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14. ABSTRACT
Obesity and metabolic diseases are known to be tightly linked to arousal-sleep cycles. Further, both metabolic disease and arousal are known to have significant impacts on cognitive function in humans and animals. Importantly, the armed forces represent a population at significant risk for increased stress and disrupted arousal-sleep cycles. Because the incidence of metabolic disease and obesity is increasing, even in these physically fit individuals, understanding the interactions between these systems is highly significant. Further, some anti-fatigue pharmacologies (e.g.,modafinil) are already used in military settings, though their long-term effects on metabolism or central nervous system function are not well-understood. We have completed Year 1 of the proposed funding period to assess the physiological and behavioral effects of this pharmacology on rat subjects. Our first year data demonstrate that chronic administration of intraperitoneal modafinil decreases food intake and body weight in rats. Additionally, we observed that acute central modafinil has deleterious effects on some hippocamapal-dependent forms of learning. These findins support out overall hypothesis that pharmacological activation of the central orexin system may modulate energy balance. Ongoing studies are assessing the effects of treatment on insulin sensitivity and also the effects of drug withdrawal on body weight regulation and cognitive function.

15. SUBJECT TERMS
Obesity, diabetes, insulin, orexin-A, arousal, stress, cognition.

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Introduction:

The incidence of obesity is escalating to epidemic proportions in all segments of society. Even among the military, with much higher levels of fitness than civilian populations, are experiencing a rapid increase in obesity and metabolic disease. Recent research has suggested an important connection between arousal/stress physiology and metabolism. One important basis for this connection may be the brain orexin-A system, which is also the principal target of the anti-fatigue drug modafinil. However, the specific molecular machinery remains unidentified, as do the behavioral effects of manipulating this system. **Objective/Hypotheses:** We have hypothesized that modafinil and other anti-fatigue drugs may act by modulating metabolic pathways in the central nervous system. We also hypothesize that chronic stress and disruption of arousal-sleep system leads to impaired metabolic function and increased susceptibility to obesity. Finally, we hypothesized that central metabolic pathways can be activated by foods and nutritional interventions, in lieu of pharmacological manipulation, with less risk of long-term metabolic complications. To assess these hypotheses, we are conducting several studies in rat subjects. The primary study deigns include administration of modafinil and assessment of the molecular and genetic effects (Project 1) as well as behavioral consequences (Project 2).

Body:

During the first year of funding, we have made significant progress toward the stated aims and objectives. All proposed studies for Year 1 have been completed and we detail the results and conclusions here. For each study, we list the statement of work task, specific objectives, methods employed, and results obtained. Figures referenced are presented in an appendix at the end of the report.

Project 1 Year 1 Tasks:

Task 1: Assess the metabolic effects of chronic orexin-A activation by modafinil (1-18 months)

- Assess chronic effects on food intake, energy expenditure, metabolic rate, and body adiposity.
- Measure insulin sensitivity and glucose homeostasis.
- Assess the effect of modafinil to exacerbate the metabolic effects of stress and circadian disruption.

Experiment Series 1.1 Methods

Adult, male, Long-Evans rats were used in all of these experiments. The choice of diets for these experiments was critical. In this case, we uses a diet that is 40% fat (by calories) with the predominant source of fat coming from saturated fat (1). We have a great deal of experience with this particular diet and it is much closer to a typical human diet than is standard rodent chow which is inconsistent in its ingredients and only 8% fat. Rats were given modafinil at a dose of 4.0 mg/kg once per day by oral gavage. These doses are approximately equivalent to doses used to increase performance in sleep-deprived humans. The key end points to be measured were food intake, body weight and body fat. .

Experiment Series 1.1 Results

We observed that chronic modafinil administration significantly reduced body weight and body adiposity in rats. Figure 1 depicts total body weight across days of treatment with either modafinil or vehicle. Figure 2 depicts that daily gavage with 4.0 mg/kg modafinil transiently decreased food intake. However, as noted in that figure experimental rats resumed food intake similar to that observed in control animals after 7-8 days of treatment. Figure 3 depicts body adiposity as measured by NMR. As can be seen in that figure, and consistent with patterns of food intake, modafinil treatment reduced (albeit slightly) the total level of body fat after 1 day of treatment, but there were no differences after 2 weeks dosing. Statistical significance was assess by repeated ANOVA using Tukey's HSD post-hoc tests. Asterisks depict significant differences between modafinil and vehicle-treated rats.

The next set of experiments will determine the effect of modafinil on propensity to gain weight when exposed to a high-fat diet after the modafinil exposure. Measures will include body weight, body fat and also measures of glucose tolerance as well. This is more analogous to the situations where modafinil is used in warfighters where they take the drug for some period of time in order to remain alert and then cycle off of it. Thus a key question becomes whether modafinil exposure increases the risk for metabolic disease.

Project 2 Year 1 Tasks:

Task 1: Identify and measure acute effects of modafinil on cognition and behavior (1-12 months)

- Measure effects of modafinil and orexin-A activation on key cognitive parameters: spatial memory, stress performance, motoric effects, and perception (months 1-6).
- Identify specific beneficial effects of acute delivery. Identify specific negative consequences of acute delivery (months 7-12).

Experiment Series 1.2 Methods

In the first series of experiments, rats received acute administration of modafinil (0.1 and 1.0 nmole/1 µl determined by pilot testing) or vehicle 1-hr prior to training in standard memory tasks for rodents: novel object recognition (NOR) and the passive avoidance task (details described below). In the second series of studies, we investigated the

NOR test: Rats will be injected intraperitoneally (i.p.) with modafinil or vehicle 1-hr prior to training session with two identical objects. For and 24-hr later, they are returned to the training/testing context and one of the objects is replaced with a novel object (e.g., paper weight or coffee cup). We use a digital-video based computer analysis to calculate percent time investigating the objects. Percent time at the novel object is used as an index of memory. This test requires 3 days habituation, 1 day for training and 1 day for test.

Passive avoidance test: Rats received ICV 0.1, 1.0 nmoles modafinil or vehicle 1-hr prior to training in the passive avoidance task. In this task, rats are presented with a moderate tone on one side of a conditioning chamber. When rats "escape" the presence of this tone by moving into the opposite chamber, the doorway is closed and rats receive a mild foot-shock. They are returned to the apparatus after 24 hours for testing. IN both instance, we measure the latency to enter the opposite side of the chamber to avoid the moderate tone stimulus. An increase in latency indicates memory of the footshock.

Elevated plus maze (EPM) and open-field tests: We have also administered icv (0.1 nmoles and 1.0 nmoles) modafinil or vehicle 1-hr prior to acute testing in critical measures of stress and

anxiety. The EPM and open field tests are standard measures of stress and anxiety in rats and mice. Here, we assessed the effects of modafinil to increase time spent in the closed arm of the EPM, and also decrease general activity in the open field (both are indicators of stress). An elevated plus maze constructed of 1/8" polypropylene plastic was used. Each of four arms (10 x 50 cm) is adjoined by a 10 x 10 cm intersection. The base of the maze is constructed such that the arms are elevated 50 cm above the ground. Animals will be placed in the center of the apparatus facing an open arm and allowed to freely explore the apparatus for 5 min for behavioral observation. Briefly, rats were treated ICV 1-hr prior to testing. Separate cohorts of rats were used for EPM and open-field. In both instance, tests were conducted at lights-out (the time of greatest activity for rodents) and both tests were 20 minutes in duration.

Intraventricular Cannulation. Animals were shaved and surgically prepped. A 2-cm midsaggital skin incision was made to expose the skull. Holes for anchoring screws and the cannula were drilled. A stainless steel (22 gauge, Plastics One) guide cannula extending into the third ventricle was permanently affixed to the skull by means of metal bone screws and quickly-drying dental acrylic. A removable 18 gauge obdurator sealed the guide cannula when not in use. All skull openings are sealed with dental acrylic. Gelfoam or bone wax followed by skin closure with suture. By manipulating the placement of the cannula, we can also put the cannula into specific brain regions for more local injection of substances.

Experiment Series 1.2 Results

We observed little or no significant effects of modafinil (either dose) to increase anxiety-like behavior in rats. In both EPM and open-field tests, experimental rats exhibited levels of anxiety-like behavior similar to those observed in vehicle treated rats. Specifically, acute modafinil did not increase significantly the amount of time spent in the closed arm (EPM, Figure 4) or significantly increase the time spent at boarders (open-field, Figure 5). Both of these measures are standards for assessing anxiety and stress in rats. However, we did observe a slight but significant effect of acute modafinil on hippocampal dependent recognition memory (Figure 6). The degree of specificity for hippocampus was indirectly assessed by passive avoidance fear conditioning, which is known to be an amygdala-dependent learning task.(Figure 7). The statistical significance of the data were analyzed by 1-way between-subjects ANOVA and Tukey's HSD post-hoc tests. Asterisks indicate statistically significant differences from vehicle treated rats.

Key Research Accomplishments:

- Chronic modafinil reduces body weight with a transient decrease in food intake.
- Chronic modafinil does not increase body adiposity.
- Acute ICV modafinil does not increase stress or anxiety levels in rats.
- Acute ICV modafinil may impair hippocamal- but not amygdale-dependent memory.

Reportable Outcomes:

1. Portions of the Year 1 data were presented at the 2006 annual meeting of the Society for the Study of Ingestive Behavior (Naples, FL USA).

2. Portions of the Year 1 data were presented at the 2007 annual European Winter Conference for Brain Research (Villars, Switzerland).

3. No patents or cells lines have been developed.

4. An animal model (mouse) of chronic variable stress based on the hypotheses generated here is currently under development in collaboration with Dr. James Herman (University of Cincinnati).

5. Derrick Choi, a student in the Benoit Lab, has selected orexin-A activation in stress and food intake for his Thesis project. This project will be officially proposed at the end of this calendar year.

Conclusions:

While, the studies of Year 1 were by design more descriptive than mechanistic, we were able to draw several important conclusions that will guide the execution of experiments proposed for the subsequent years' funding periods. First, we conclude that chronic intraperitoneal administration of modafinil does not increase body adiposity in rats. This was a key hypothesis for the overall proposal. Importantly, we are now assessing the effects of chronic modafinil administration *and withdrawal* on the rate of body weight gain and increased risk for the development of obesity. Second, we conclude that *acute* administration of modafinil directly into the central nervous systems *does not* in itself increase stress or anxiety-like behaviors. These data were critical for the correct interpretation of data to be colleted in experiments during the subsequent funding periods. However, we did observe a significant reduction in hippocampal-dependent memory function, in rats receiving the highest dose of ICV modafinil. These data will be conceptually replicated by ICV administration of orexin-A and also by testing in the classic Morris water maze task. These experiments are ongoing. The next phase of these studies will assess the effects of *chronic* drug-treatment an *withdrawal*.

References:

1. Woods, S.C., Seeley, R.J., Rushing, P.A., D'Alessio, D.A., and Tso, P. 2003. A controlled high-fat diet induces an obese syndrome in rats. *Journal of Nutrition* 133:1081-1087.

Appendices:

1. Supporting Data:

See attached

Figure 1. Daily modafinil administration significantly reduced body weight in rats.

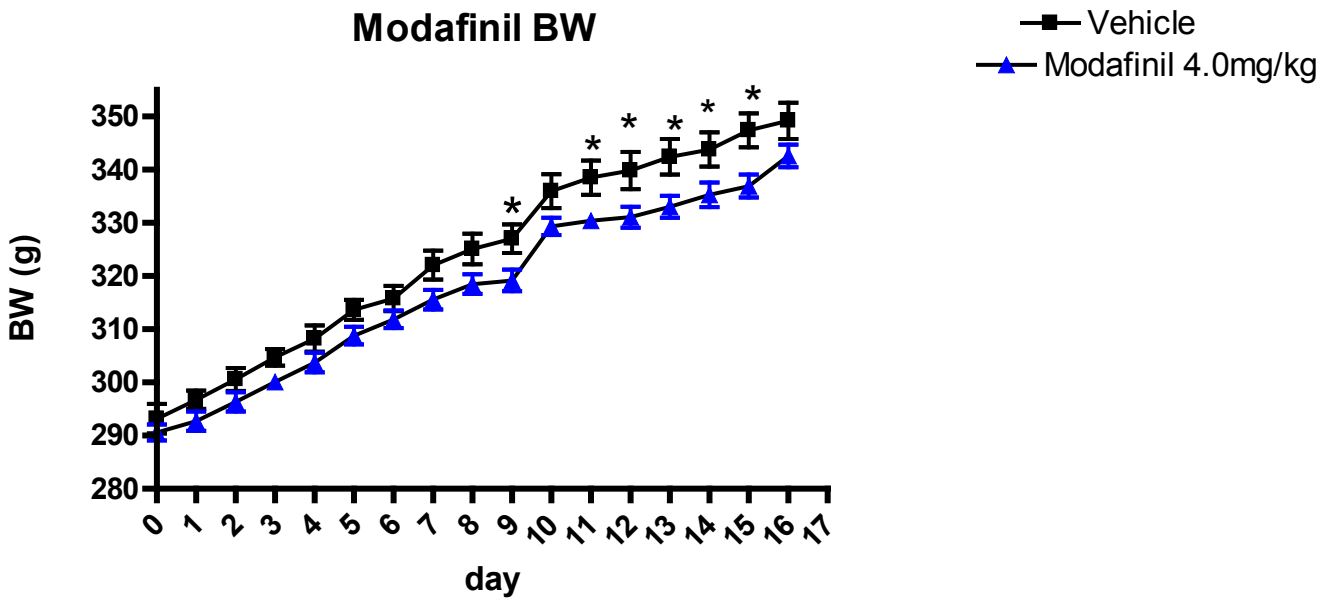


Figure 2. Daily modafinil transiently decreased cumulative food intake in rats.

