High fat diets induce a rapid loss of the insulin anorectic response in the amygdala

Stéphane Boghossian, Karalee Lemmon, MieJung Park and David A. York
Center for Advanced Nutrition, Utah State University, Logan UT 84321, USA

Running title: Dietary fats induce amygdala insulin resistance

Corresponding author: Stéphane Boghossian, PhD
Center for Advanced Nutrition
Utah State University
4715 Old Main Hill
Logan, UT 84321
USA

Phone: +1-435-797-2160
Fax: +1-435-797-1114
e-mail: stephane.boghossian@usu.edu
ICV insulin decreases food intake (FI). The central bed nucleus of the amygdala (CeA), as other regions of the brain regulating feeding behavior, expresses insulin receptors. Our objectives were to show an insulin anorectic response in the amygdala, study the effect of high fat diets on this response and map the neural network activated by CeA insulin using fos immunohistochemistry. Sprague-Dawley (SD) rats fitted with unilateral CeA cannulas were adapted to a low fat (LFD) diet before they were fed with high fat diet (HFD). Their feeding response to CeA saline or Insulin (8 mU) was tested after 24h, 72h or 7 days of HFD. In a second experiment, SD rats were fed the HFD for 3, 7 or 49 days then refed with the LFD. They were tested for their insulin response before and after HFD and every 3 days for the following weeks. Insulin tolerance tests were performed in a parallel group of rats. The CeA insulin stimulation c-Fos expression was studied to identify the distribution of activated neuronal populations. Feeding HFD for 72h or more induced a CeA, but not peripheral, insulin resistance that was slowly reversed by LFD refeeding. Duration of HFD feeding determined the time frame for reversal of the insulin resistance. CeA insulin increased fos in multiple brain regions including the arcuate/PVN region of the hypothalamus. We conclude that the amygdala may be an important site for insulin regulation of food intake and may have a significant role in determining susceptibility to HFD-induced obesity

**Key words:** Dietary fats, insulin resistance, amygdala, rat
The increasing incidence of obesity and type 2 diabetes presents a major health challenge as well as a huge economic burden upon health care systems. Understanding the etiology of this “diabesity pandemic” (45), the insulin resistance (23) and associated pathologies of the metabolic syndrome, is essential if this epidemic is to be reversed. Environmental factors, such as the lack of exercise or the excessive consumption of food, associated with modern life styles, have been linked to the development of insulin resistance. Food intake regulation and energy balance is achieved through a complex coordination of peripheral signals and central regulatory circuitry (8, 25). These processes determine the initiation, termination, size, composition and frequency of meals and the long term regulation of food intake in relation to body energy requirements. The hypothalamus has a central role in the regulation of feeding and of energy expenditure (52). It receives a variety of endocrine, neural or metabolic information, relating the current status of body energy stores and feeding activity and integrates this information to help regulate the efferent pathways and match energy intake to energy expenditure. Central to these control mechanisms are the arcuate nuclei, the paraventricular nucleus (PVN) and the lateral hypothalamus (LH) which are particularly important for the control of food intake (8, 25, 32, 52). However, many other regions of the central nervous system are involved in the regulation of energy balance and the nucleus accumbens, the amygdala, the brain stem and many others, form together with the hypothalamic areas a complex circuitry regulating all levels of ingestive behavior (9, 11). The amygdala, a brain region traditionally studied for its role in behaviors such as fear and anxiety, is becoming of interest for its role in influencing ingestive behaviors (17, 55). It has an important role in the development of conditioned taste aversion or reward responses to ingested food (18, 53, 56), both behaviors playing an important part in the non-homeostatic
regulation of feeding. It is also involved in the regulation of macronutrient selection as lesions of this part of the brain alter macronutrient selection (29). In addition, several neuropeptides/hormones administered to the central bed nucleus of the amygdala (CeA) are able to regulate the intake of palatable feed, mainly diets rich in dietary fats. A specific reduction in dietary fat intake has been observed after administration into the CeA of enterostatin (36) or melanocortin 4 agonists (4, 57), whereas mu-opioids (27, 38, 55) as well as melanocortin 4 antagonists (3, 4, 57) increase consumption of dietary fats. Intra-amygdala NPY reduces the preference for dietary fat but does not influence total caloric intake in rats given a choice of diets (48).

