thought to

be involved with the regulation of body weight and long term food intake regulation, it is plausible that the short term regulators of food intake (initiation and termination of a meal) are functioning normally and that only the sensitivity to these short term regulators is changed. Two possible mechanisms for modifying the sensitivity, would be to alter the extracellular ion concentration or change the number of synapses between neurons.

Small changes in the extracellular potassium can cause large changes in the excitability of a neuron. As an action potential travels down a neuron, potassium moves from inside the neuron to the outside. One of the functions of glial cells, the structural cells in the brain, is maintaining a constant extracellular potassium concentration (Kandel, 1985). Postreceptor effects of insulin could affect ion channels in the glial cells to either increase or decrease the amount of potassium taken up by the glial cell.

Glial cells in the pituitary can insert and retract processes between neurons and thereby change the number of synapses between neurons (Hatton et al., 1988). There are more than 10 times the number of glial cells as compared to neurons in the brain (Kandel, 1985). Different peptides have been reported to alter cAMP or inositol phospholipid turnover in glial cells in culture, although none of these peptides were insulin (Cholewinski and Wilkin, 1988). Insulin receptors have been identified on glial cells

(Bouhaddi et al., 1988). Insulin has been shown to increase phospholipid incorporation in membranes derived from the whole brain (Figlewicz et al., 1990) (considering the proportion of neurons to glial cells, it is a good possibility that this finding occurred in glial cells). It is possible, insulin is causing post-receptor effects on glial cells to alter the number of synapses between neurons.

Therefore there is evidence to suggest that insulin could be causing long term effects on food intake by interacting with glial cells. Increasing or decreasing neuron sensitivity could either increase or decrease the amount of information making it through the various synaptic relays or checkpoints in the brain. This could either increase or decrease the probability of a "satiety peptide" terminating a meal.

Determining the "Hardware"

An area that is crucial to determine the 'physiological' mechanisms for food intake, are the neural pathways involved with food intake. By using different dyes and nerve tract staining techniques, it can be determined where all the nerve fibers from a particular structure go. For example, injection of a dye into the stomach wall will label neurons in the dorsal motor nucleus of the vagus (Yoshida et al., 1988). Injecting label into the VMH, (Fahrbach, et al., 1989) identified over 12 major areas of the brain that have neurons that terminate in the VMH. But

just knowing that neurons connect two structures, does not explain what kind of information is carried by those neurons.

Starting with structures where it is relatively easy to determine what type of information is being carried, like the vagus, single unit recording (to monitor electrical activity) and microdialysis (to monitor chemical activity) need to be done in the awake animal. The pathways must be carefully mapped out, one step at a time, moving up into higher structures of the brain.

In the not to distant future, the technology will probably be available where recording from several neurons simultaneously, in different connecting areas of the brain, in an awake animal will be possible. With this type of information, associating behavior with specific neuronal tracts will be greatly facilitated.

Once the normal activity within the neuronal pathway has been described over the duration of a meal; the neural activity elicited by various infused peptides could be compared to what normally happens during a meal. This would be stronger support that a peptide is or is not involved in physiological hunger or satiety, than any type of evidence available to date.

Comparing the effects of an infused substance on neural activity, to the normal activity that occurs during ingestive behavior, could work as a physiological screening

method for identifying novel compounds that might be useful in modifying intake.

APPENDICES

APPENDIX A

MANUFACTURE OF EQUIPMENT

Luer Plug (see Figure 3)

The luer plug (perfectum adapter; Popper and Sons) is solid stainless steel (a). The luer plug was made to fit into the hub of a needle. A small hole is drilled through the luer plug so that the ventricular needle can just pass through easily. Another hole was drilled perpendicular (b) to accommodate a stainless steel screw. When the screw was tightened, the ventricular needle could not be moved.

Guide Cannulas

Guide cannulas (c) were made from 16 gauge stainless steel hypodermic needles. A rotary grindstone was used to cut the needle at 1.5 cm from the bottom of the hub of the needle. The ventricular needles needed to be passed through the guide cannula to test for patency prior to surgery. In about 30% of the 16 gauge needles, there was a stricture where the needle part was attached to the hub. To open this stricture, a 1" safety pin was used. The bottom of the safety pin was held in a vice grips. The hub end of the 16 gauge needle was twisted on and off of the pointed end of the safety pin. This was enough to allow the ventricular needle to pass easily.

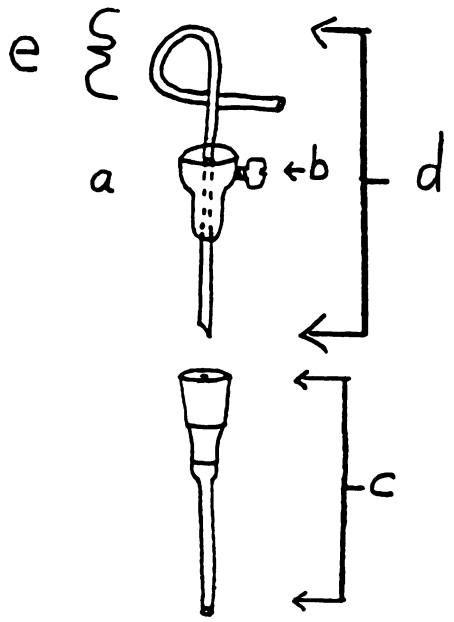


FIGURE 3 INFUSION APPARATUS

Ventricular Needle

The ventricular needle (d) was put into the luer plug. The luer plug was put snugly into the hub of the guide cannula. The ventricular needle was pushed down through the guide cannula until the distance from the bottom of the hub of the guide cannula to the point of the ventricular needle was 4 cm. A black line was marked on the ventricular needle just on top of the luer plug as a guide for depth during surgery.

The top of the ventricular needle sticking out of the luer plug was wrapped 3/4 times around a pencil, taking care not to crimp the needle. This left a loop at the top (e), and the blunt end sticking out perpendicular to the pointed end. The loop allowed the cap to fit on, and acted like a spring so that small movements were less likely to put a hole in the tubing or pull the ventricular needle out.