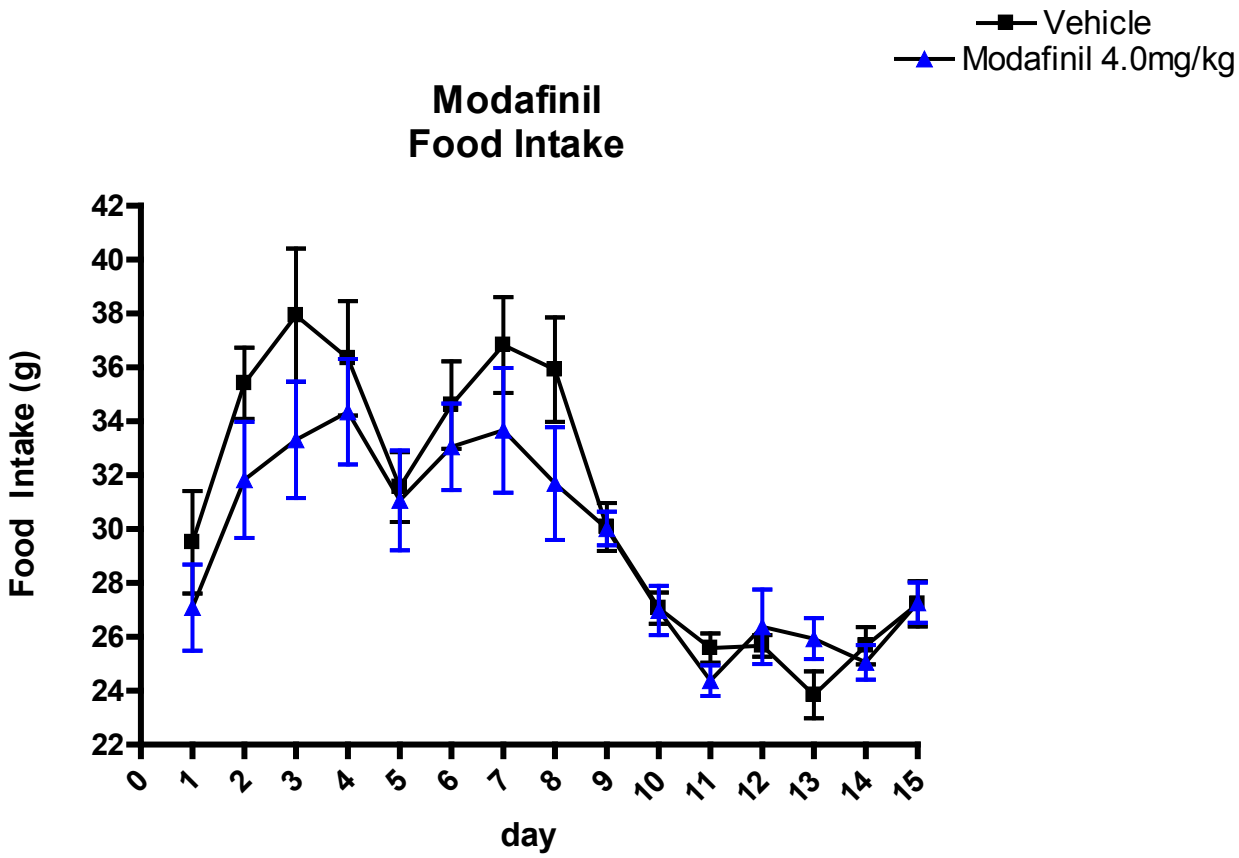


Figure 3. Chronic modafinil transiently reduced body adiposity as measured by NMR.

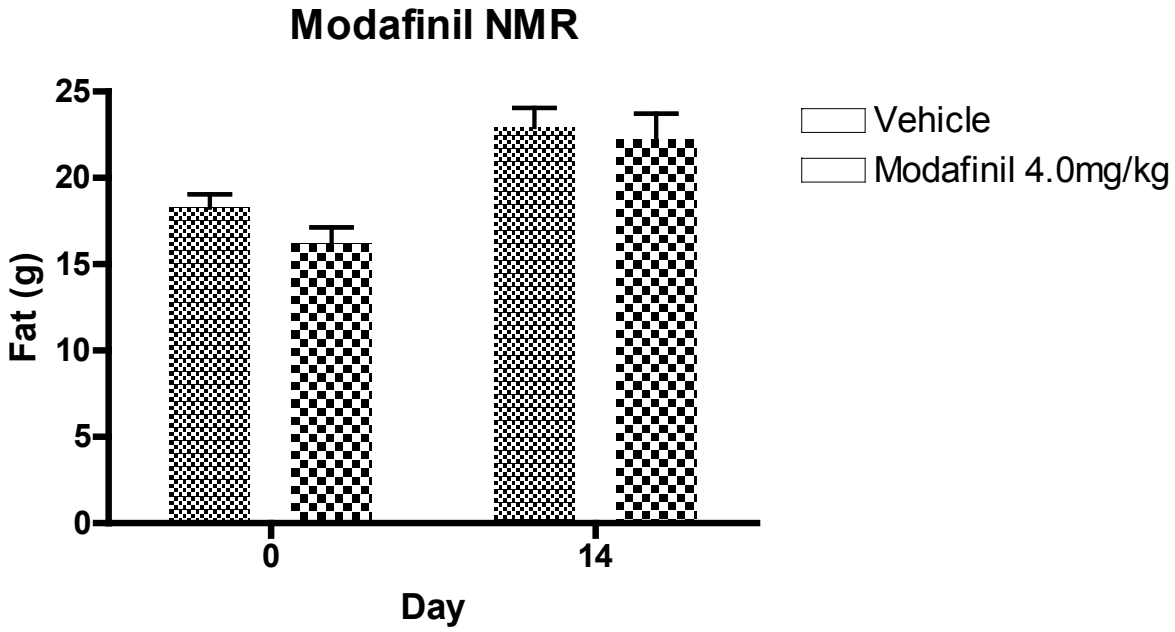


Figure 4. Acute modafinil does not significantly increase anxiety as measured by elevated plus maze (EPM) test.

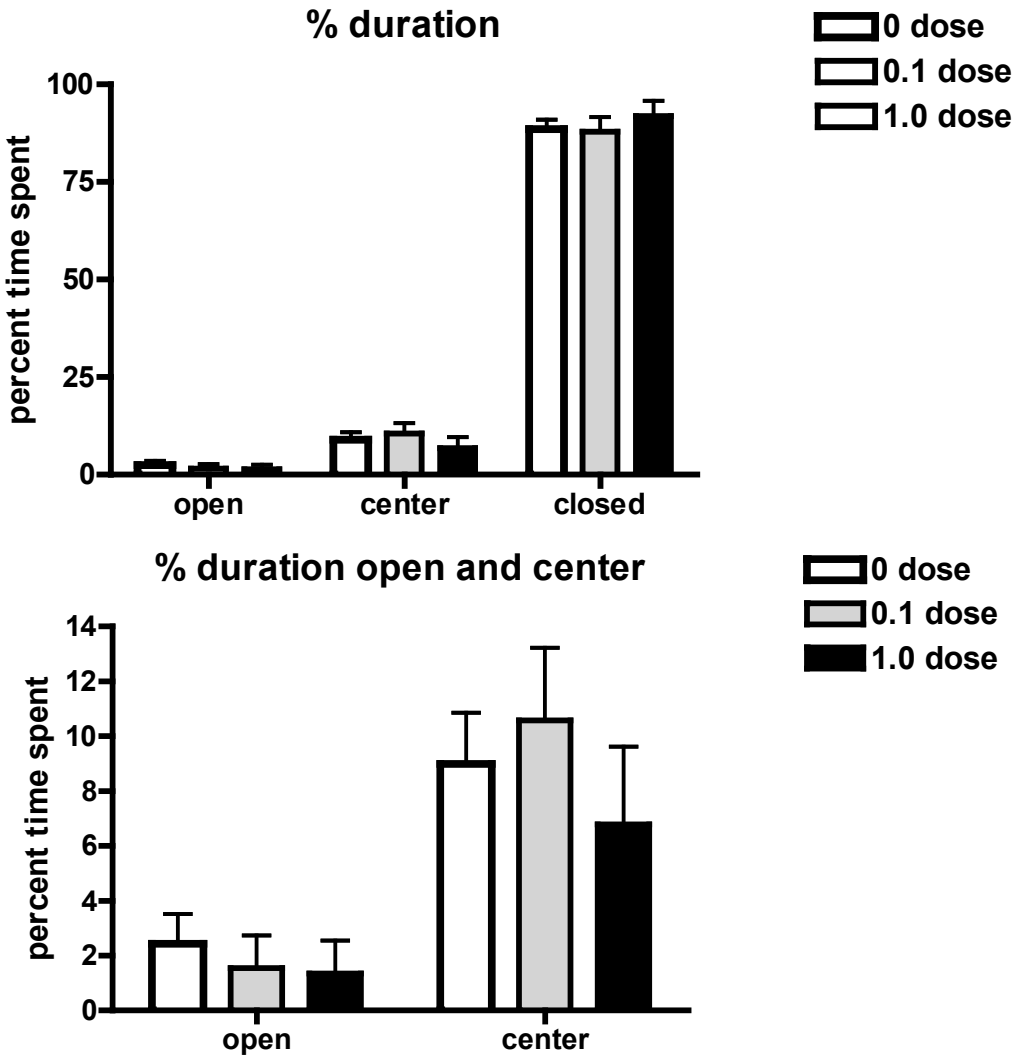


Figure 5. Acute modafinil does not significantly increase anxiety behaviors as measured by open field.

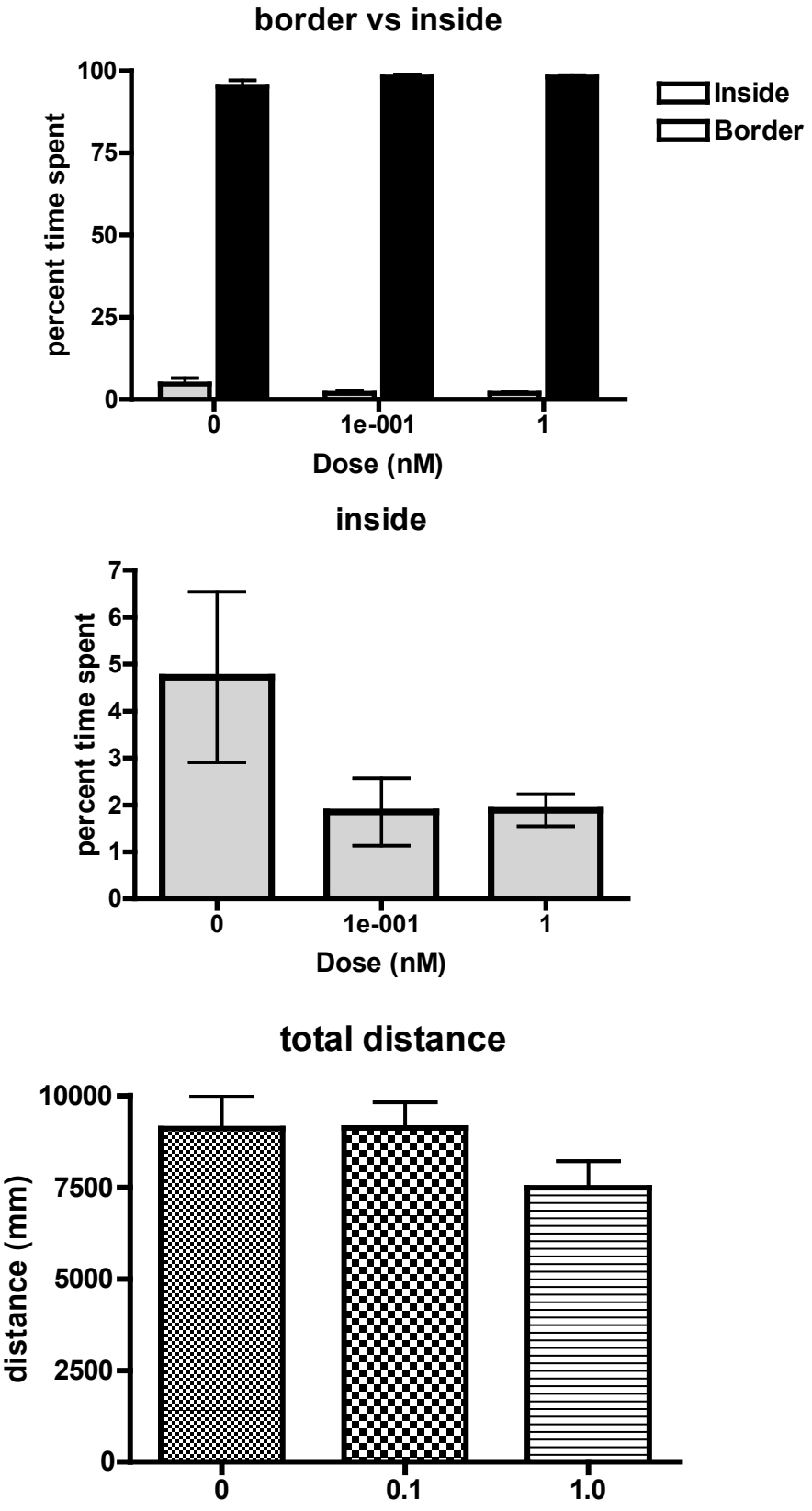


Figure 6. High dose of acute ICV modafinil significantly impairs object recognition memory.

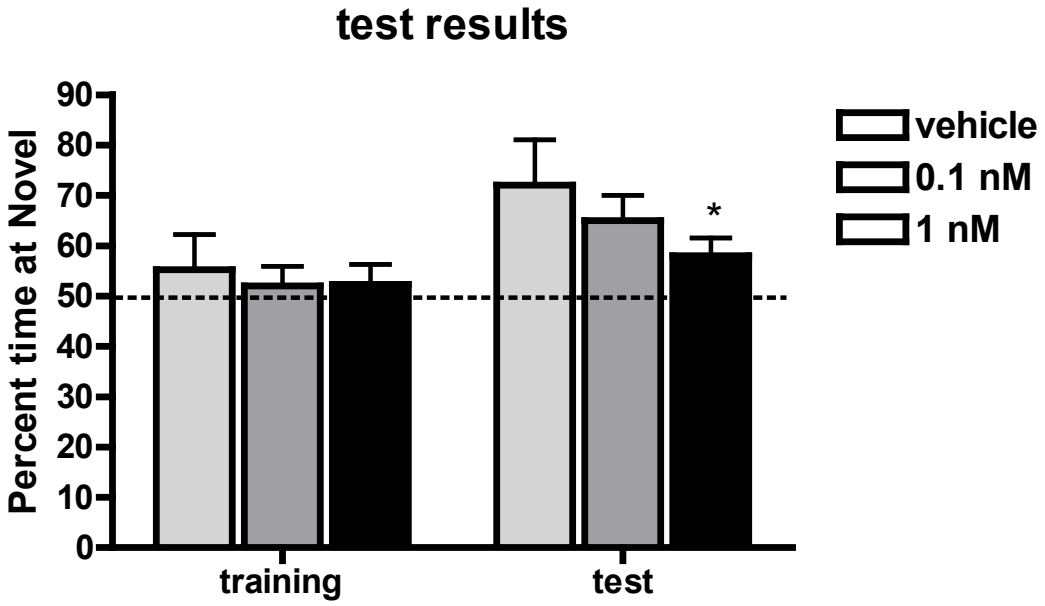


Figure 7. Acute ICV modafinil does not impair amygdale-dependent fear learning, as assayed by the passive avoidance task.