It appears that dietary fat may have a critical role in the development of central as well as peripheral insulin resistance. Studies have found that a short-term HFD feeding was sufficient to initiate mechanisms of insulin resistance in the liver (31, 51) and in the hypothalamus (41) whereas more prolonged exposure to HFD is necessary to induce peripheral insulin resistance. Insulin has been proposed as a long-term lipostatic regulator of energy balance through actions on the anorexic and orexigenic neurons in the arcuate nucleus (52). Despite increasing levels of leptin and insulin as body fat levels increase, they fail to prevent the development of obesity indicating the development of resistance or insensitivity to their effects (for review see (49)). Insulin receptors are present throughout the CNS (54). It is unclear whether they are involved in feeding behavior mechanisms. However, male and female NIRKO (Neuron specific Insulin Receptor Knock-Out) mice lacking insulin receptors in the CNS become sensitive to diet induced obesity with increased body fat, moderate insulin resistance or hyperinsulinemia, demonstrating a functional role of CNS insulin receptors in the regulation of body weight and energy homeostasis (12).
The purpose of the present study was to further explore the role of insulin in the control of food intake by extra-hypothalamic structures. We focused our study on the amygdala as a site for insulin effects on food intake. Our results demonstrate that insulin has a strong anorectic effect when injected into the CeA and activates a neural circuit that includes both hypothalamic and extra hypothalamic sites and that the CeA becomes rapidly insulin resistant in animals fed a high fat diet.

RESEARCH DESIGN AND METHODS

General conditions to the experiments

Animals

A total of 170 male Sprague-Dawley rats (body weight at surgery: 240-280 g; Charles River) were used in our experiments. They were individually housed in hanging wire mesh cages in a temperature- (22-24 C) and light-controlled (lights off 1900-0700 h) room. Food and water were available ad libitum. The animals were handled daily for body weight and food intake measurements in order to habituate them and minimize the stress associated with injections. The Institutional Animal Care and Use Committee of Utah State University approved the animal protocols.

Cannula implantation

Rats anesthetized with pentobarbital sodium (Nembutal; 50 mg/Kg body weight, ip) were stereotaxically implanted with one unilateral stainless steel cannula (Plastics One, Roanoke, VA) aimed to the central nucleus of the amygdala: [coordinates (AP/L/DV to bregma) -2.4/-
3.8/-6.0 mm] according to Paxinos and Watson (44) and previous experiments (36). Each cannula was secured in place with 3 anchor screws and dental acrylic and occluded with a 26 gauge wire stylet. The injector was designed to project 2 mm beyond the guide cannula tip. Each rat received an injection of the analgesic drug Carprofen (Rimadyl® 5 mg/kg, s.q.) before returning to their home cage.

**Diffusion of injected doses.**

Four male Sprague-Dawley rats were implanted with CeA indwelling cannulas as described above. After recovery, they received a 0.5 μL injection of a water-soluble dye (methylene blue solution) in the same conditions described in our experimental protocol. The rats were sacrificed 5h after the injection and brains were collected on ice. We chose to determine the diffusion of the dye at a time when we observe an effect on 2h-food intake (5h post injection). The dye solution was prepared in saline, the vehicle used for all injections, so it would have similar diffusion properties to the insulin solution used in our experiments. Brains were sectioned (50μm) 1mm rostral and caudal to the location of the cannula and injection site and the diffusion area of the dye mapped on diagrams matching the brain atlas from Paxinos and Watson (36).

**c-Fos immunochemistry.**

Rats were deeply anesthetized with sodium pentobarbital and transcardially perfused with isotonic phosphate buffered saline (PBS) followed by 4% paraformaldehyde in 0.1M phosphate buffer. The brains were removed, and coronal sections (50 μm thick) were obtained with a vibrating microtome. Tissue was processed for Fos-like
immunohistochemistry as previously described. Briefly, the brain sections were placed in 3% H$_2$O$_2$ and absolute methanol solution for 20 min, rinsed with PBS and placed in 1% gelatin / 3% normal goat serum solution for 60 min. Without rinsing, the tissue was then incubated at 4°C for 36–48 h with the primary antibody (1:20,000 c-fos polyclonal rabbit IgG (Santa Cruz, CA, USA) in 1% normal goat serum). The tissue was rinsed with PBS and processed using the standard avidin–biotin complex (ABC) procedure (Vectastain Elite, Vector Laboratories, Burlingame, CA, USA) followed by 3,3’-diaminobenzidine (DAB) with nickel chloride enhancement (Vector Laboratories) technique.

A representative section of the area of interest was selected for data analysis and an observer blind to experimental groups counted the c-Fos positive neurons defined as cells with nuclei containing reaction product that was solid black either throughout the nucleus or covered at least half of the nucleus.