Stylets

Stylets were made by soldering 1.7 cm long, 0.635 mm diameter, stainless steel rod to a luer plug.

Protective Caps

The protective caps were made from 8 oz plastic containers. The container was thick but non brittle. The bottom was cut off with a hack saw; a bunsen burner was used to smooth the bottom where it was jagged from being cut and to roughly shape it for the sheep's head. A solder iron was used to make small holes for stainless screws in the bottom

so that the plastic cap would stick to the dental acrylic. A larger (~1 cm) hole was made in the back of the cap for the poly ethylene (PE) -tubing to go through to connect to the osmotic minipump.

APPENDIX B

SURGICAL PROCEDURE: Step by step and helpful hints

The sheep were anesthetized with injectable sodium thiopental, intubated and anesthesia was maintained with isoflurane gas. A stereotaxic apparatus was used to stabilize and fix the position of the sheep's head. In order to make the proper size and shape of incision, the protective cap was positioned 2 cm cranial to the ligamentum nuchae crest (where the neck muscles attach to the back of the skull) on the midline. Three stab incisions were made just inside of the cap, one on each side and one at the caudal end. The stab incisions were guides to form a U shaped skin flap. The initial incision was made smaller than the cap because the skin would stretch once the hole was made. The skin and underlying tissues were scraped off the skull with a periosteal elevator and pulled rostrally until an area of the top of the skull had been cleared that extended about 2 cm cranial to the bregma suture line. An 8 mm hole was trephined 1 cm caudal to the bregma suture line and 1 cm lateral to the midline.

While drilling a hole through the skull, the bone seemed to be made up of 3 layers based on the ease of drilling. A thin hard layer on the top and bottom and a

soft wider layer in between. Therefore a lot of pressure could be put on the trephine at the start, but when the drilling became hard again for the second time, care and time needed to be taken so that the bone did not break off and the trephine pith the sheep. Typically the skull was approx. 0.8 cm thick. The soft part of the skull usually did not end till after 0.5 cm. White faced sheep had softer skulls than black faced sheep.

After the hole was drilled, four 1/2" stainless steel screws were placed in the skull around the hole. I used a 1.5 mm in diameter intra- medullary pin to put small holes in the skull to start the screws. The screws were only tightened to approx. 9 mm (3/8") so that the dental acrylic could flow under the screw heads.

The ventricular needle in the luer plug was inserted into the guide cannula so that just the point of the ventricular needle stuck out past the end of the guide cannula. The needle point would puncture the dura mater when I inserted the guide cannula through the hole into the brain parenchyma.

Once the guide cannula was in the brain parenchyma, I would withdraw the ventricular needle (being careful not to move the guide cannula) and replace the ventricular needle with a stylet.

The protective cap was put over the screws and guide cannula and dental acrylic was poured in the protective cap. Enough acrylic was poured in to cover the screws in the

skull and protective cap, but not cover the guide cannula.

As the acrylic hardened, PE-60 tubing was filled with the appropriate test solution. The syringe was left attached to the tubing, to keep the solution from running out.

Once the acrylic was hard, the ventricular needle was inserted into the guide cannula. As soon as the tip of the ventricular needle entered the brain parenchyma, the syringe was removed from the tubing and the free end of tubing was held up above the sheep's head. When the ventricular needle entered the ventricle, the fluid level would drop in the tubing, then the tubing was lowered below the sheep's head. If the ventricular needle was actually in a ventricle, there was a steady drip of CSF siphoned out of the ventricle.

An osmotic minipump was inserted subcutaneously in the neck. The PE tubing, that was connected to the ventricular needle, was cut and attached to the osmotic minipump so there was enough slack to allow the sheep free movement. A helmet and halter was placed on the sheep after the sheep was disconnected from the anesthetic gas machine but before the sheep woke up. After recovery the sheep would be returned to the metabolism cage.

During the days that followed, any air bubbles in tubing would be an indication of a hole or interruption in the line between the pump and the ventricular needle.

APPENDIX C

PRELIMINARY METHODS: What Worked And What Did Not

In the process of finding a method that worked, I found a lot of things that did not work. When I realized that a method was not working, I would try and come up with all the possibilities why. Then I would change as many of the potential problems as I could all at once. I was not really interested in finding out why the technique was not working, I merely wanted a technique that worked. This section is an overview of the different things I tried and why I thought they did not work.

Housing

Initially, 4 ft by 8 ft by 3.5 ft high pens for the sheep were made out of woven wire hog panels. In order that each pen could be opened individually, scrap hog panels were cut 4 ft wide and used as a gate for the front of the pen. Wood shavings were used for bedding. The sheep pushed the shavings out of the pens through the hog panels. Therefore holes were drilled in scrap pieces of wood, mostly 2" X 6"s, and were wired to the bottom of the panels to keep the shavings from coming out. The 2 X 6's also made the panels more stable.

The sheep were fed and watered in separate 8 gal black rubber buckets with metal o-rings on top. Both buckets were

wired in the corners of the pen so that the sheep could not tip them over. The sheep stood in and on the buckets and pawed their feed; either spilling or adding bedding material to their feed.

To stop the sheep from spilling feed, wooden gates were made from 1" X 6" commercial grade boards for the front of the pens. Two 1'X 1' windows were cut out of each gate 1 ft from the bottom. Wooden boxes were made from commercial grade boards and secured to the gate below the windows. The food and water buckets were fastened to the boxes with rubber straps with S hooks on each end. The sheep stuck their heads through the window into the buckets to eat or drink. This set up worked well for keeping the sheep from climbing in their feed. The wood was cheap and a few times a sheep jumped through the hole and broke the gate and the box.

There were no major problems with this setup. Minor problems include it was not convenient to clean out; the sheep were in the way and there were to many corners to clean easily with a scoop shovel. Shavings would get between the back panel and the wall. Once in a while a sheep would jump over the panels. The sheep would occasionally butt the sides of the pen, especially during and after cleaning. A few sheep damaged their infusion set-up in this manner.