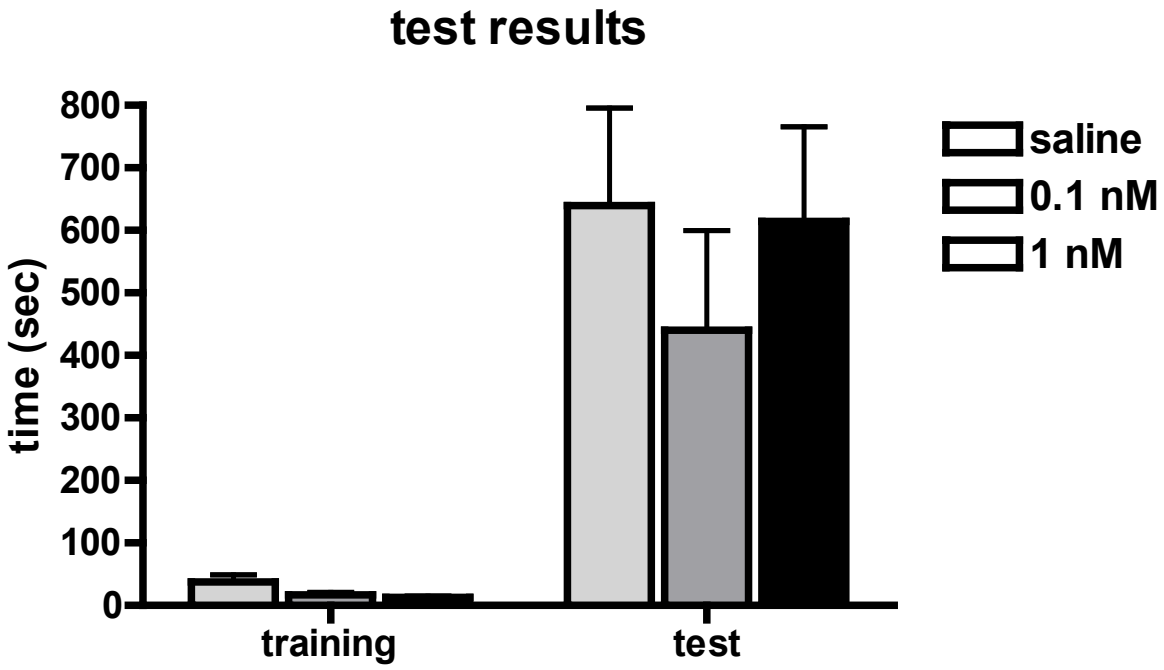


Figure 1.

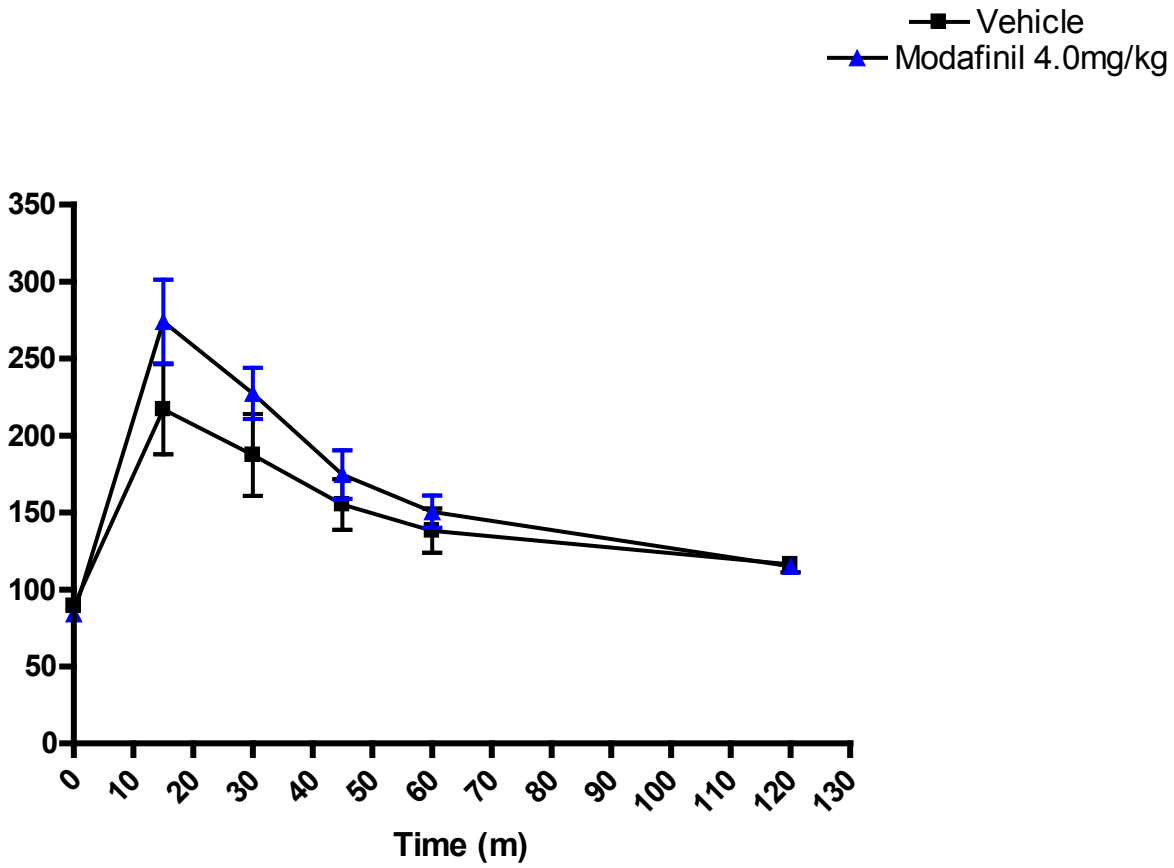

Vehicle
Modafinil 4.0mg/kg
350
300
250
200
150
100
50
0
0
10
20
30
40
50
60
70
80
90
100
110
120
130
Time (m)

Figure 2.

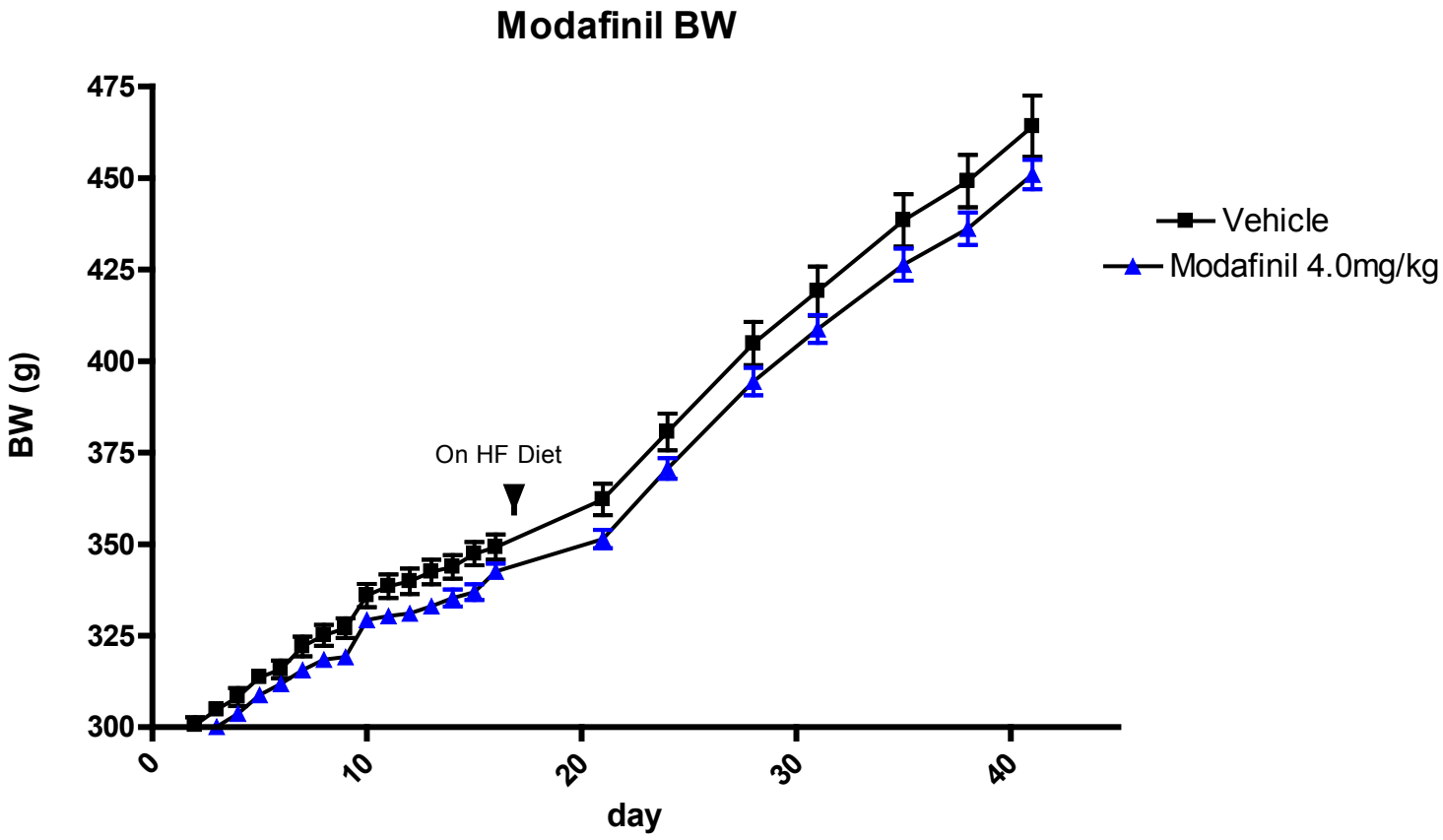

Modafinil BW
BW (g)
475
450
425
400
375
350
325
300
0
10
20
30
40
day
On HF Diet
Vehicle
Modafinil 4.0mg/kg

Figure 3.

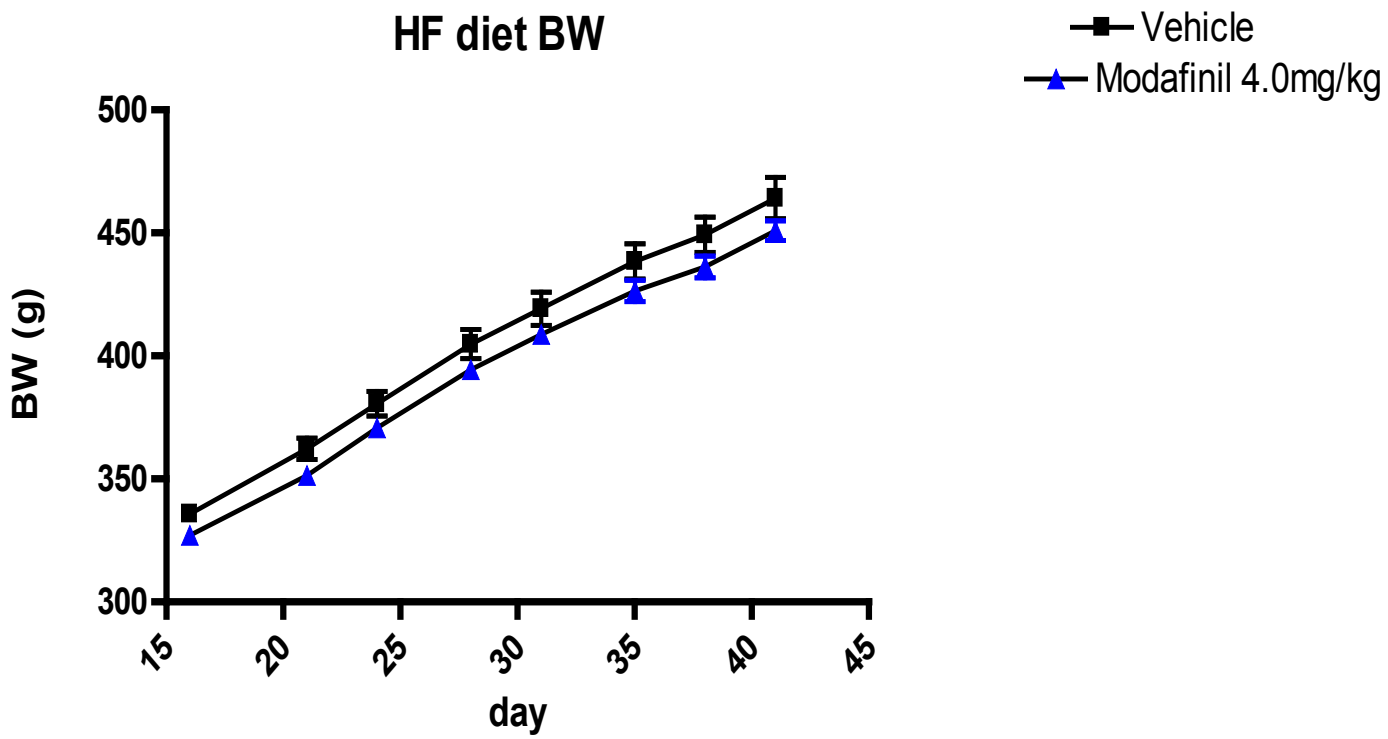

HF diet BW
Vehicle
Modafinil 4.0mg/kg
BW (g)
500
450
400
350
300
15
20
25
30
35
40
45
day

Figure 4.