**Diets**

After recovery from the cannula implant (7days), rat diets were changed from chow (11% energy as fat; 3.30 kcal/g; Harlan Teklad 8604, Madison, WI) to either a low fat or a high fat diet. The high fat (HF; 45% energy as fat; 4.73 kcal/g D12451 Research Diets, New Brunswick, NJ) and low fat (LF; 10% energy as fat; 3.85 kcal/g D12450B; Research Diets) diets were provided in individual feeding cups. For the 2-choice diet experiment, rats were adapted progressively to the new diets according to the schedule shown in Table 1. Body weight and food intake were recorded daily during the adaptation period.

**Insulin administration**
After adaptation to the diet, rats were weight matched and assigned to experimental groups. On the day of testing, three hours prior to lights off, the food cups were removed from the cages and the rats were injected into the amygdala with vehicle (saline) or insulin (Humulin®R; Eli Lilly and Co, Indianapolis IN, USA). The doses of insulin used in our experiments were selected based on previous experiments taken from the literature for icv or intra neuropil administrations (15, 16, 40, 46). Solutions were prepared fresh before injection. Fresh food was reintroduced when lights went off and food intake was recorded after 1, 2, 4, 12, 24 hours correcting for spillage. Spillage was minimal and no difference in the amount of spilled food was noticeable between groups. Insulin or vehicle was administered in 0.5 µL volume over a 1 minute time period. The injector was left in place for an additional 1 minute to prevent backflow. At the end of the experiment cannula localization was confirmed as previously described (35).

**Insulin Tolerance Test (ITT)**

In the middle of the light period, a blood sample was collected from the tail tip (0 min) of satiated rats. Insulin (0.75 U/kg) was injected intraperitoneally and additional blood samples taken at 15, 30, 60 and 120 min. Blood glucose levels were measured with a glucose meter (Glucometer Elite XL; Bayer, Elkhart, IN).

**Experiment 1: Amygdala insulin effect of food intake : dose response**

Thirty-two male Sprague-Dawley rats fed a regular chow diet were fitted with unilateral amygdala cannula and assigned to one of the experimental groups based on their BW:
Control (saline; n=8); Insulin 4mU (n=8); Insulin 8mU (n=8); Insulin 16mU (n=8). Food intake was measured on 3 hour-fasted rats as described above.

**Experiment 2: Amygdala insulin resistance induced by consumption of dietary fat.**

A. **Time course**

Thirty-two male Sprague-Dawley rats fitted with unilateral amygdala cannula were used. Sixteen were fed with a HFD and tested for their insulin (8mU) amygdala response after 24h, 72h and 7 days exposure to the HFD (HFD saline n=8; HFD Insulin n=8); the remaining 16 rats were kept on LFD and tested at the same times (LFD Saline n=8; LFD Insulin n=8). One rat from the HFD Insulin group lost his cannula after 6 days and was removed from all data analysis.

B. **Reversal of insulin resistance**

Thirty-two male Sprague-Dawley rats fitted with unilateral amygdala cannula and adapted to the LFD were weight matched and assigned to 4 experimental groups: LF (Saline n=8; Insulin n=8) and HF-3d (Saline n=8; Insulin n=8). One rat from the HF-3d saline group lost his cannula after 10 days and was removed from all analysis. Rats from the LF group were tested for their amygdala insulin (8mU) response at day 0, 3, 6, 9 and 15 while maintained on the LFD. Rats from the HF-3d group were tested at the same times, but after an initial testing for their insulin response at day 0 while still on the LFD, they were fed a HFD for 3 days and then switched back to LFD for the remaining period of the experiment.
In parallel to this experiment, 18 intact Sprague Dawley rats were tested for peripheral insulin sensitivity according to the ITT test described above. They were assigned to the same feeding schedule as described above (LF n=9; HF-3d n=9).

A second experiment was conducted where the rats were fed the HFD for 1 or 6 weeks before returning to the LFD. Thirty six rats fitted with amygdala cannulas were submitted to the protocol described above, but were exposed to the HFD for 7 days (HF-7d; Saline n=6; Insulin n=6) or 42 days (HF-42d; Saline n=6; Insulin n=6) instead of the 3 days, before being switched back to the low fat diet. The high fat diets were introduced 5 weeks apart such that all rats were switched back to the low fat diet at the same age. The response to amygdala insulin (8mU) was tested at days 0, 3, 7, 14, 21 and 28 while maintained on the LFD. A third group of rats (Saline n=6; Insulin n=6) remained on the LFD throughout the experiment. One rat from group HF-7d was removed from the experiment after it lost its cannula.