Due primarily to changes in the method of infusing (see infusion) I needed to restrict the movement of the sheep.

Ten calf metabolism crates were obtained from: the veterinary research building; ULAR; and the blacksmith at farm maintenance. In 2 crates the floor was replaced with 1/2" cold rolled steel. This looked good but the surface was rough; it was heavy; and I was concerned that it might be abrasive on the sheep's legs. Several coats of "rubber paint" were brushed on the steel to make it smoother and softer, but it came off in long strips after the sheep had been in the crate for a few weeks. I ended up just painting them with four coats of metal paint. The floor still felt rather rough but none of the sheep became lame or sore.

In the front of the metabolism cages, one wooden box was made for each metabolism crate to hold both buckets for feed and water. There was no support for the front of the boxes and they did not hold up well.

Because of the problem with the boxes, wire bucket holders (from Dairy supply) which fit on a two inch wide board were purchased to hold buckets for water and feed. A 2 X 6 board was attached with 2 hinges to the metabolism crate. Rubber straps with an S hook on each end were again used to keep the sheep from pulling the buckets out of the bucket holders.

This setup worked well, however when some of the sheep ate, they would take a large mouthful of feed, than lift their heads out of the bucket and look around while they ate. Some food would spill out of the sides of their mouths. It was difficult to quantify this amount. Therefore

12" diameter PVC pipe was cut in 20" lengths, with a 7" wide slot cut out along the length of pipe. The PVC pipe was bolted in the bucket so that the slot faced the front. This raised the sides of the bucket 18", with a slot in front for the sheep to stick their heads in. Now when the sheep were eating, the sheep could not move their heads from side to side unless they backed up first. This worked well for avoiding spillage.

Feed

In the beginning I bought grain from Mason elevator and mixed a 2 wk supply with chopped hay. The mix was not very uniform and settling would occur. The feed was switched to a maintenance pellet (feed # 1730, Countrymark, Columbus, OH) and that worked well.

Infusion

I started using auto syringe pumps and infused 2.7 ml/day. Sheep ate less when infused with artificial CSF (A-CSF). The amount infused was decreased to 2 ml/day. The sheep still did not eat as well as they had before surgery.

A 3 ml syringe is the smallest syringe that will fit securely in the auto syringe pump. In order to decrease the infusion rate further osmotic minipumps (2ml2, Alzet, Palo Alto, CA) that delivered 140 ul/day for 2 wk were tried. At necropsy it was found that what appeared to be the choroid plexus, had completely walled off the tip of the cannula from the ventricle. The dye, that was infused just prior to euthanizing the sheep, went out of the cannula; stained the

choroid plexus; then was shunted back up along the outside of the cannula to the top of the brain. So I decided I needed a faster infusion rate, and went back to the Auto-syringe's.

Polyvinyl (tygon) tubing was placed around tuberculin syringes (1 ml capacity), and then the tuberculin syringes fit securely in the Auto-syringe. Using tuberculin syringes, I infused 0.7 ml/day. Controls still went off feed. I went back to the minipumps, but to the 2ml1 which infuse at a rate of 280 ul/day for 1 wk.

Angiotensin II (Ang II)

When Ang II is injected into the lateral ventricle an abrupt drinking response is elicited. This effect of Ang II has been used by other investigators as support that their solutions were going into the ventricle (Woods et al., 1979). However, the response of Ang II in other areas of the brain has not been reported; Ang II in other areas of the brain might elicit water intake also. I found it difficult to be objective on what constituted a response. Sometimes immediately after infusing the Ang II a sheep would go to the water bucket and drink a 1/2 kg of water; another sheep would just stand there for a minute and then drink 4 kg of water all at once. There seemed to be a variation in response. Plus I was concerned about what effects Ang II might have on food intake, and if Ang II might change the response to insulin. In the final method I did not use a response to Ang II as a criteria for patency

of the infusion system.

Surgical Techniques

I first used a method described in Pappenheimer et al., (1962) to implant a permanent cannula into the cisterna magna. Later I learned from Dr. Heisey (a co-author in the paper by Pappenheimer et al., 1962; and a professor here at Michigan State Univ. in the physiology department), that there were some major differences between their technique and ours (Foster et al.,1987).

The cannulas used to access the cisterna magna were made from 14 gauge hypodermic needles cut to a length of 5 cm. A 1 cm crossbar was made with 16 gauge baling wire. Copper wire was used to tie the crossbar 1.7 cm from the tip of the hypodermic needle. A 90% metal epoxy was used to cement the crossbar to the hypodermic needle.

These cannulas were placed in holes drilled through the occipital bone directly above the cisterna magna. Two metal screws were placed into the occipital bone, one above and one below the crossbar. Then dental acrylic was used to cement the cannula to the skull. The screws held the dental acrylic to the skull. This type of cannula was used on 12 sheep. All sheep recovered for 2 wk before any experiments started. When I went to collect CSF I found that the cannulas had come loose on seven of the sheep. What I think happened is an electric current developed because of all the different metals that I used to make the cannulas. This weakened the metal epoxy and allowed the crossbar to come

loose from the 16 gauge needle. I tried doing three of the sheep over again, but the occipital bone seemed to have become restructured also. These changes in the skull supported the idea that I might have set up an electric current.

In the sheep that did not lose their cannulas, there was a cellular infiltration around the obturator up the guide cannula.

The second model of cisterna magna cannulas were made from stainless steel hypodermic needles, the crossbars were made from stainless steel wire. The crossbars were soldered on the hypodermic needle. The solder was coated with rubber paint. An obturator was made of stainless steel wire, and coated with rubber paint for a tighter fit in the cannula to stop the cellular infiltration.