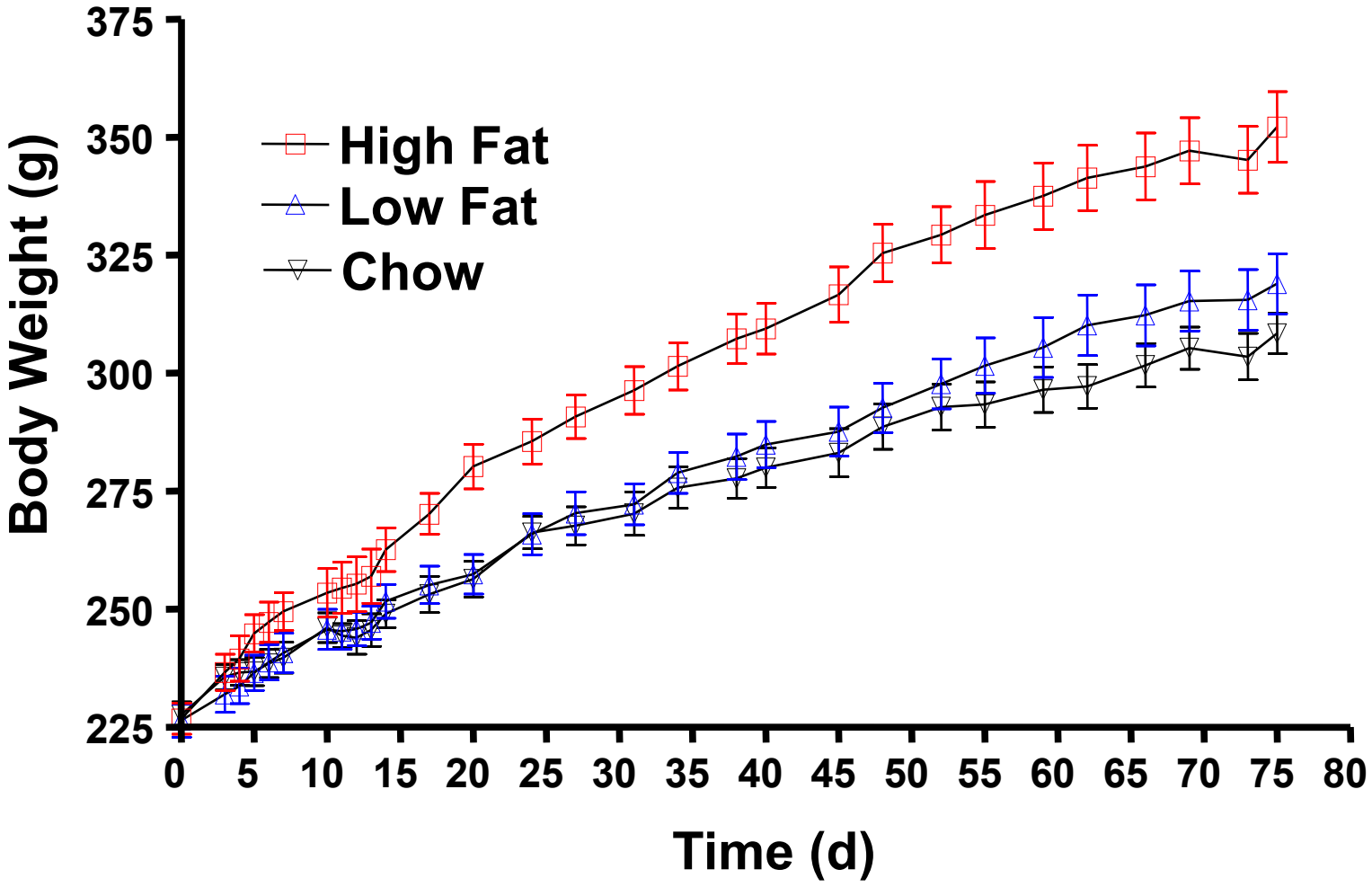

Body Weight (g)
375
350
325
300
275
250
225
High Fat
Low Fat
Chow
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
Time (d)

Figure 5.

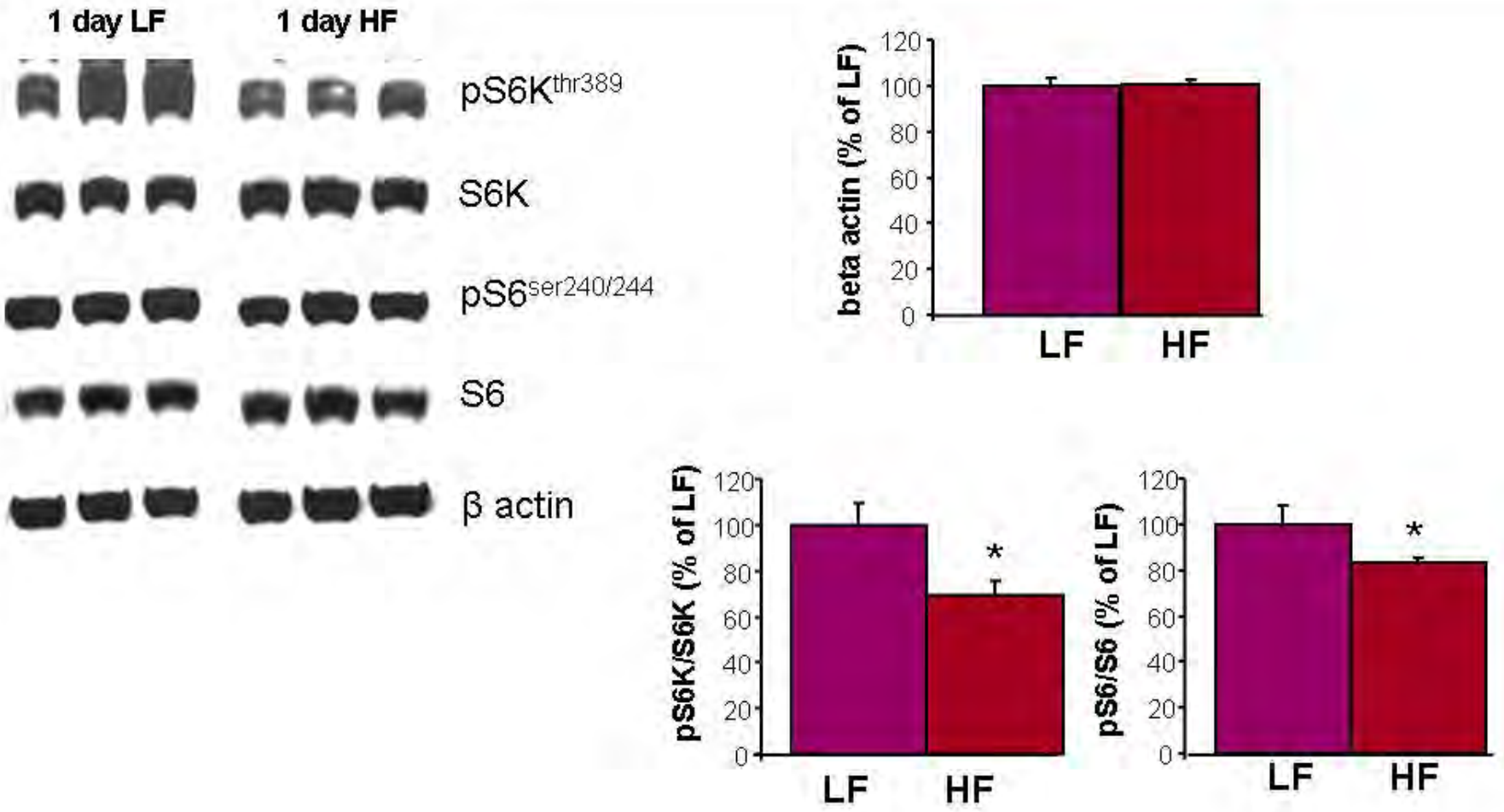

1 day LF
1 day HF
pS6K thr389
S6K
pS6 ser240/244
S6
β actin
beta actin (% of LF)
120
100
80
60
40
20
0
LF
HF
pS6K/S6K (% of LF)
pS6/S6 (% of LF)
*

Figure 6.

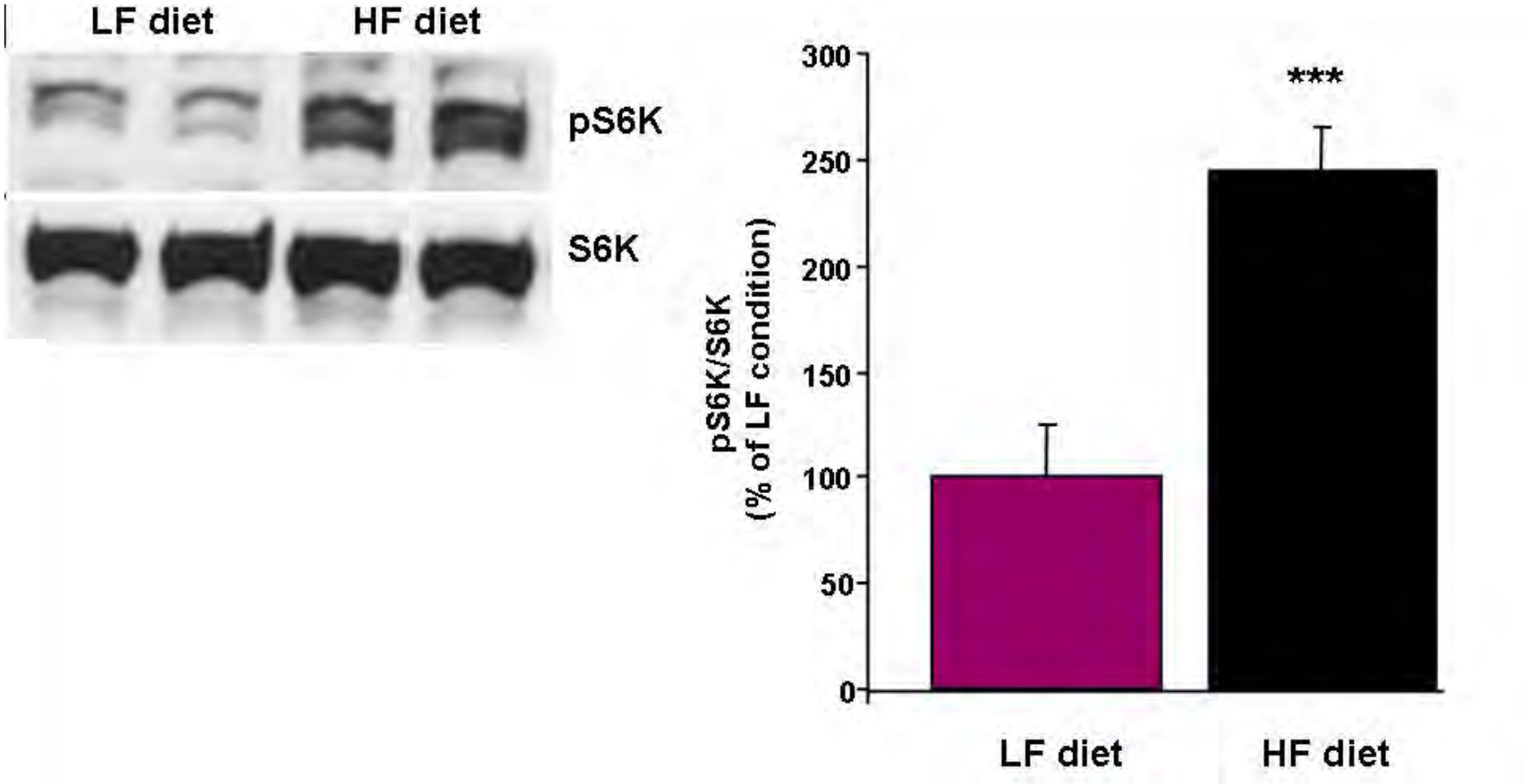

LF diet
HF diet
pS6K
S6K
pS6K/S6K
(% of LF condition)
300
250
200
150
100
50
0
***
LF diet
HF diet

Figure 7.

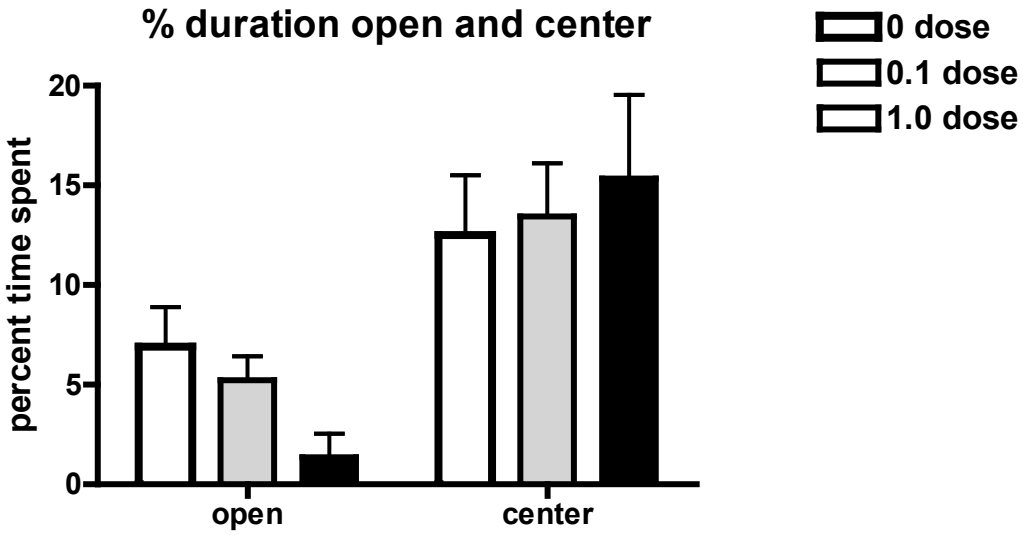
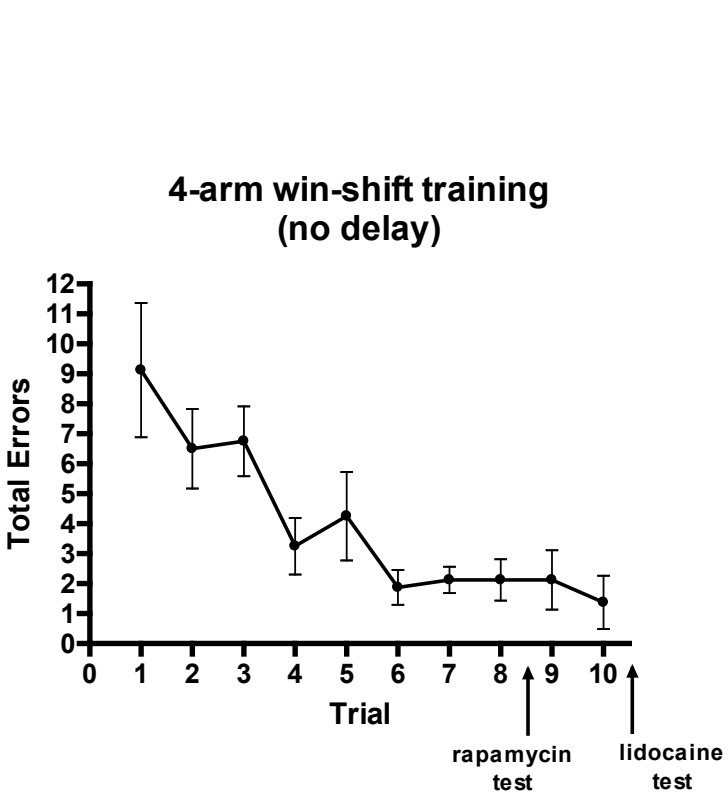
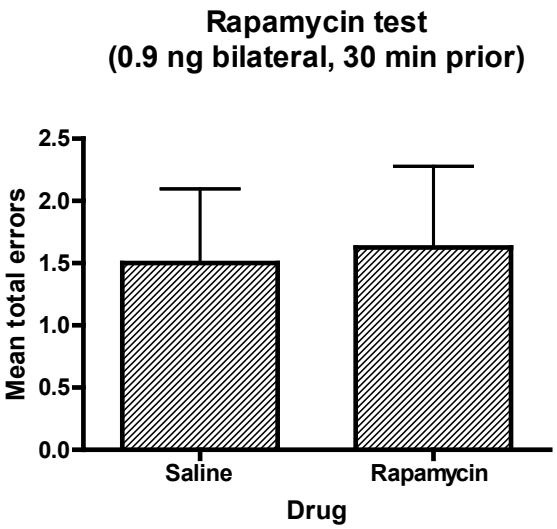

% duration open and center
percent time spent
20
15
10
5
0
open
center
0 dose
0.1 dose
1.0 dose

Figure 8.