At the end of the experiment, half the rats were checked for proper cannula placement as described, and the other half were injected with saline or insulin 8mU into the amygdala. They were then deeply anaesthetized and perfused with isotonic PBS followed by 4% paraformaldehyde solution between 4 and 5 hours after the initial insulin stimulation. Brains were removed and processed for c-Fos immunohistochemistry as described above.

**Experiment 3: Effects of amygdala insulin on dietary choice**

Sixteen male Sprague-Dawley rats fitted with unilateral amygdala cannula were adapted to a 2-choice HF/LF diet according to the paradigm described (Table 1). At the end of the adaptation period, they received an injection of either saline (n=8) or insulin (n=8; 8mU) and food intake was measured over the subsequent 24 hour period as described above.
Statistical Analyses

Food intake data are expressed as mean ± SEM. BW and FI were analyzed using either two-way analysis of variance (ANOVA) with Neumann-Keuls post hoc tests or t-tests when appropriate. Significance was set at p<0.05 for all analyses.

RESULTS

Experiment 1: Dose response effect of amygdala insulin on food intake of rats fed chow diet.

Amygdala insulin at the 8 and 16 mU doses (Figure 1) induced a decrease in caloric intake as early as 1 hour (-56.2 and -52.6% respectively, p<0.05 vs. saline) after the reintroduction of food. The decrease in food intake lasted for at least 24h (-15.0 and -12.1% respectively, p<0.05 vs. saline). The 4 mU dose had no significant inhibition of food intake. Two-way ANOVA showed an effect of the dose (F(3,112)=5.037; p<0.05) with no interaction with time. Bilateral injections of insulin had an effect comparable to the unilateral injections in both the magnitude and time course of the response (data not shown). This suggested a non additive effect from the 2 amygdale. Based on this observation, we chose to use unilateral injections in all our following experiments.

Experiment 2A: Time course of the development of amygdala insulin insensitivity.

Insulin injected to SD rats fed a HFD for only 24 h decreased food intake at 2, 4, and 12h after presentation of food (Figure 2). The rats lost the sensitivity to amygdala insulin after they were fed the HFD for 72 h and 7 days. A cohort of rats tested in parallel, and kept on the
LF diet maintained their insulin response for the duration of the study (7 days). BW was not significantly different between groups even after 7 days on the HFD.

Experiment 2B: Time course of the reversal of HF diet induced amygdala insulin insensitivity.

In this experiment, one group of rats was fed with LFD throughout the experimental period and was injected with insulin (8mU) or saline in the amygdala at days 0, 3, 6, 9 and 15. They responded to insulin administration at each time point as shown on figure 3A. Amygdala insulin consistently inhibited FI by 30 to 50% (p<0.05). A second group of rats (figure 3B) were tested at day 0 while fed the LFD, showing a 50% inhibition of FI following insulin administration (p<0.05). After eating a HFD for 3 days they lost the response to amygdala insulin. When refed with the LFD and tested again for their amygdala insulin response after 3, 6 and 12 days, they regained sensitivity to amygdala insulin after 6 days of refeeding with the LFD.

In a parallel experiment, we performed an insulin tolerance test to study the peripheral insulin sensitivity of rats submitted to the same feeding schedule (Figure 5). Our results show that there was no difference in the response to the peripheral insulin tolerance test when the rats were fed with the HFD for 3 days.

We also considered the possibility that long term exposure to a HFD might impair the restoration of insulin sensitivity when returning to a LFD. Rats exposed to a HFD for 7 or 42 days were returned to the LFD and tested for their anorectic response to insulin at various subsequent time intervals. The data presented in figure 4 show that normal insulin sensitivity
was not restored 28 days after the restoration of the LFD suggesting that the length of HFD exposure determines the rate of restoration of insulin sensitivity.

The rats received 7 or 8 injections during the course of this experiment. They were carefully habituated to handling prior to the experiments. ANOVA performed on the LFD saline group showed no effect of the repeated injections on the 4h (and 24h; data not shown) caloric intake suggesting that the altered response in the HFD group was not due to an habituation to repeated injections.

Experiment 3: Effect of amygdala insulin on choice of HF and LF diets

In this experiment, SD rats adapted to a 2-choice HF/LF diet received either saline or insulin (8mU) in the amygdala. Figure 6 shows that amygdala insulin decreased the total caloric intake at 4, 12 and 24h, although the 24h decrease in food intake did not reach a significant level. While there was no significant effects in the consumption of either diet, it appear to be a differential time course, the decrease in the HFD consumption being observed at later time points (4, 12 and 24h) than the decrease in the LFD consumption (2 and 4h). Figure 7 shows the proportion of HFD and LFD selected by the animals after amygdala insulin administration. Despite a non significant decrease in the preference for HFD in the insulin treated animals at 12 and 24h, it appears that the relative intake of both diets does not change over the 24h period.