The second model was used on 6 sheep. I was able to get a CSF sample on all these sheep. However the day after collecting several samples from each sheep, 4 of the 6 sheep were showing abnormal clinical signs, like the spinal cord had been damaged. The first sample of CSF was easy to get, but regardless of the amount of CSF I removed, or even the length of time between samples on the same day, each successive sample was considerably more difficult to collect. When I had trouble collecting a sample of CSF I would move the needle around. I used 18 gauge 4" spinal needles to puncture the dura and collect the CSF. The diameter of the spinal needles was smaller than the guide

cannula. The up and down movement probably did not cause a problem. The side to side movement, that I was not even aware of, could easily have damaged the spinal cord.

At the time I thought that this technique would not work. If I had used the luer plug with a side screw to hold the ventricular needle solid that I used for the last experiments, this technique might have worked.

The second technique I tried was cannulating the subarachnoid space of the spinal cord. A 14 gauge 1" needle was inserted into the subarachnoid space between the lumbar vertebrae. Polyethylene (PE) tubing was pushed through the needle and subarachnoid space to the first cervical vertebrae. The tubing went easy until the thoracic cervical junction. A fine wire (initially I used a guitar wire) that would fit inside the PE tubing was used as a stylet. I could verify the location of the tip of the tubing by leaving the wire in the tubing and taking an x-ray.

(A subjective observation was that this method increased the respiratory rate of sheep without affecting their temperature.)

The subarachnoid cannulation was performed on two sheep initially. When A-CSF (control solution) was infused into the subarachnoid cannulas, their food intake immediately dropped by 50%. The infusion was stopped, and the sheep returned to eating the same amount they had been eating before the infusion. New A-CSF was made and the infusion rate was decreased. Three days elapsed before the infusion

began again. This time food intakes did not decrease. After two weeks 400 uU/kg/day of insulin was added to the infusion. There was no response in food intake after 2 weeks, so the dose of insulin was doubled. Three weeks later the dose was increased 10 times. None of the doses affected food intake. The sheep were anesthetized and a radio-opaque dye was injected into the tubing under a fluoro-scope. The dye seemed to leak out of the tube everywhere the tubing rubbed against the vertebrae of the spinal cord (three places). No dye was observed in the ventricle system.

At necropsy I found that connective tissue had been laid down over the entire PE tubing I was infusing through. I do not know how long it took for this connective tissue growth to occur, but I thought that once it was formed that it would be unlikely that the infusion would reach the ventricular system. This also reinforced the importance of being able to verify where the infusion was actually going. That the mere physical presence of the tubing in the proper location at necropsy did not prove the infusion had been going in the right place. Since it seemed that glial cells or some type of fibroblast (connective tissue forming cells) were so active within the spinal cord and ventricular system. I wanted a method of verifying the patency of the tubing that could be used throughout the experimental period and not just at necropsy.

The third technique came from Breuhaus et al., (1989).

A 0.5 cm hole was drilled in the skull over the lateral ventricle. Polyvinyl tubing (.085" OD) was lowered by hand into the brain parenchyma. A manometer filled with A-CSF was connected to the tubing; once the tubing entered the ventricle the level of A-CSF in the manometer would drop. Sometimes CSF would drip from the end of the tubing. I felt this was a good indication that I was actually in the ventricle. For the final method a consistent drip of CSF out of the tube was one of the criteria for patency of the infusion system.

Sometimes the fluid did not drop while the tubing was pushed through the brain because of either: a tissue plug in the end of the tubing; or the tubing did not penetrate a ventricle.

When the tube was pulled back out, sometimes there was an obvious plug, other times it was hard to tell. Since the tube was lowered by hand, the second time the tubing was lowered, it was impossible to ensure the tube went down the same tract. Another concern was that the tubing made a large enough tract to act like a false ventricle or to leave a tract to the ventricle that would close up later. In either case the A-CSF would drop in the manometer even though the end of the tubing was not actually in the ventricle.

Another potential problem area was when the tube was cemented in. While one person held the tube, another person would press the dental acrylic around the hole and tubing.

The person holding the tube could pull the tube out of the ventricle and not realize it.

Another potential problem with this method was the tubing that stuck out of the hole was bent down and cemented flat against the skull. Over the next several days the portion of the tube that was in the brain may have partially straightened out, removing the tube from the ventricle.

A vacuum pac (Olympia Washington) was used to support the head. The vacuum pac feels like a small bean bag chair. When vacuum is applied to the vacuum pac it becomes rigid. The vacuum pac worked well for support, but if the sheep moved during surgery, the vacuum pac would not hold the head in the same position. The movement itself was a very minor problem but a small rotation of the head could easily go unnoticed because of the drapes. Just a small rotation could make a big difference in where the tip of the cannula ended up. This problem was eliminated by the use of a stereotaxic instrument.

I do not know which of these potential problems were actually problems. I do know that sometimes I saw a drop in the level of A-CSF in the manometer at the time of surgery and sometimes I saw a dramatic drinking response to Ang II at the beginning of the experiment but a poor or no drinking response at the end of the experiment and/or the cannula out of the ventricle at necropsy.

To get around the problem of the tube straightening out after the tube had been cemented to the skull, and the

problem of it being so easy to move the tube while cementing it in, I switched from polyvinyl to silicone tubing (silastic). A 14 gauge 2" needle and obturator were used to make a hole. The proper depth of the hole was determined by connecting a manometer filled with A-CSF to the needle. When the pressure dropped in the manometer: the manometer was disconnected; the silastic tubing was pushed into the needle; and the needle was pulled out. The problem with this was keeping the silastic tubing at the same depth while pulling the needle out (ie pulling the silastic tubing out with the needle). Plus if there was not a flow of CSF out of the end of the tube, the tube could not be put in deeper. The problem of a tissue plug that I encountered with the polyvinyl technique was made worse by this technique.

Next a model of a cannula was obtained from Dr. Latteman in Seattle WA that was used with baboons. Dr. Latteman's model had six guide cannulas arranged in 2 rows of three. The guide cannulas were attached to a 2 cm in diameter flat round piece of stainless steel (approx. the size of a quarter) with holes around the perimeter for screws to be placed into the skull. Engineering said they could make one for something like 200 -300$. I also obtained a model from Dr. Della- Ferra at St. Louis, MO. that was used with sheep. Dr. Della-Ferra's model consisted of a single guide cannula, a luer plug with a side screw so that the depth of the ventricular needle could be changed.