4-arm win-shift training
(no delay)
Total Errors
0
1
2
3
4
5
6
7
8
9
10
11
12
0 1 2 3 4 5 6 7 8 9 10
Trial
rapamycin test
lidocaine test


Rapamycin test
(0.9 ng bilateral, 30 min prior)
Mean total errors
0.0
0.5
1.0
1.5
2.0
2.5
Saline
Rapamycin
Drug

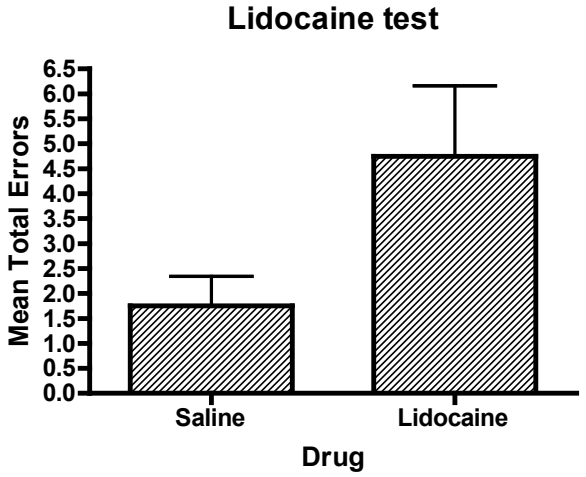
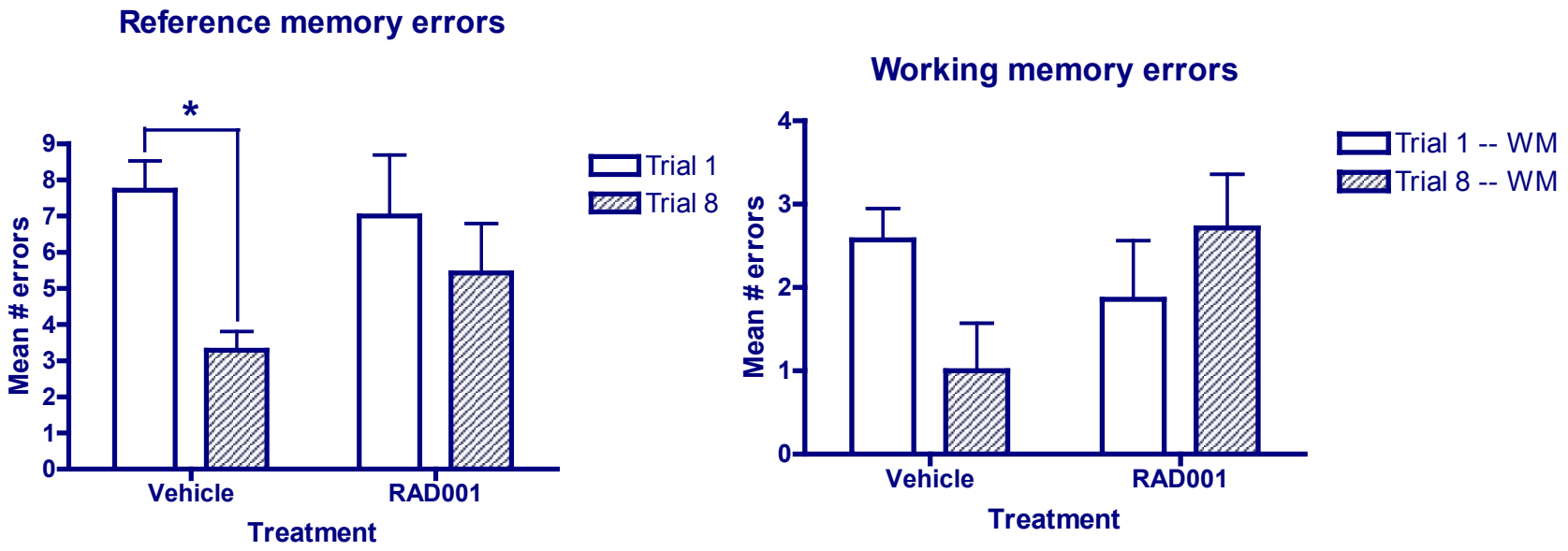

Lidocaine test
Mean Total Errors
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
Saline
Lidocaine
Drug

Figure 9.


Reference memory errors
*
Mean # errors
0
1
2
3
4
5
6
7
8
9
Vehicle
RAD001
Treatment
Trial 1
Trial 8
Working memory errors
Mean # errors
0
1
2
3
4
Vehicle
RAD001
Treatment
Trial 1 -- WM
Trial 8 -- WM

Introduction:

The incidence of obesity is escalating to epidemic proportions in all segments of society. Even among the military, with much higher levels of fitness than civilian populations, are experiencing a rapid increase in obesity and metabolic disease. Recent research has suggested an important connection between arousal/stress physiology and metabolism. One important basis for this connection may be the brain orexin-A system, which is also the principal target of the anti-fatigue drug modafinil. However, the specific molecular machinery remains unidentified, as do the behavioral effects of manipulating this system. **Objective/Hypotheses:** We have hypothesized that modafinil and other anti-fatigue drugs may act by modulating metabolic pathways in the central nervous system. We also hypothesize that chronic stress and disruption of arousal-sleep system leads to impaired metabolic function and increased susceptibility to obesity. Finally, we hypothesized that central metabolic pathways can be activated by foods and nutritional interventions, in lieu of pharmacological manipulation, with less risk of long-term metabolic complications. To assess these hypotheses, we are conducting several studies in rat subjects. The primary study deigns include administration of modafinil and assessment of the molecular and genetic effects (Project 1) as well as behavioral consequences (Project 2). During the second year of funding we have also pursued a suspected underlying metabolic pathway that might mediate the effects of nutrients on energy regulation as well as behavior. The mammalian target of Rapamycin (mTOR) kinase is a key regulator of several cellular functions, including cell growth and differentiation.

Body:

During the second year of funding, we have made significant progress toward the stated aims and objectives. All proposed studies for Year 2 have been completed and we detail the results and conclusions here. For each study, we list the statement of work task, specific objectives, methods employed, and results obtained. Figures referenced are presented in an appendix at the end of the report.

Project 1 Year 2 Tasks:

Task 1: Assess the metabolic effects of chronic orexin-A activation by modafinil (1-18 months)

- Assess chronic effects on food intake, energy expenditure, metabolic rate, and body adiposity.
- Measure insulin sensitivity and glucose homeostasis.
- Assess the effect of modafinil to exacerbate the metabolic effects of stress and circadian disruption.

Task 2: Identify metabolic pathways involved in modafinil action (19-36 months)

- Identify key nutrient sensitive molecules in orexin producing neurons.
- Determine whether nutrient sensitive pathways alter orexin neuronal activity.

Experiment Series 1.2 Methods

Adult, male, Long-Evans rats were used in all of these experiments. The choice of diets for these experiments was critical. In this case, we uses a diet that is 40% fat (by calories) with the predominant source of fat coming from saturated fat (1). We have a great deal of experience with this particular diet and it is much closer to a typical human diet than is standard rodent chow which is inconsistent in its ingredients and only 8% fat. Rats were given modafinil at a dose of 4.0 mg/kg once

per day by oral gavage. These doses are approximately equivalent to doses used to increase performance in sleep-deprived humans. The key end points to be measured were measures of glucose tolerance.

Experiment Series 1.2 Results

We observed that chronic modafinil administration significantly reduced body weight and body adiposity in rats. However, what this experiment does not tell us is whether modafinil might lead to other disruptions in metabolic functioning that might leave the warfighter using modafinil at greater risk for metabolic disease. This is particularly true because a large data base links increased orexin activity to metabolic dysfunction. Interestingly, when rats were given an IP glucose tolerance test, rats given modafinil showed a strong trend towards higher glucose levels (see Figure 1). This is particularly interesting because rats given modafinil actually had lower body weights and body fats. Thus, we would have expected glucose tolerance to actually improve in these animals making even the small observed degradation potentially quite important.

The next experiment was to test whether exposure to modafinil might result in increased susceptibility to weight gain when rats are subsequently exposed to a high-fat diet. This experiment would be analogous to a warfighter using the drug during training or combat and then potentially experiencing negative side-effects after returning to civilian life where exposure to high-fat foods are plentiful. While weight gain clearly accelerates when rats are exposed to the high-fat diet, there is no difference in weight gain between the rats that had been exposed to a high-fat diet from those that had not (see Figures 2 and 3).

Experiment Series 2.1 Methods and Results

Because hypothalamic mTORC1 signaling has been implicated as a target of leptin in the regulation of energy balance, we investigated its role in obesity-induced leptin resistance. In contrast to rats maintained on a low-fat (LF) diet for 3 weeks, rats maintained on a HF-diet had no anorexic response to icv leptin (Figure 4). Western blot analysis revealed that leptin was unable to modulate hypothalamic mTORC1 signaling in the HF group, whereas it significantly induced phosphorylation of both S6 Kinase 1 (S6K1) and S6 ribosomal protein (S6) in the LF group. Similar to leptin, the cytokine ciliary neurotrophic factor (CNTF) induces hypophagia and increases STAT3 phosphorylation. However, CNTF and its analogue CNTFAx15 activate leptin-like pathways in the hypothalamus even in leptin-resistant states, including diet-induced obesity. Icv CNTFAx15 decreased 24-h food intake and body weight in rats on HF or LF diet and increased the phosphorylation of hypothalamic S6K1 and S6 in a comparable way on both diets. Importantly, mice lacking the expression of S6K1 (S6K1-/-) did not respond to the anorectic action of either leptin or CNTFAx15, implying a crucial role for S6K1 in modulating the actions of these two cytokines. Finally, exposure to HF diet decreased mTORC1 signaling within the hypothalamus (Figure 5) and increased mTOW signaling in hippocamous (Figure 6). Overall, these findings strongly point to the possibility that reduced hypothalamic mTORC1 signaling contributes to the development of hyperphagia, weight gain and leptin resistance during diet-induced obesity.

<u>Project 2 Year 2 Tasks:</u>

Task 2: Measure cognitive and behavioral effects of chronic orexin-A activation (months 13-24)

- Assess the effects of chronic delivery of modafinil on memory, stress, motoric ability and perception (months 17-24)
- Identify and assess the effects of chronic delivery of secondary pharmacological targets from Project 1 (months 17-36).

Experiment Series 1.2 Methods

In the first series of experiments, rats received acute administration of modafinil (0.1 and 1.0 nmole/1 μl) or vehicle 1-hr prior to training in standard memory tasks for rodents: radial arm maze task and the passive avoidance task (details described below). In the second series of studies, we investigated the effects of chronic modafinil on response to stressful stimuli.

Passive avoidance test: Rats received daily ICV 0.1, 1.0 nmoles modafinil or vehicle for 2 weeks prior to training in the passive avoidance task. In this task, rats are presented with a moderate tone on one side of a conditioning chamber. When rats "escape" the presence of this tone by moving into the opposite chamber, the doorway is closed and rats receive a mild foot-shock. They are returned to the apparatus after 24 hours for testing. In both instances, we measure the latency to enter the opposite side of the chamber to avoid the moderate tone stimulus. An increase in latency indicates memory of the footshock.

Elevated plus maze (EPM): We have also administered daily ICV (0.1 nmoles and 1.0 nmoles) modafinil or vehicle for 2 weeks prior to acute testing in critical measures of stress and anxiety. The EPM test is a standard measures of stress and anxiety in rats and mice. Here, we assessed the effects of modafinil to increase time spent in the closed arm of the EPM (as an index of stress). An elevated plus maze constructed of 1/8" polypropylene plastic was used. Each of four arms (10 x 50 cm) is adjoined by a 10 x 10 cm intersection. The base of the maze is constructed such that the arms are elevated 50 cm above the ground. Animals were placed in the center of the apparatus facing an open arm and allowed to freely explore the apparatus for 5 min for behavioral observation. Tests were conducted at lights-out (the time of greatest activity for rodents) and both tests were 20 minutes in duration.

Intraventricular Cannulation. Animals were shaved and surgically prepped. A 2-cm midsaggital skin incision was made to expose the skull. Holes for anchoring screws and the cannula were drilled. A stainless steel (22 gauge, Plastics One) guide cannula extending into the third ventricle was permanently affixed to the skull by means of metal bone screws and quickly-drying dental acrylic. A removable 18 gauge obdurator sealed the guide cannula when not in use. All skull openings are sealed with dental acrylic. Gelfoam or bone wax followed by skin closure with suture. By manipulating the placement of the cannula, we can also put the cannula into specific brain regions for more local injection of substances.

Experiment Series 2.1 Methods

Working / Reference Memory Assessment: In this task, 4 of the 8 arms in an 8-arm radial arm maze are baited with food (see diagram), all arms are open for the animal to enter and remain open for the entire trial. All arms are identical, requiring the animal to utilize spatial cues external to the maze to identify and recall the location of the food. The animals were placed in the center of the maze and allowed to explore freely until all 4 food pellets have been consumed. Rats received chronic ICV infusions of the mTOR inhibitor, rapamycin or vehicle during training.