Experiment 4: Diffusion of injected doses.

Since the insulin inhibition of food intake is tested 3 hours after the insulin injections, there is concern that the insulin might diffuse to extra-amygdalar areas to have its effects. Figure 8 shows the diffusion distance of 0.5µl of an aqueous injected dye after 5 hours. This showed a
very limited diffusion within the amygdala region but no diffusion outside of this area at a
time when the insulin anorectic activity is already evident.

Experiment 5: c-Fos activation in response to amygdala insulin.
The early onset gene c-fos has been used widely to identify neuronal activation. c-Fos
staining was limited to neuronal nuclei and visible as black staining, round or oval in
appearance (Fig. 9; representative micrographs). Five hours after injection of insulin (8 mU)
onto the CeA, c-Fos expression was increased in several regions of the brain that are
involved in the regulation of feeding behavior, including the ARC, PVN and amygdala,
indicating that those areas were activated in response to amygdala insulin (Fig. 10).

DISCUSSION
The studies presented here extend our continuing attempts to identify the functional pathways
that regulate food intake, energy expenditure, adiposity or food preferences, focusing on the
role of the amygdala as a relay, modulator or source of these pathways (34-36, 47). In this
report, we present experimental support for a role of insulin in the amygdala to regulate food
intake and provide data to show that high fat diets induce a rapid loss of this insulin response
before any evident changes in peripheral insulin sensitivity.

Insulin has been described as a long term regulator of energy balance acting through a
mechanism involving the Arcuate-PVN system. Insulin given into the third ventricle, it
suppresses the orexigenic signals from the NPY/AgRP neurons and favors the anorexigenic
signals from the POMC/CART neurons resulting in an inhibition of food intake (10, 30, 52).
However, lateral intracerebroventricular infusion of insulin does not alter food intake in rodents (37). The amygdala, as part of the cortico-limbic system, is involved in the control of several cognitive and emotive functions such as learning and memory, reward, aversion, fear and anxiety (17, 19, 26, 56). Numerous neuropeptidergic systems are active in the amygdala allowing influence on ingestive behaviors. Because the amygdala, as other parts of the brain, is rich in insulin receptors (54), we investigated the effects of insulin on food intake when directly administered in the amygdala. Our results show that the CeA responds to an acute injection of insulin in a dose dependent manner to inhibit appetite for rat chow. The anorectic effect is present as early as 1h after the food was reintroduced (4 hours after insulin injection) and lasted for at least 24h. Because microinjected peptides can diffuse to areas adjoining the site of injection for their responses it is possible that the insulin responsive site is not the CeA but a different brain area. Our demonstration that the diffusion volume of 0.5 µL of a water-soluble dye was very limited and still restricted within the amygdala at a time when the insulin anorectic response was evident lends credence to the thesis that a discrete band of neural tissue encompassing the central bed nucleus of the amygdala is the locus of the amygdala insulin effect on feeding behavior observed in these experiments. In this context, our findings demonstrate for the first time the existence of neural elements in the amygdala exerting an anorectic control on food intake in response to insulin. However, the dose of insulin required to elicit the feeding response was high for an injection directly into the neuropil. At this time, to our best knowledge, it is unclear what the levels of endogenous insulin are in the brain or in the amygdala and the earlier studies reporting concentrations of 2mU/g of wet weight in the hypothalamic areas (21) or around 17 µU/mL in the CSF (42) are showing some variability probably attributed to different methods used for measurement (6).
The dose of 8mU (280 ng) that was used in our experiments seem to be much higher than these numbers and over 1000 times greater than the dose of insulin that has been shown to induce an acute short term signaling response after injections into the mediobasal hypothalamus (41). However, it is in the same range than the dose required for a feeding response to third ventricular insulin. We also note that Figlewicz and colleagues likewise found that a dose of 5mU insulin directly into the ventral tegmental area was ineffective in reducing food intake (15). There could be a number of explanations for the need for such high doses for the anorectic behavioral response compared to the lower doses that induce an acute short-lived signaling response. First, the food intake response is only observed after a delay of 3 hours from the time of injection. This suggests that continued long term stimulation of the insulin signaling pathway may be needed to induce a significant and prolonged increase in the downstream effectors sufficient to reduce food intake and that a low dose of insulin only gives a short pulsatile change in downstream effectors that is insufficient to effect a behavioral response over several hours. Alternatively, it is possible that, despite the presence of abundant insulin receptors in the amygdala, the food intake response is mediated through alternative receptors such as IGF-1 receptors.