Initially I tried combining Dr. Latteman's and Dr.

Della-Ferra's models. I had engineering put a 16 gauge stainless steel needle through a 2 cm in diameter round flat piece of stainless steel. The stainless steel disk had three holes to screw it to the skull. Although the screws through the disk and into the skull made the guide cannula very solid, while turning the screws in, the disk would wobble on the skull, increasing the risk of complications due to trauma. In the last experiment, I relied on the dental acrylic for holding the guide cannula.

There were in particular, 3 very expensive lessons learned during this project about the development of a new technique. First, keep the experimental turnover time as short as possible. For the first several experiments I had four months invested in a group of animals before finding out if the cannulas were in the ventricles or not. Included in keeping the experimental turnover time down are: keep the number of animals to a minimum; keep the design very simple and short; do the things that you think will cause the maximum response in the shortest time (once you know your technique works you can refine it later). Second, go the "best route first"; you can evaluate the performance of cheaper substitutes once you know how the "right stuff" works. Third, if anyone is doing anything similar, go watch them and have them watch you.

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Adolph, E.F. Urges to eat and drink in rats. Am. J. Physiol. 151:110-125; 1947.

3. Amir, S.; Lagiorgia, M.; Pollock, R. Intra-ventromedial hypothalamic injection of insulin suppresses brown fat thermogenesis in the anaesthetized rat. Brain Res. 480:340-343; 1989.

4. Arase, K.; Fisler, J.S.; Shargill, N.S.; York, D.A.; Bray, G.A. Intracerebroventricular infusions of 3-OHB and insulin in a rat model of dietary obesity. Am. J. Physiol. 255:R974-R981; 1988.

5. Banting, F.; Best, C.H.; Collip, J.B.; Macleod J.J.R.; Noble, E.C. The effect of pancreatic extract (insulin) on normal rabbits. Am. J. Physiol. 62:162-176; 1922.

6. Baskin, D.G.; Porte, D.; Guest, K.; Dorsa, D.M. Regional concentrations of insulin in the rat brain. Endocrinology 112:898-903; 1983.

7. Berelowitz, M.; Szabo, M.; Frohman, L.A.; Firestone, S.; Chu, L. Somatomedin-C mediates growth hormone negative feedback by effects on both the hypothalamus and the pituitary. Science 212:1279-1281; 1981.

8. Bergman, E.N.; Roe, W.E.; Kon, K. Quantitative aspects of propionate metabolism and gluconeogenesis in sheep. Am. J. Physiol. 211:793-799; 1966.

9. Bernardis, L.L.; McEwen, G.; Kodis, M.; Feldman, M.J. Pair-feeding of sham-operated controls to rats with dorsomedial hypothalamic lesions: new evidence for an 'organismic' set point. Behav. Brain Res. 26:99-108; 1987.

10. Bouhaddi, K.; Thomopoulos, P.; Fages, C.; Khelil, M.; Tardy, M. Insulin effect on GABA uptake in astroglial primary cultures. Neurochem. Res. 13:1119-1124; 1988.

11. Boyd, F.; Clarke, D.W.; Raizada, M.K. Insulin inhibits specific norepinephrine uptake in neuronal cultures from rat brain. Brain Res. 398:1-5; 1986.

12. Breuhaus, B.A.; Demarest, K.T.; Chimoskey, J.E. Comparison

of intracerebroventricular and intracarotid infusions of PGFE2 in conscious sheep. Am. J. Physiol. 256:R685-R693; 1989.

13. Brief, D.J.; Davis, J.D. Reduction of food intake and body weight by chronic intraventricular insulin infusion. Brain Res. Bull. 12:571-575; 1984.

14. Brockman, R.P.; Laarveld, B. Hormonal regulation of metabolism in ruminants; a review. Livest. Prod. Sci. 14:313-334; 1986.

15. Cain, D.P. Effects of insulin injection on responses of olfactory bulb and amygdala single units to odors. Brain Res. 99:69-83; 1975.

16. Carr, G.D.; White, N.M. Contributions of dopamine terminal areas to amphetamine-induced anorexia and adipsia. Pharmacol. Biochem. Behav. 25:17-22; 1986.

17. Chiodo, L.A. Dopamine-containing neurons in the mammalian central nervous system: electrophysiology and pharmacology. Neurosci. Biobehav. Rev. 12:49-91; 1988.

18. Cholewinski, A.J.; Wilkin, G.P. Peptide receptors on astrocytes:evidence for regional heterogeneity. Biochem. Soc. Trans. 16:429-432; 1988.

19. Cohn, C.; Joseph, D. Influence of body weight and body fat on appetite of "normal" lean and obese rats. Yale J. Biol. Med. 34:598-607; 1962.

20. Cserr, H. Potassium exchange between cerebrospinal fluid, plasma, and brain. Am. J. Physiol. 209:1219-1226; 1965.

21. Danguir, J. Sleep deficits in diabetic rats: restoration following chronic intravenous or intracerebroventricular infusions of insulin. Brain Res. Bull. 12:641-645; 1984.

22. Davidson, D.A.; Bohannon, N.J.; Corp, E.S.; Lattemann, D.P.; Woods, S.C.; Porte, D.; Dorsa, D.M.; Baskin, D.J. Evidence for separate receptors for insulin and insulin-like growth factor-I in choroid plexus of rat brain by quantitative autoradiography. J. Histochem. Cytochem. 38:1289-1294; 1990.

23. de Boer, G.; Trenkle, A.; Young, J.W. Glucagon, insulin, growth hormone, and some blood metabolites during energy restriction ketonemia of lactating cows. J. Dairy Sci. 68:326-337.; 1985.

24. de Jong, A.; Strubbe, J.H.; Steffens, A.B. Hypothalamic influence on insulin and glucagon release in the rat. Am. J. Physiol. 233:E380-E388; 1977.