Experiment Series 1.2 Results

We observed little or no significant effects of modafinil (either dose) to increase anxiety-like behavior in rats. In the EPM test, experimental rats exhibited levels of anxiety-like behavior similar to those observed in vehicle treated rats (Figure 7). Specifically, chronic modafinil did not increase significantly the amount of time spent in the closed arm. Chronic modafinil did not impair performance in the passive avoidance test (Data not shown). The statistical significance of the data were analyzed by 1-way between-subjects ANOVA and Tukey's HSD post-hoc tests. Asterisks indicate statistically significant differences from vehicle treated rats.

Experiment Series 1.2 Results

Finally, we did observe that chronic inhibition of the mTOR pathway attenuated reference, but not working memory (Figures 8 & 9). The statistical significance of the data were analyzed by 1-way between-subjects ANOVA and Tukey's HSD post-hoc tests. Asterisks indicate statistically significant differences from vehicle treated rats.

Key Research Accomplishments:

- Chronic modafinil reduces body weight with a transient decrease in food intake.
- Chronic modafinil does not attenuate glucose tolerance or insulin sensitivity..
- Acute ICV modafinil does not increase stress or anxiety levels in rats.
- Acute ICV modafinil may impair hippocamal- but not amygdale-dependent memory.
- Nutrients acutely activate the mTOR pathways in the CNS.

Reportable Outcomes:

1. Portions of the Year 1 data were presented at the 2007 annual meeting of the Society for Neuroscience in San Deigo, CA.

2. Portions of the Year 2 data were presented at the 2008 annual European Winter Conference for Brain Research (Les Arc, France).

3. No patents or cells lines have been developed.

4. An animal model (mouse) of chronic variable stress based on the hypotheses generated here is currently under development in collaboration with Dr. James Herman (University of Cincinnati).

5. The data collected in Years 1 and 2 are contained in a manuscript that is being prepared for submission.

References:

1. Woods, S.C., Seeley, R.J., Rushing, P.A., D'Alessio, D.A., and Tso, P. 2003. A controlled high-fat diet induces an obese syndrome in rats. *Journal of Nutrition* 133:1081-1087.

Appendices:

1. Supporting Data:

See attached

Introduction:

The incidence of obesity is escalating to epidemic proportions in all segments of society. Even among the military, with much higher levels of fitness than civilian populations, are experiencing a rapid increase in obesity and metabolic disease. Recent research has suggested an important connection between arousal/stress physiology and metabolism. One important basis for this connection may be the brain orexin-A system, which is also the principal target of the anti-fatigue drug modafinil. However, the specific molecular machinery remains unidentified, as do the behavioral effects of manipulating this system. **Objective/Hypotheses:** We have hypothesized that modafinil and other anti-fatigue drugs may act by modulating metabolic pathways in the central nervous system. We also hypothesize that chronic stress and disruption of arousal-sleep system leads to impaired metabolic function and increased susceptibility to obesity. Finally, we hypothesized that central metabolic pathways can be activated by foods and nutritional interventions, in lieu of pharmacological manipulation, with less risk of long-term metabolic complications. To assess these hypotheses, we are continuing several studies in rat subjects. During the third year of funding we have also pursued a suspected underlying metabolic pathway that might mediate the effects of nutrients on energy regulation as well as behavior. The mammalian target of Rapamycin (mTOR) kinase is a key regulator of several cellular functions, including cell growth and differentiation.

Body:

During the third year of funding, we have made significant progress toward the stated aims and objectives. All proposed studies for Year 3 have been completed and we detail the results and conclusions here. For each study, we list the statement of work task, specific objectives, methods employed, and results obtained. Figures referenced are presented in an appendix at the end of the report. We note that there is overlap between Year 2 and Year 3 progress as the proposed tasks overlap these timeframes.

Project 1 Year 3 Tasks:

Task 2: Identify metabolic pathways involved in modafinil action (19-36 months)

- Identify key nutrient sensitive molecules in orexin producing neurons.
- Determine whether nutrient sensitive pathways alter orexin neuronal activity.

Experiment Series 2.1 Methods and Results

Because hypothalamic mTORC1 signaling has been implicated as a target of leptin in the regulation of energy balance, we investigated its role in obesity-induced leptin resistance. In contrast to rats maintained on a low-fat (LF) diet for 3 weeks, rats maintained on a HF-diet had no anorexic response to icv leptin (Figure 1). Western blot analysis revealed that leptin was unable to modulate hypothalamic mTORC1 signaling in the HF group, whereas it significantly induced phosphorylation of both S6 Kinase 1 (S6K1) and S6 ribosomal protein (S6) in the LF group. Similar to leptin, the cytokine ciliary neurotrophic factor (CNTF) induces hypophagia and increases STAT3 phosphorylation. However, CNTF and its analogue CNTFAx15 activate leptin-like pathways in the hypothalamus even in leptin-resistant states, including diet-induced obesity. Icv CNTFAx15 decreased 24-h food intake and body weight in rats on HF or LF diet and increased the

phosphorylation of hypothalamic S6K1 and S6 in a comparable way on both diets. Importantly, mice lacking the expression of S6K1 (S6K1-/-) did not respond to the anorectic action of either leptin or CNTFAx15, implying a crucial role for S6K1 in modulating the actions of these two cytokines. Finally, exposure to HF diet decreased mTORC1 signaling within the hypothalamus (Figure 1) and increased mTOR signaling in hippocampus (Figure 2). Overall, these findings strongly point to the possibility that reduced hypothalamic mTORC1 signaling contributes to the development of hyperphagia, weight gain and leptin resistance during diet-induced obesity.

Experiment Series 2.2 Methods and Results

In a separate set of studies, we have assessed the effects of food presentation on activation of the orexin system as well as context-based expectations of palatable foods. Briefly, rats were exposed to a novel context where they either received a palatable HF diet, no HF diet, *or in which they expected HF diet to be delivered.* They were then sacrificed by perfusion for c-fos immunohistochemistry in hypothalamus and cortical circuits. While the analyses are still underway, we have thus far observed that 1) palatable foods increase orexin neuron activation to a greater extent than do non palatable foods and 2) *even the expectation of a palatable food* increases activation of orexin expressing cells (Figure 3). Finally, we are in the process of a CNS-wide extensive quantification of regions that express fos under these conditions. We have thus far observed food and expectation-induced neuronal activation in the PFC, PVT, hypothalamus, and VTA (Figure 4). Importantly, many of the cells in these regions that express fos also express receptors for orexin. A manuscript describing portions of these data is currently in preparation.

Project 2 Year 3 Tasks:

Task 2:

- Identify and assess the effects of chronic delivery of secondary pharmacological targets from Project 1 (months 17-36).

Task 3: Assess dietary interventions (months 13-36).

- Identify any key beneficial effects of dietary activation on arousal, memory systems, stress and behavior (months 24-30).
- Compare dietary administration and activation to pharmacological interventions (months 30-36).
- Assess dietary consequences on cognitive performance and behavior (months 30-40).

Experiment Series 2.1 Secondary targets: Methods and Results

We have observed that chronic inhibition of the mTOR pathway attenuated reference, but not working memory (Figures 5 & 6). Briefly, rats were exposed to a pharmacological mTOR inhibitor (RAD, a rapamycin inhibitor) and trained in a spatial radial arm maze task. Inhibition of mTOR signaling blocked the formation of long-term memories, but had no affect on acute behavioral responses. The statistical significance of the data was analyzed by 1-way between-subjects ANOVA and Tukey's HSD post-hoc tests. Asterisks indicate statistically significant differences from vehicle treated rats.

Experiment Series 3.1 Dietary effects on behavior and cognition: Methods and Results

In collaboration with our colleague Terry L. Davidson, we have also confirmed that diets high in fatty acids exert deleterious effects on cognition. Briefly, rats were maintained on either a high-fat (40% fat by kcal) diet or standard low-fat chow. They then underwent a reversal learning paradigm in which they first leaned that on CS (light or tone) predicted the delivery of sucrose pellets and another CS (again, light or tone) meant no sucrose would be delivered. After this training, the conditions were reversed, such that the CS previously paired with sucrose was no longer followed by sucrose pellets. Control rats acquire this "reversal learning" phase without difficulty. Rats with damage to the hippocampus, however, exhibit deficits. Therefore we predicted that the HF diet would attenuate the "reversal" or this task. Indeed this was the outcome as demonstrated by Figure 7. In order to assess whether the chronic HF affected downstream targets of mTOR signaling, we performed western blots on phsophorylated S6. As depicted in Figure 8, we observed no differences in the levels of hippocampal pS6 protein. This was confirmed by immunohistochemistry for pS6 in the hippocampus (e.g., Figure 9). While disappointing, these stat are important in that they suggest a potential target lies upstream of pS6 and we are currently assaying for pS6K as well as other markers of mTOR signaling.

Experiment Series 3.2 Effects of stress and nutrients on orexin signaling: Methods and Results

In another series of experiments we have begun to assess the effects of stress on orexin signaling. Briefly, rats were first exposed to a 3-week chronic social stress, the visible burrow system (VBS). In the VBS, male rats develop a dominance hierarchy with some rats becoming "dominant" and some becoming "submissive." Both DOM and SUB rats exhibited altered HPA axis function relative to home-cage controls as has been previously published. Also consistent with previous reports, we found that DOM rats spent a greater amount of time in the open-arms of an elevated plus maze (Figure 10, left panel). However, we also observed that DOM rats exhibited significantly increased motivation to obtain a palatable food (Figure 10, right panel). Further, we observed that DOM rats have significantly increased expression orexin mRNA and also orexin-1 receptor in the pre-frontal cortex (Figure 11). These novel findings have recently been accepted for publication in *Neuroscience.*

Experiment Series 3.2 Comparison of pharmacological and dietary interventions: Methods and Preliminary Results

We are in the process of completing analyses from rats that have been maintained on several different diets (i.e., a 40% fat diet, low or high-protein diets, and standard lab chow). We are assaying brains from these rats for expression of orexin, orexin-receptor and genes related to the mTOR signaling pathways. Importantly, we are in the process of comparing body weight and behavioral activity responses of these rats to rats treated chronically with the orexin-receptor antagonist or rapamycin. The analyses are expected to be complete within the next 2-3 months.

Key Research Accomplishments:

- Dietary nutrients and the amino acid leucine specifically acutely activate the mTOR pathways in the hypothalamus
- Context and memory dependent expectation of nutrients and palatable food activates lateral hypothalamic orexin neurons.
- Context and memory dependent expectation of nutrients and palatable food also activates a network of cortical circuits critical for memory and cognition.
- Pharmacological manipulation of the orexin system alters non-homeostatic ingestive behaviors.
- Chronic stress up-regulates expression of orexin and orexin-receptor mRNA.
- Orexin activation may mediate some of the behavioral and cognitive responses following chronic stress exposure.

Reportable Outcomes:

Manuscripts

1. Davis JF, Krause EG, Melhorn SJ, Sakai RR, Benoit SC (in press). Dominant rats display increased risk taking and augmented operant responding. *Neuroscience.*
2. Choi DL, Davis JF, Sakai RR, Benoit SC (in prep). Context-dependent activation of orexin neurons in food intake and stress.

Published abstracts

3. Tracy AL, Krueger DA, Clegg DJ, Seeley RJ, Benoit SC (2008). The role of mammalian target of rapamycin (mTOR) in nutrient-mediated long-term memory processes. Society for Neuroscience.
4. Choi DJ, Davis JF, Benoit SC (2008). Orein-1 receptor blockade attenuates non-homeostatic food intake. Society for Neuroscience.
5. Choi DL, Davis JF, Benoit SC (2009). Context-dependent expectation of food activates the orexin system. Society for the Study of Ingestive Behavior, to appear in *Appetite.*

Meeting presentations

6. Choi DL, Davis JF, Schurdak JD, Clegg DJ, Benoit SC (2008). Role for orexin-A signaling on agouti-related peptide-induced hyperphagia. Cincinnati Neurofest.

Conclusions:

We have concluded that mTOR likely plays an important role in cognitive behaviors and long-term memory formation. Further, we have concluded that access to palatable foods increased orexin activation as well as activation of orexin-target neurons. We are in the process of assessing whether orexin directly activates mTOR signaling or whether these are parallel cellular events. Important, we have also concluded that orexin plays a role in the response to chronic social stress and may mediate the effects of chronic stress on ingestive behaviors. That is, we are beginning to understand that orexin and the cell-signaling molecule, mTOR play important roles in these cognitive and behavioral outcomes. Both can be manipulated by nutrients, both are responsive to stress and both may prove useful targets for pharmacological or nutrient-related treatments for improving cognitive performance in the face of stress and metabolic challenges.

References:

n/a

Appendices:

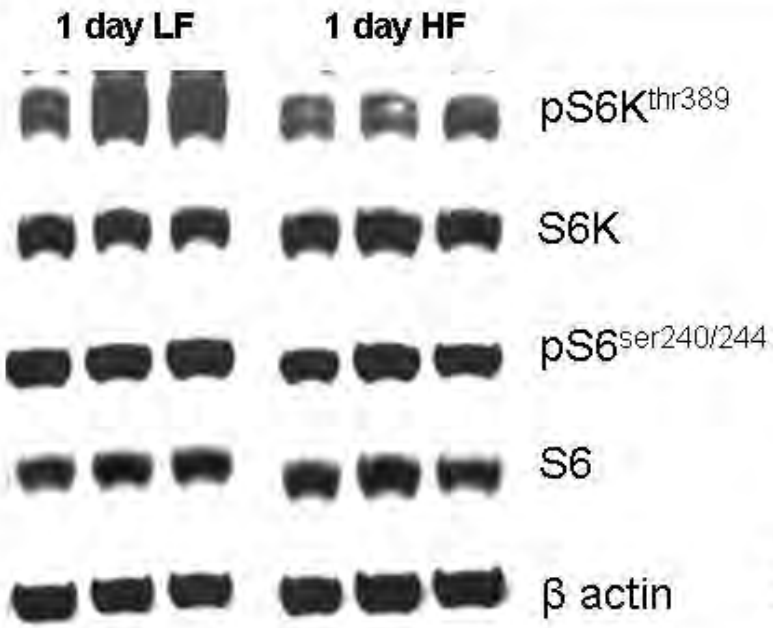
n/a

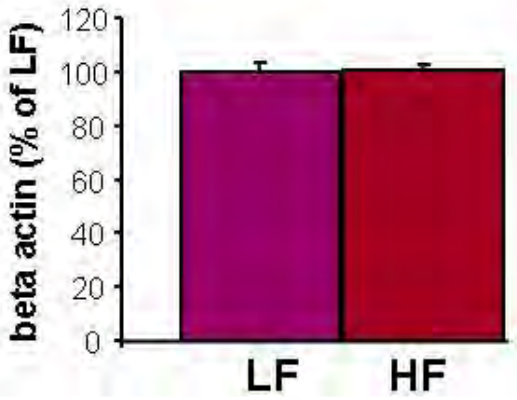
Supporting Data:

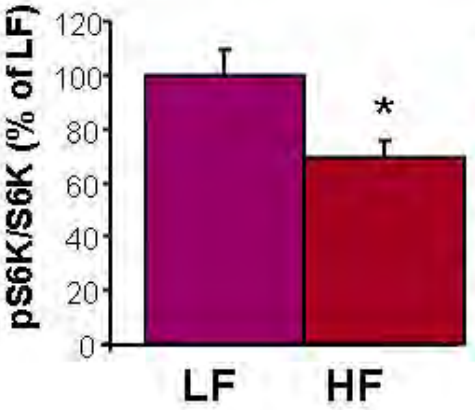
See next pages.