Amygdala insulin administration resulted in significant modifications in the activity of hypothalamic areas that are involved in appetite control, as shown by c-fos immunohistochemistry. This result is consistent with the anorectic response observed after CeA insulin administration. The activation of neurons in the ARC-PVN axis suggests that the amygdala insulin inhibition of feeding might be mediated by POMC neurons. While the hypothalamus is usually associated with the metabolic regulation of food intake (homeostatic
control), it is unclear if the amygdala system has a direct or indirect (non homeostatic control) role in the regulation of appetite. The cortico-limbic system including the amygdala has recognized cognitive functions thought to be important in some aspects of feeding. These involve reward, pleasure or choice and can override the hypothalamic homeostatic control pathways. Indeed, the intake of specific nutrients including carbohydrates, fats and alcohol can be affected by neuropeptide activity within the amygdala (3, 4, 36, 47, 48, 55). The amygdala system is also well connected to other areas of the brain through numerous projections from and towards the cortex, other areas of the limbic system, areas of the hypothalamus or the brainstem (39). Thus it is unlikely that the c-fos expression induced by amygdala insulin is only limited to pathways affecting feeding and, while the amygdala-PVN pathway is affected by amygdala insulin, it is possible that other regions activated by the amygdala insulin are involved in autonomic and endocrine responses.

A second new finding is that the amygdala insulin response is modulated by diet, and that HFDs induce a loss of insulin response within 3 days. It is well established that diets with a high fat content induce weight gain, obesity and insulin resistance in rodent (22). However, while peripheral insulin resistance can be shown in animals that have significant weight gain, there is little information to show if this is a primary cause of the obesity or a condition that develops with increasing fat deposition. In our study, we observed the development of the central insulin resistance before any significant body weight gain. Others have also reported that the loss of insulin sensitivity appeared before the markers of obesity such as enlargement of abdominal fat cells, or elevation of circulating free fatty acids (5). Food intake as well as body weight are known to be affected by intracerebroventricular infusions of insulin. Acute
or chronic ventricular infusions of insulin have an anorexigenic effect when animals are fed a standard (high carbohydrates/low fat) diet (7). In contrast, other studies have shown no effect of chronic ventricular infusions of insulin in rats fed a high fat diet, concluding that the brain loses its sensitivity to insulin when fatty acid utilization is high (2). Dietary fats have been implicated in the susceptibility to develop insulin resistance. Levin and colleagues have shown that a relative insulin and leptin resistance is present in dietary susceptible rats even before introduction of high fat diets but that 3-4 weeks or longer of feeding HF diets is associated with further deterioration and complete loss of the anorectic responses to both hormones (33). A decrease in central insulin sensitivity in response to high fat diets was observed in the arcuate nucleus of both diet resistant and diet sensitive rats (14) suggesting that other brain areas may be involved in insulin’s anorectic response and that these other areas may be having a role in the development of obesity. Our data suggest the amygdala may be one such region.

Our third observation emanates from the insulin tolerance test. We show that rats submitted to a HFD for 3 days are still responding to a peripheral insulin administration in the same way as our controls LFD fed rats. The lack of development of overt peripheral insulin resistance after only 3 days of HFD exposure is not surprising and in accord with other studies that reported that insulin resistance was induced after feeding with high fat diets for several weeks (28). Recent studies by others (41), report that short term (< 3 days) exposure to HFD is sufficient to induce signaling changes consistent with the development of hepatic insulin resistance and insulin resistance mechanisms in hypothalamic areas. It is not clear, however, if these changes are sufficient to affect feeding behavior. These data taken together
suggest the development of a central/amygdala insulin resistance is established far earlier than the peripheral insulin resistance, and suggests it may have a role in the subsequent development of obesity. Further studies will have to be conducted to evaluate the effects of HFD on insulin signaling pathways within the amygdala and the impact of these changes on feeding.

When Sprague-Dawley rats were submitted to a 2-choice HF/LF diet regimen, we were unable to identify any major effect of insulin on their food preferences. Based on the previous data obtained with rats fed single diets (HF or LF only), it might have been expected that there would be no effect on food consumption since the intake of the HF diet would induce insulin insensitivity. These rats were predominantly selecting the diet rich in fat before the experiment started and where exposed to dietary fats for more than 2 weeks (adaptation period). Nevertheless, amygdala insulin induced a small but non-significant reduction in intake of both diets resulting in an overall caloric loss of appetite. The simultaneous consumption of LFD might have protected or prevented against the deleterious effect of dietary fats. Alternatively, it is possible that there is a threshold level of intake of dietary fat that was not reached by the combined intake of HF and LF diets.