25. Deetz, L.E.; Wangsness, P.J.; Kavanaugh, J.F.; Griel, L.C. Effect of intraportal and continuous intrajugular administration of insulin on feeding in sheep. J. Nutr. 110:1983-1991; 1980.

26. Della-Fera, M.A.; Buonomo, F.C.; Baile, C.A. Growth hormone secretory responsiveness to multiple injections of growth hormone-releasing factor in sheep. Dom. Anim. Endocrinol. 3:153-164; 1986.

27. Drewry, M.M.; Harris, R.B.S.; Martin, R.J. The effect of increased adiposity on food intake of juvenile rats. Physiol. Behav. 45:381-386; 1989.

28. Duffy, K.R.; Pardridge, W.M. Blood-brain barrier transcytosis of insulin in developing rabbits. Brain Res. 420:32-38; 1987.

29. Edholm, O.G.; Fletcher, J.G.; Widdowson, E.M.; McCance, R.A. The energy expenditure and food intake of individual men. Brit. J. Nut. 9:286-301; 1955.

30. Fahrbach, S.F.; Morrell, J.J.; Pfaff, D.W. Studies of ventromedial hypothalamic afferents in the rat using three methods of HRP application. Exp. Brain Res. 77:221-233; 1989.

31. Figlewicz, D.; Szot, P.; Greenwood, M.R.C. Insulin stimulates inositol incorporation in hippocampus of lean but not obese zucker rats. Physiol. Behav. 47:325-330; 1990.

32. Fonberg, E. Amygdala, depression and drug treatment. Acta Physiol. Hungarica 74:103-114; 1989.

33. Foster, L.A.; Ames, N.K.; Emery, R.S. Technique for continuous access to cerebrospinal fluid (CSF) in sheep to quantify peptides related to feed intake. J. Anim. Sci. 65(S1):390; 1987.

34. Fregley, M.J.; Garper, J.M.; Radford, E.P. Regulation of sodium chloride intake by rats. Am. J. Physiol. 209:287-292; 1965.

35. Giza, B.K.; Scott, T.R. Intravenous insulin infusions in rats decrease gustatory-evoked responses to sugars. Am. J. Physiol. 252:R994-R1002; 1987.

36. Goodner, C.J.; Berrie, M.A. The failure of rat hypothalamic tissues to take up labeled insulin in vivo or to respond to insulin in vitro. Endocrinol. 101:605-612; 1977.

37. Grill, H.J.; Norgren, R. The taste reactivity test. II. mimetic responses to gustatory stimuli in chronic thalamic

and chronic decerebrate rats. Brain Res. 143:281-297; 1978a.

38. Grill, H.J.; Norgren, R. Chronically decerebrate rats demonstrate satiation but not bait shyness. Science 201:267-269; 1978b.

39. Grizard, J.; Champredon, C.; Aina, E.; Sornet, C.; Debras, E. Metabolism and action of insulin and glucagon in goat during lactating and dry period. Horm. Metabol. Res. 20:71-76; 1988.

40. Guler, H.; Schmid, C.; Zapf, J.; Froesch, E.R. Effects of recombinant insulin-like growth factor I on insulin secretion and renal function in normal human subjects. Proc. Natl. Acad. Sci. 86:2868-2872; 1989.

41. Hatton. G.I.; Yang, Q.Z.; Smithson, K.G. Synaptic inputs and electrical coupling among magnocellular neuroendocrine cells. Brain Res. Bull. 20:751-755; 1988.

42. Havrankova, J.; Brownstein, M.; Roth, J. Insulin and insulin receptors in rodent brain. Diabetologia 20:268-273; 1981.

43. Havrankova, J.; Roth, J. Insulin receptors are widely distributed in the central nervous system of the rat. Nature 272:827-829; 1978.

44. Heitmann, R.N.; Sensenig, S.C.; Reynolds, C.K.; Fernandez J.M.; Dawes, D.J. Changes in energy metabolite and regulatory hormone concentrations and net fluxes across splanchnic and peripheral tissues in fed and progressively fasted ewes. J. Nutr. 116:2516-2524; 1986.

45. Hernandez, L.; Hoebel, B.G. Feeding and hypothalamic stimulation increase dopamine turnover in the accumbens. Physiol. Behav. 44:599-606; 1988.

46. Hervey, G.R. Physiological factors involved in long-term control of food intake. Internat. J. Vit. Nutr. Res. 58:477-490; 1988.

47. Hill, J.M.; Lesniak, M.A.; Pert, C.B.; Roth, J. Autoradiographic localization of insulin receptors in rat brain: prominence in olfactory and limbic areas. Neuroscience 17:1127-1138; 1986.

48. Hoebel, B.G.; Teitelbaum, P. Hypothalamic control of feeding and self-stimulation. Science 135:375-377 1962.

49. Hove, K.; Blom, A.K. Plasma insulin and growth hormone in dairy cows; diurnal variation and relation to food intake and plasma sugar and acetoacetate levels. ACTA Endocrinol.

73:289-303; 1973.

50. Ikeda, H.; West, D.B.; Pustek, J.J.; Figlewicz, D.P.; Greenwood, M.R.C.; Porte, D.; Woods, S.C. Intraventricular insulin reduces food intake and body weight of lean but not obese zucker rats. Appetite 7:381-386; 1986.

51. Jacobs, K.M.; Mark, G.P.; Scott, T.R. Taste responses in the nucleus tractus solitarius of sodium-deprived rats. J. Physiol. 406:393-410; 1988

52. Kandel, E.R. In: Principles of Neural Science. Eds Kandel, E.R. and Schwartz. 2nd ed. Elsevier New York; p17; 1985.

53. Kasuga, M.; Zick, Y.; Blithe, D.L.; Crettaz, M.; Kahn, C.R. Insulin stimulates tyrosine phosphorylation of the insulin receptor in a cell-free system. Nature 298:667-669; 1982.