Figure 1.

Benoit, Stephen C., Ph.D.







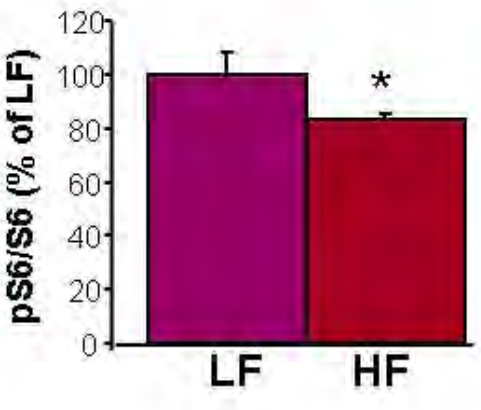


Figure 2.

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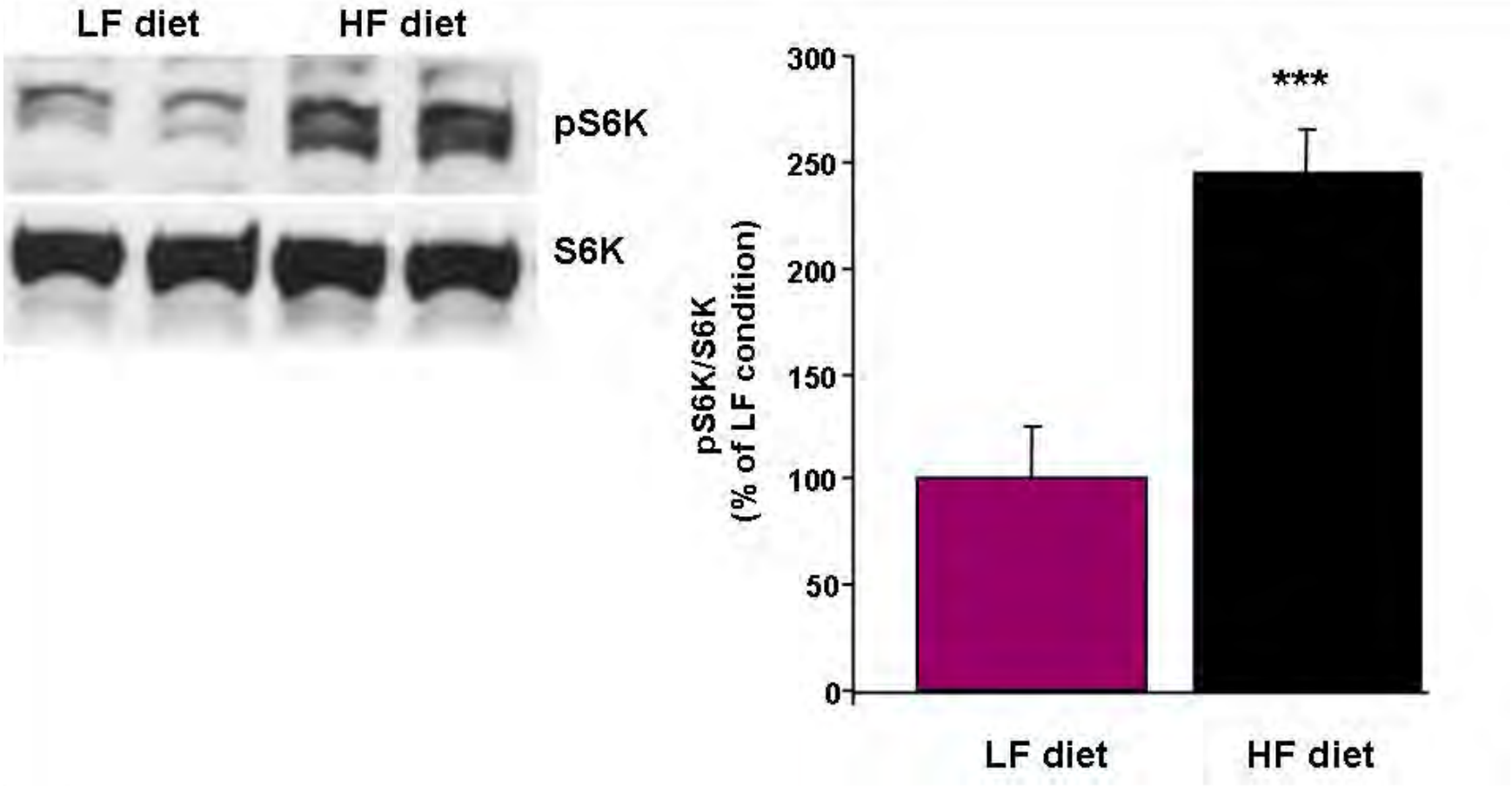

Figure 3.

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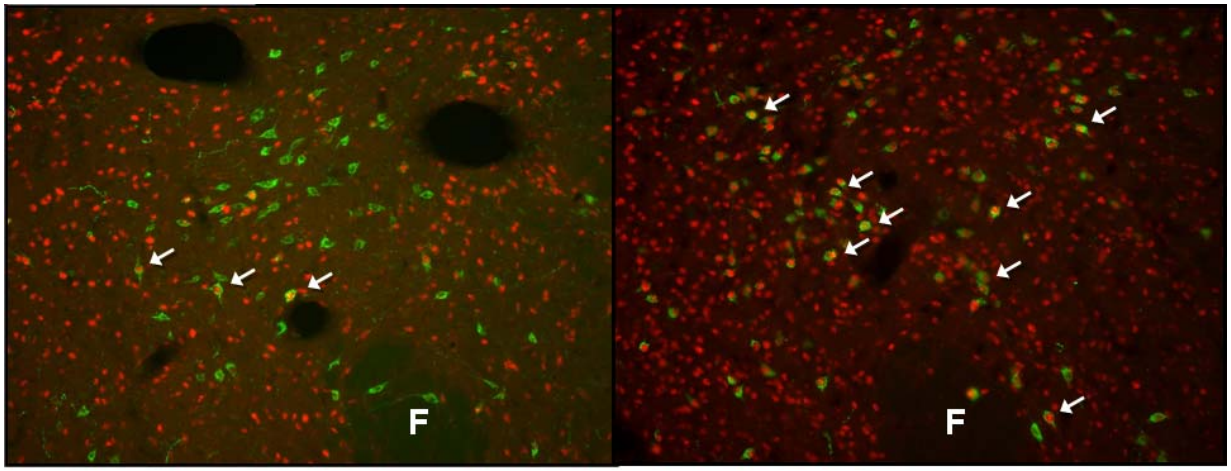

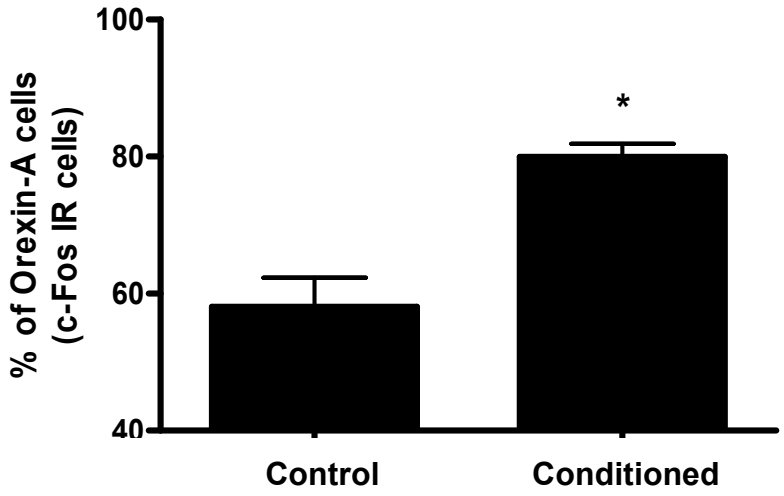

Figure 4.

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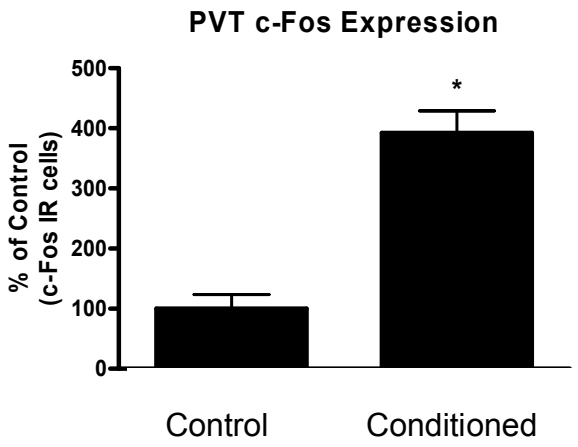

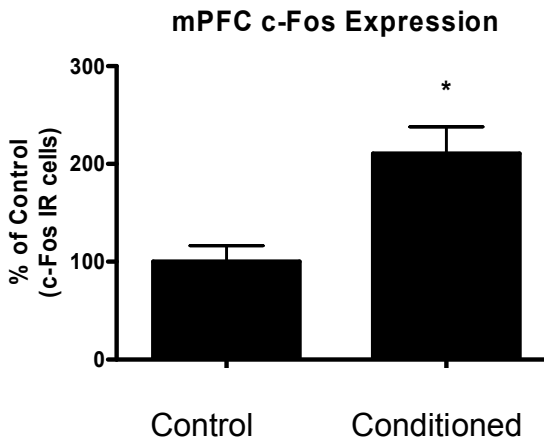

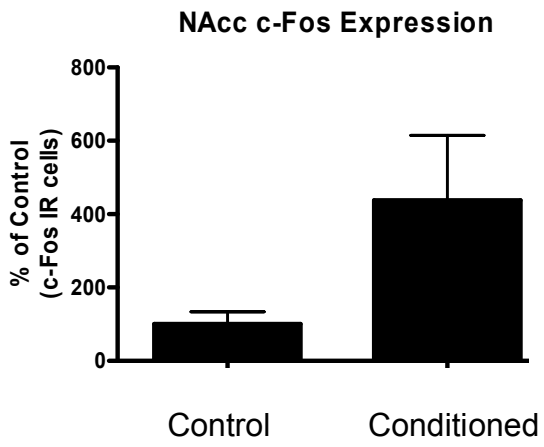

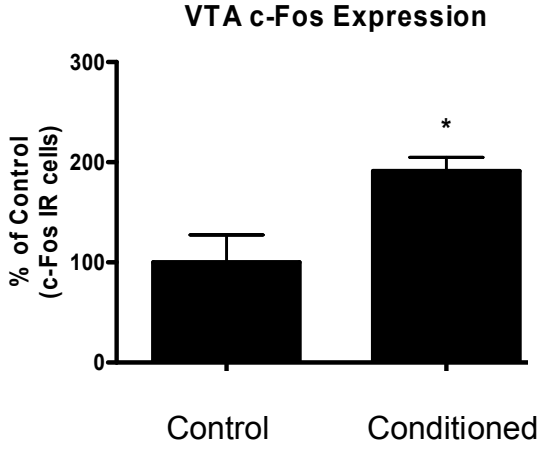

Figure 5.

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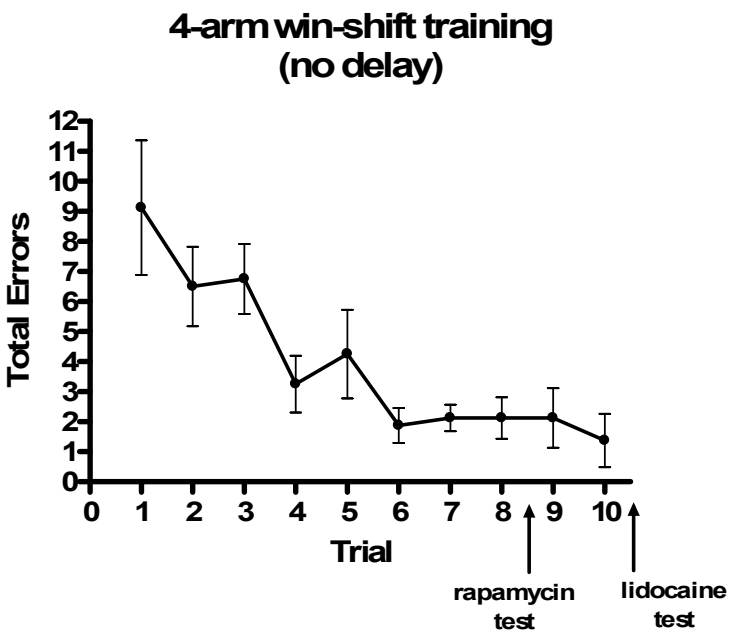

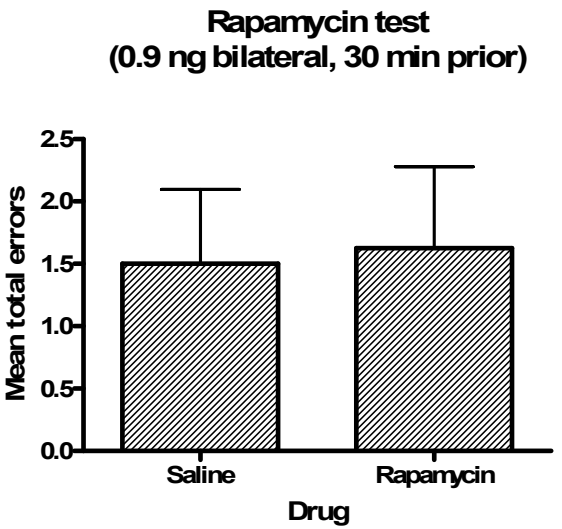

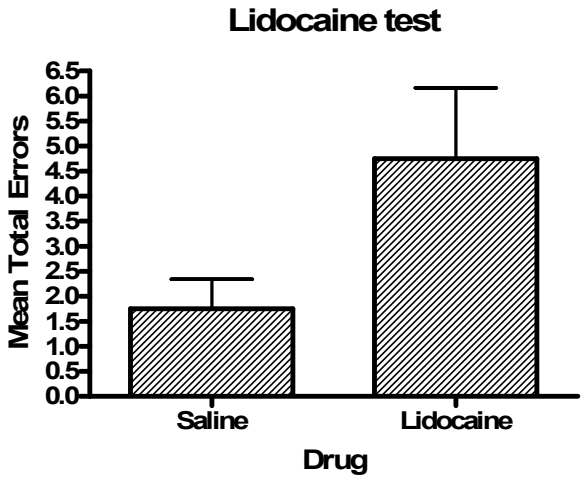

Figure 6.