The amygdala insulin resistance acquired after 3 days of HFD feeding was not immediately reversed by refeeding with a LFD. It required at least 6 days of refeeding before an anorectic effect of amygdala insulin was restored. It is well established that weight loss and/or exercise (20) will improve insulin sensitivity but life style changes are difficult to maintain and control in the long term. For example, acute physical exercise has been linked to improved
glucose homeostasis and enhanced peripheral insulin sensitivity in humans (58) and rodents, including DIO rats (50). High-fat diet consumption-related increase in the serine phosphorylation of IRS-1 is reversed by physical activity in parallel with a reduction in JNK activity. Many drugs have been developed to lower blood glucose and control type 2 diabetes (such as metformin or troglitazone). In addition, dietary supplements are being developed and thoroughly studied for their effects in improving insulin sensitivity, lowering fat accumulation, and thus reducing the risk of developing diabetes (1). However these studies are focused on peripheral insulin resistance. In our case, we observed a restoration of the response to amygdala insulin after a period close to 6 days of refeeding the LFD. However, when rats were maintained on HFDs for longer periods, the restoration of normal insulin responses was significantly delayed. Our data suggest a very rapid onset of amygdala insulin resistance when fed a HF diet but a slower restoration of normal insulin sensitivity when dietary fat is reduced that is dependent upon the time of exposure to the HFD. If this is also true in the human condition, it is of particular significance to individuals that predominantly eat high fat diets. These data suggest that the mechanisms involved in development of insulin resistance in the short term might be different from those associated with long term insulin resistance and that this could explain the differing time requirements for its reversal.

Numerous mechanisms have been proposed to explain the development of insulin insensitivity. These studies involving cell culture, peripheral tissue metabolism and central mechanisms have investigated the effects of local accumulation of fat metabolites such as triacylglycerols or ceramides, the direct effect of fatty acids on gene transcription and cell signaling and responses to inflammation, oxidative stress or activation of the endoplasmic
reticulum unfolded protein stress (UPR) response (43). The central resistance to insulin associated with high fat diet consumption, probably results from a number of effects including a reduction in insulin transport into the brain (24), reduced insulin signaling (14) and through an effect of dietary fat on the response to the downstream targets of insulin signaling such as the melanocortin responses (13). Our experiments bypassed any effect of diet on insulin transport into the brain by using direct injections onto the amygdala. At this time, we do not know if the signaling changes associated with short and long term insulin resistance in the amygdala are different or if mechanisms other than those shown in peripheral tissues are involved. In conclusion, we show that the amygdala is insulin responsive and that dietary fat induces insulin resistance in the amygdala before the onset of peripheral insulin resistance.

**Perspectives and significance.**

The main focus of this study was to investigate the interrelationship between dietary fat and insulin responsiveness in the amygdala. The data establishes that the amygdala is an insulin responsive area for the control of food intake. It also provides new insight into the role of dietary fat in regulating feeding behavior and insulin sensitivity of the amygdala. Since the pleasurable and rewarding aspects of food ingestion modulated by the cortico-limbic system are possibly more important in man than the homeostatic controls of the hypothalamus, these data might be of particular significance to our understanding of the current obesity epidemic. The rapid loss of insulin sensitivity when fed a high fat diet and only slow restoration of normal insulin sensitivity on return to a low fat diet suggests that insulin can only exert its anorectic effect when fat diets are ingested. While the level of fat necessary to induce complete insulin insensitivity is not clear, a similar situation in man might explain the failure
of long term regulation of energy balance in the face of the typical western high fat diets. The physiological importance of the insulin anorectic effects in the amygdala relative to the effects in the hypothalamus remains to be clarified. While the recent focus on hypothalamic feeding pathways has yielded great insight into the neurobiology of feeding, it may be the pathways in the corticolimbic system that are more important determinants of feeding in man.

ACKNOWLEDGEMENTS

This work was supported by funding from the Utah Science Technology and Research (USTAR) Program. Part of the results presented here has been presented in a poster entitled “Amygdala Insulin suppresses food intake” at the European Congress of Obesity 2008 meeting.
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**LEGENDS TO FIGURES**

**Figure 1:** Effect of 3 doses of amygdala insulin (4, 8, 16 mU) on food consumption (kcal) in male Sprague-Dawley rats. * p<0.05 compared to saline control group.

**Figure 2** Effect of amygdala insulin (8 mU) on food consumption (kcal) in male Sprague-Dawley rats fed either a LF or HFD for 24h, 72h or 7 days. Food intake was measured 1, 2, 4, 12 and 24 h after reintroduction of food.