54. Katoh-Semba, R.; Keino, H.; Kashiwamata, S. A possible contribution by glial cells to neuronal energy production: enzyme-histochemical studies in the developing rat cerebellum. Cell Tissue Res. 252:133-139; 1988.

55. Kennedy, G.C. The role of depot fat in the hypothalamic control of food intake in the rat. Proc. Roy. Soc. B. (London) 140:578-592; 1953.

56. King, B.M.; Dallman, M.F.; Esquerre, K.R.; Frohman, L.A. Radio-frequency vs. electrolytic VMH lesions: differential effects on plasma hormones. Am. J. Physiol. 254:R918-R924; 1988.

57. Kirchgessner, A.L.; Sclafani, A.; Nilaver, G. Histochemical identification of a PVN-hindbrain feeding pathway. Physiol. Behav. 42:529-543; 1988.

58. Lauterio, T.J.; Marson, L.; Daughaday, W.H.; Baile, C.A. Evidence for the role of insulin-like growth factor II (IGF-II) in the control of food intake. Physiol. Behav. 40:755-758; 1987.

59. Leibowitz, S.F. Hypothalamic paraventricular nucleus: interaction between α_2-noradrenergic system and circulating hormones and nutrients in relation to energy balance. 12:101-109; 1988.

60. Manin, M.; Balage, M.; Larue-Achagiotis, C.; Grizard, J. Chronic intracerebroventricular infusion of insulin failed to alter brain insulin-binding sites, food intake, and body weight. J. Neurochem. 51:1689-1695; 1988.

61. Manin, M.; Broer, Y.; Balage, M.; Rostene, W.; Grizard, J. Metabolic clearance of insulin from the cerebrospinal

fluid in the anesthetized rat. Peptides 11:5-12; 1990.

62. Mayer, J. Glucostatic mechanism of regulation of food intake. New Engl. J. Med. 249:13-16; 1953.

63. McCann, J.P.; Bergman, E.N.; Reimers, T.J. Effects of obesity and ovarian steroids on insulin secretion and removal in sheep. Am. J. Physiol. 256:E116-E128; 1989.

64. McElduff, A.; Poronnik, P.; Baxter, R.C.; Williams, P. A comparison of the insulin and insulin-like growth factor I receptors from rat brain and liver. Endocrinol. 122:1933-1939; 1988.

65. McGarry, J.D.; Foster, D.W. Regulation of hepatic fatty acid oxidation and ketone body production. Ann. Rev. Biochem. 49:395-420; 1980.

66. McGowan, M.K.; Andrews, K.M.; Kelly, J.; Grossman, S.P. Effects of chronic intrahypothalamic infusion of insulin on food intake and diurnal meal patterning in the rat. Behav. Neurosci. 104:373-385; 1990.

67. Minano, F.J.; Peinado, J.M.; Myers, R.D. Profile of NE, DA and 5-HT activity shifts in medial hypothamus perfused by 2-DG and insulin in the sated or fasted rat. Brain Res. Bull. 22:695-704; 1989.

68. Myers, R.D.; Peinado, J.M.; Minano, F.J. Monoamine transmitter activity in lateral hypothalamus during its perfusion with insulin or 2-DG in sated and fasted rat. Physiol. Behav. 44:633-643 1988.

69. Myers, R.D.; Swartzwelder, H.S.; Peinado, J.M.; Lee, T.F.; Hepler, J.R.; Denbow, D.M.; Ferrer, J.M.R. CCK and other peptides modulate hypothalamic norepinephrine release in the rat: dependence on hunger or satiety. Brain Res. Bull. 17:583-597; 1986.

70. Ono, T.; A.B. Steffens; Sasaki, K. Influence of peripheral and intracerebroventricular glucose and insulin infusions on peripheral and cerebrospinal fluid glucose and insulin levels. Physiol. Behav. 30:301-306; 1983.

71. Oomura, Y. Chemical and neuronal control of feeding motivation. Physiol. Behav. 44:555-560; 1988.

72. Oomura, Y.; Kita, H. Insulin acting as a modulator of feeding through the hypothalamus. Diabetologia 20:290-298 1981.

73. Owen, O.E.; Reichard, G.A.; Boden, G.; Shuman, C. Comparative measurements of glucose, beta-hydroxybutyrate, acetoacetate, and insulin in blood and cerebrospinal fluid

during starvation. Metabolism 23:7-14; 1974.

74. Pappenheimer, J.R.; Heisey, S.R.; Jordan, E.F.; Downer, J. deC. Perfusion of the cerebral ventricular system in unanesthetized goats. Am. J. Physiol. 203:763-774; 1962.

75. Plata-Salaman, C.R.; Oomura, Y. Effect of intra-third ventricular administration of insulin on food intake after food deprivation. Physiol. Behav. 37:735-739; 1986.

76. Plata-Salaman, C.R.; Oomura, Y; Shimizu, N. Dependence of food intake on acute and chronic ventricular administration of insulin. Physiol. Behav. 37:717-734; 1986.

77. Porte, D.; Woods, S.C. Regulation of food intake and body weight by insulin. Diabetologia 20:274-280; 1981.

78. Rechler, M.M.; Nissley, S.P. Insulin-like growth factor (IGF)/somatomedin receptor subtypes:structure, function, and relationships to insulin receptors and IGF carrier proteins. Hormone Res. 24:152-159; 1986.

79. Recio-Pinto, E.; Ishii, D.N. Insulin and related growth factors: effects on the nervous system and mechanism for neurite growth and regeneration. Neurochem. Int. 12:397-414; 1988.

80. Rinderknecht, E.; Humbel, R.E. Primary structure of human insulin-like growth factor II. FEBS Let. 89:283-287; 1978.

81. Rusk, I.N.; Cooper, S.J. The selective dopamine D1 receptor agonist SK&F 38393: its effects on palatability- and deprivation-induced feeding, and operant responding for food. Pharmacol. Biochem. Behav. 34:17-22; 1989.

82. Sakaguchi, T.; Bray, G.A.; Eddlestone, G. Sympathetic activity following paraventricular or ventromedial hypothalamic lesions in rats. Brain Res. Bull. 20:461-465; 1988

83. Sasaki, N.; Rees-Jones, R.W.; Zick, Y.; Nissley, S.P.; Rechler, M.M. Characterization of insulin-like growth factor I-stimulated tyrosine kinase activity associated with the β-subunit of type I insulin-like growth factor receptors of rat liver cells. J. Biol. Chem. 260:9793-9804; 1985.

84. Schwartz, M.W.; Sipols, A.; Kahn, S.E.; Lattemann, D.F.; Taborsky, G.J.; Bergman, R.N.; Woods, S.C.; Porte, D. Kinetics and Specificity of insulin uptake from plasma into cerebrospinal fluid. Am. J. Physiol. 259:E378-383; 1990.

85. Shemer, J.; Raizada, M.K.; Masters, B.A.; Ota, A.; Le-

Roith, D. Insulin-like growth factor I receptors in neuronal and glial cells. J. Biol. Chem. 262:7693-7699; 1987.

86. Shimazu,T.; Ogasawara, S. Effects of hypothalamic stimulation on gluconeogenesis and glycolysis in rat liver. Am. J. Physiol. 228:1787-1793; 1975.

87. Sims, E.A.H.; Goldman, R.F.; Gluck, C.M.; Horton, E.S.; Kelleher, P.C.; Rowe, D.W. Experimental obesity in man. Trans. Assoc. Am. Physicians 81:153-170; 1968.

88. Shor-Posner, G.; Grinker, J.A.; Marinescu, C.; Brown, O.; Leibowitz, S.F. Hypothalamic serotonin in the control of meal patterns and macronutrient selection. Brain Res. 17:663-671; 1986.

89. Stanley, B.G.; Schwartz, D.H.; Hernandez, L.; Hoebel, B.G.; Leibowitz, S.F. Patterns of extracellular norepinephrine in the paraventricular hypothalamus: relationship to circadian rhythm and deprivation-induced eating behavior. Life Sci. 45:275-282; 1989

90. Steffens, A.B.; Scheurink, A.J.W.; Luiten, P.G.M. Interference of the nutritional condition of the rat with peripheral glucose regulation determined by CNS mechanisms. Physiol. Behav. 35:405-410; 1985.

91. Steffens, A.B.; Scheurink, A.J.W.; Porte, D.; Woods, S.C. Penetration of peripheral glucose and insulin into cerebrospinal fluid in rats. Am. J. Physiol. 255:R200- R204; 1988.

92. Strubbe, J.H.; Mein, C.G. Increased feeding in response to bilateral injection of insulin antibodies in the VMH. Physiol. Behav. 19:309-313; 1977.

93. Tannenbaum, G.S.; Guyda, H.J.; Posner, B.I. Insulin-like growth factors: a role in growth hormone negative feedback and body weight regulation via brain. Science 220:77-79; 1983.

94. Tsujii, S.; Bray, G.A. Effects of glucose, 2- deoxyglucose, phlorizin, and insulin on food intake of lean and fatty rats. Am. J. Physiol. 258:E476-E481; 1990.

95. van Houten, M.; Posner, B.I. Cellular basis of direct insulin action in the central nervous system. Diabetologia 20:255-267; 1981.

96. Wallum, B.J.; Taborsky, G.J.; Porte, D. Jr.; Figlewicz, D.P.; Jacobson, L.; Beard, J.C.; Ward, W.K.; Dorsa, D. Cerebrospinal fluid insulin levels increase during intravenous insulin infusions in man. J. Clin. Endocrinol. Metab. 64:190-194; 1987.

97. Wilcox, B.J.; Corp, E.S.; Dorsa, D.M.; Figlewicz, D.P.; Greenwood, M.R.C.; Woods, S.C.; Baskin, D.G. Insulin binding in the hypothalamus of lean and genetically obese zucker rats. Peptides 10:1159-1164; 1989.

98. Woods, S.C.; Lotter, E.C.; McKay, L.D.; Porte, D. Chronic intracerebroventricular infusion of insulin reduces food intake and body weight of baboons. Nature 282:503-505; 1979.

99. Woods, S.C.; McKay, L.D.; Stein, L.J.; West, D.B. Insulin in the cerebrospinal fluid (CSF) of the baboon: response to feeding and intravenous (iv) infusions. Diabetes 30:119A; 1981.

100. Woods, S.C.; McKay, L.D.; Stein, L.J.; West, D.B.; Lotter, E.C.; Porte, D. Neuroendocrine regulation of food intake and body weight. Brain Res. Bull. 5(S4):1-5; 1980.

101. Woods, S.C.; Porte, D. Effect of intacisternal insulin on plasma glucose and insulin in the dog. Diabetes 24:905-909; 1975.

102. Woods, S.C.; Porte, D. Relationship between plasma and cerebrospinal fluid insulin levels of dogs. Am. J. Physiol. 233:E331-E334; 1977.

103. Woods, S.C.; Porte, D.; Bobbioni, E.; Ionescu, E.; Sauter, J.F.; Rohner-Jeanrenaud, F.; Jeanrenaud, B. Insulin: its relationship to the central nervous system and to the control of food intake and body weight. Am. J. Clin. Nutr. 42:1063-1071; 1985.

104. Wright, E.M. Secretion and circulation of the cerebrospinal fluid. In: Rodriquez E.M.; van Wimersma Greidanus, T.B. eds. Cerebrospinal Fluid (CSF) and Peptide Hormones, volume 9. Frontiers of hormone research. Basel, New York, Karger; 1982:4-15.

105. Wurtman, R.J.; Wurtman, J.J. Carbohydrate craving, obesity and brain serotonin. Appetite 7:99-103; 1986.

106. Yoshida, J.; Polley, E.H.; Nyhus, L.M.; Donahue, P.E. Labeling of nerve cells in the dorsal motor nucleus of the vagus of rats by retrograde transport of Fluoro-Gold. Brain Res. 455:1-8; 1988.