The insert show that BW of the rats before insulin injections was not significantly affected by the diet. * p<0.05 compared to saline control group.

**Figure 3:** The amygdala insulin (8mU) response of rats fed LFD (A) or HFD for 3 days then refed LFD (B). The figure shows the food intake of saline (black bars) and insulin (white bars) treated rats 4h after the food was given back on the test days.

* p<0.05 compared to saline control group.

**Figure 4:** The amygdala insulin (8mU) response of rats fed LFD (A) or HFD for 1 (B) or 6 (C) weeks then refed LFD. The figure shows the food intake of saline (black bars) and insulin (white bars) treated rats 4h after the food was given back on the test days.

* p<0.05 compared to saline control group.
**Figure 5:** Insulin tolerance test on rats fed HFD for 7 days. Rats were fasted for 6 hours and then injected with 0.75U/kg body weight and tail blood sampled for assay of blood glucose at times shown.

**Figure 6:** Effects of amygdala insulin (8mU) on dietary preferences of SD male rats adapted to a 2-choice diet. Graphs represent calories consumed from the HFD (A), the LFD (B) and the total calories ingested (C) 2, 4, 12 and 24 h after reintroduction of food. *p<0.05 vs. Saline controls

**Figure 7:** Effects of amygdala insulin (8mU) on dietary preferences of SD male rats adapted to a 2-choice diet. Figure represents the HFD and LFD consumed as a percent of the total caloric intake.

**Figure 8:** Area of diffusion of a 0.5 µL bolus injection of water-soluble ink, 5h after injection. PC pyriform cortex; GP globus pallidus, BMA basomedial amygdala; VMH ventromedial hypothalamus; CeA Central nucleus amygdala; BLA basolateral amygdala

**Figure 9:** Representative micrographs showing c-Fos expression in different areas of the brain after saline (left panel) or insulin (8mU; center panel) injection into the central nucleus of the amygdala. ARC; arcuate nucleus, VMH; ventromedial hypothalamus, PVN; paraventricular nucleus of hypothalamus, AMYG; Basolateral/central amygdala nuclei. The right panel shows localization on the rat brain atlas from Paxinos and Watson (36).
Figure 10: c-Fos positive cells in brain areas after saline or insulin (8mU) injection into the amygdala. ARC; arcuate nucleus, VMH; ventromedial hypothalamus, PVN; paraventricular nucleus of hypothalamus, AMYG; Basolateral/central amygdala nuclei

*p<0.05 vs Saline
Table 1: Two-choice diet adaptation schedule.

<table>
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<th>Time</th>
<th>4 days</th>
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<th>2 days</th>
<th>2 days</th>
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<th>1 day</th>
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<tbody>
<tr>
<td>Diet</td>
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<td>HFD</td>
<td>LFD</td>
<td>HFD</td>
<td>LFD</td>
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<tr>
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<td>2</td>
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<td>2</td>
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</tr>
</tbody>
</table>

LFD, Low Fat Diet; HFD, High Fat Diet
Figure 1

Insulin Dose response

Food intake (Kcal)

Time (hours)

Saline
Insulin 4 mU
Insulin 8 mU
Insulin 16 mU
Figure 2
Figure 3

A

B

4h-food intake (kcal)

Time (days)

Saline

Insulin 8mU

LFD

HFD

LFD

**Saline**

**Insulin 8mU**
Figure 4

A

B

C

-42 -7 0 3 7 14 21 28

Time (days)

4h-food intake (kcal)

Saline

Insulin 8mU

LFD

HFD

LFD

-42 -7 0 3 7 14 21 28

Time (days)

4h-food intake (kcal)

LFD

HFD

LFD

N/D

-42 -7 0 3 7 14 21 28

Time (days)

4h-food intake (kcal)

Saline

Insulin 8mU
Figure 5

A. LFD

B. LFD-HFD

C. LFD-HFD-LFD
Figure 6

(A) HFD

(B) LFD

(C) Total Kcal
Figure 8

CeA
GP
PC
BLA
VMH
BMA
LH

Bregma -2.30 mm
Figure 9

Saline   Insulin

ARC

VMH

PVN

Cortex

Hippocampus

AMYG
Figure 10

Comparison of the number of c-fos positive neurones in different brain regions under saline and insulin conditions. The graph shows a significant increase in the number of c-fos positive neurones in the VMH and PVN regions following insulin administration, as indicated by the * symbol. No significant differences were observed in the ARC, Cortex, Hippocampus, and AMYG regions between the saline and insulin conditions.