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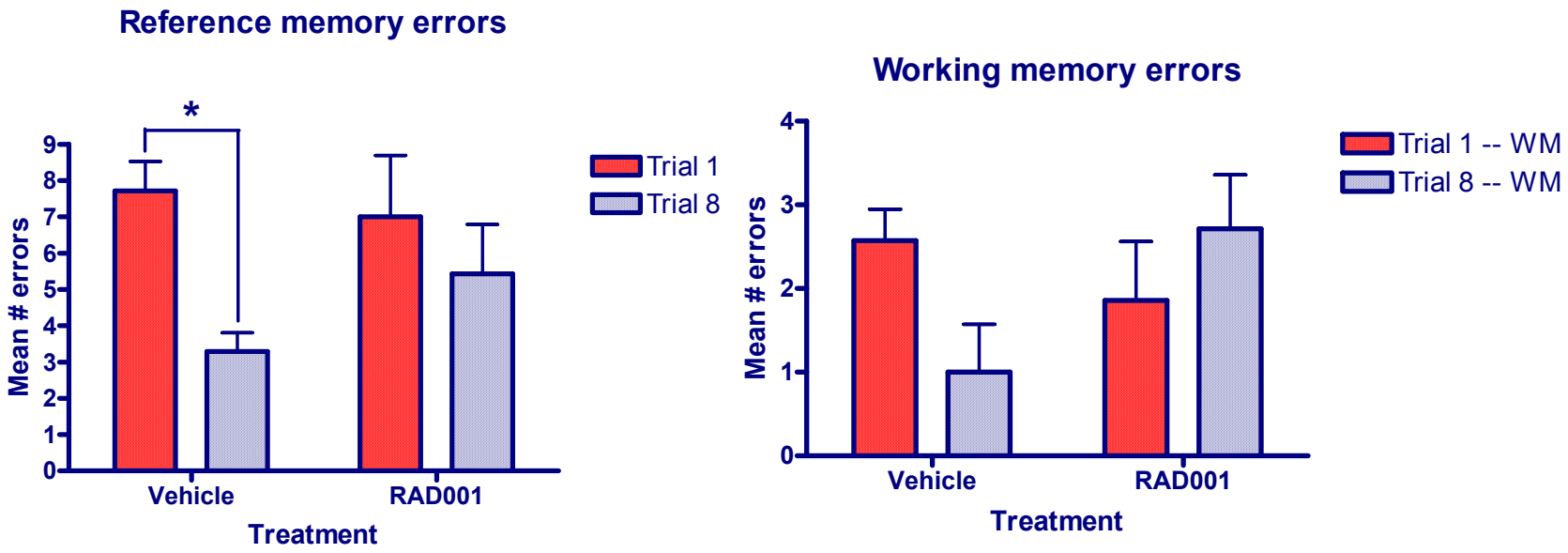

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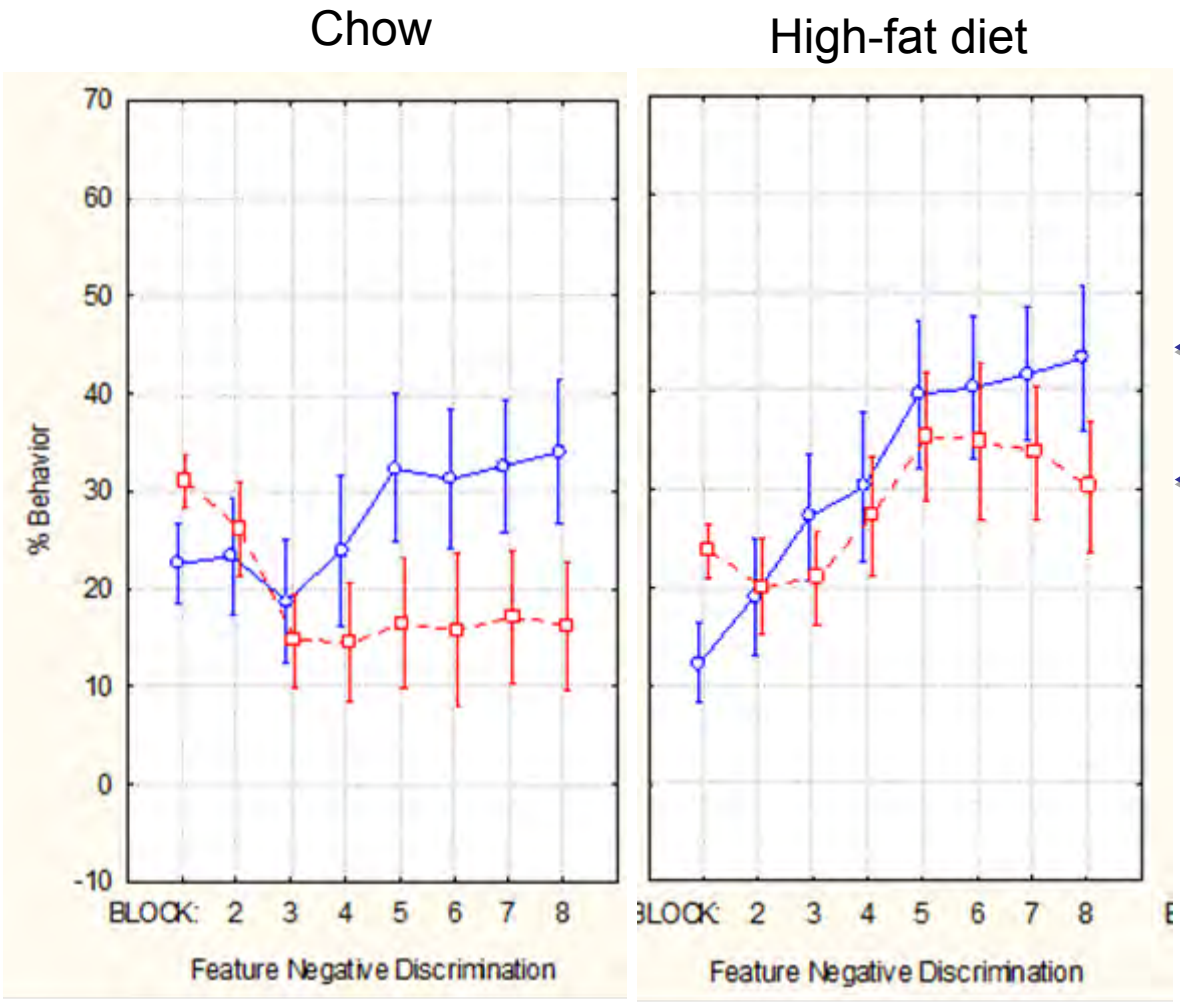
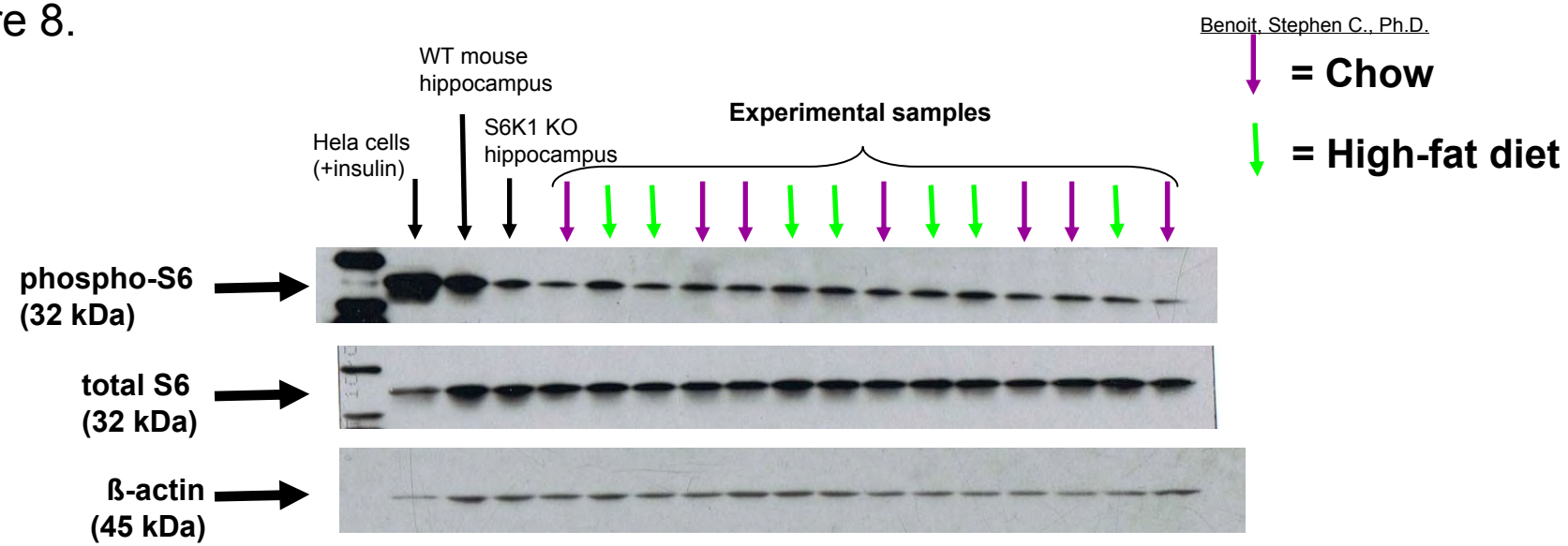


Figure 8.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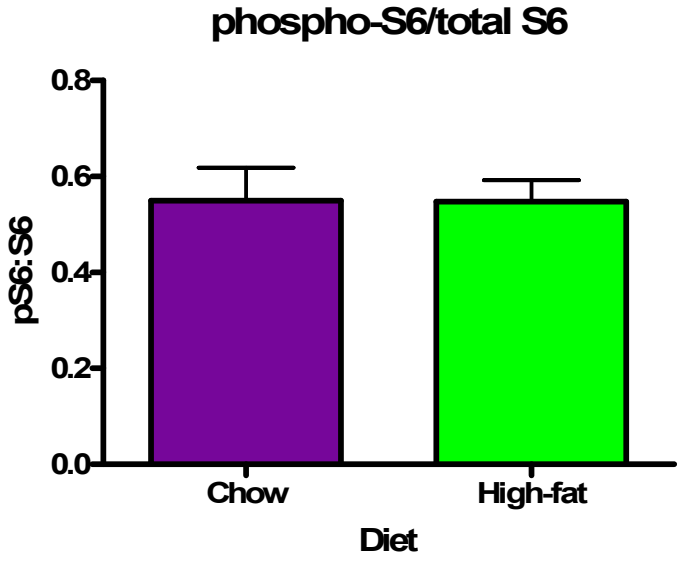
= Chow
= High-fat diet
WT mouse hippocampus
Hela cells (+insulin)
S6K1 KO hippocampus
Experimental samples
phospho-S6 (32 kDa)
total S6 (32 kDa)
ß-actin (45 kDa)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0.6
0.4
0.2
0.0
Chow
High-fat
Diet

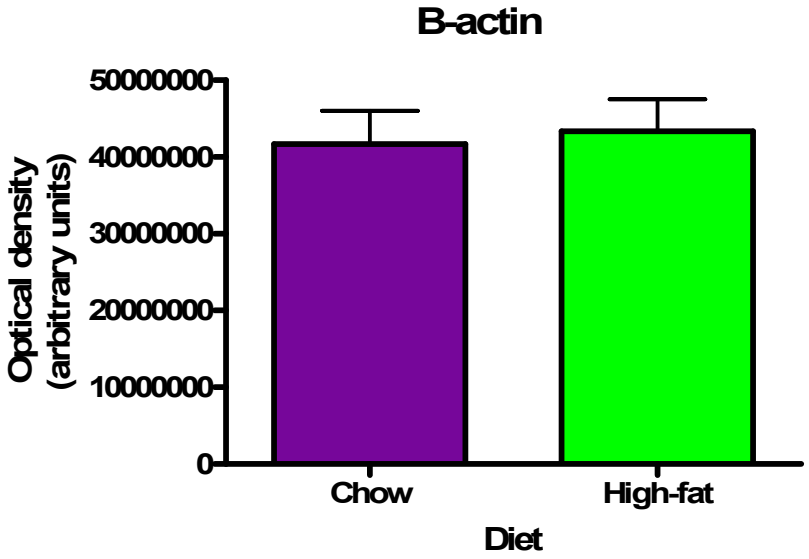
B-actin
Optical density (arbitrary units)
50000000
40000000
30000000
20000000
10000000
0
Chow
High-fat
Diet

Figure 9.

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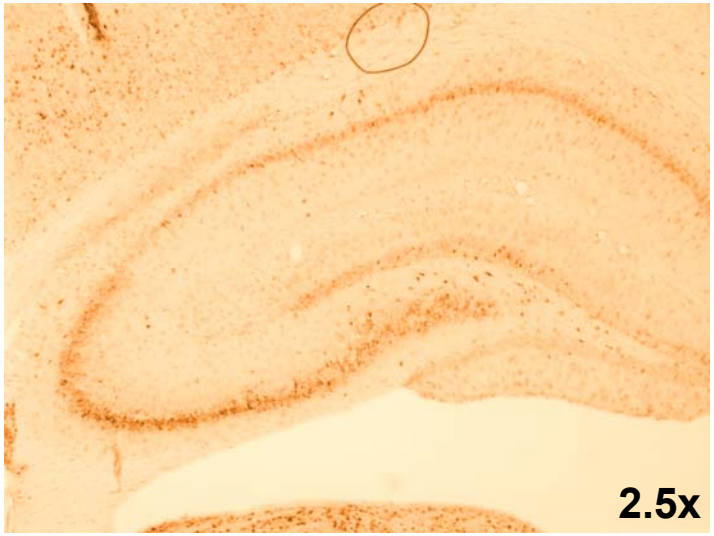
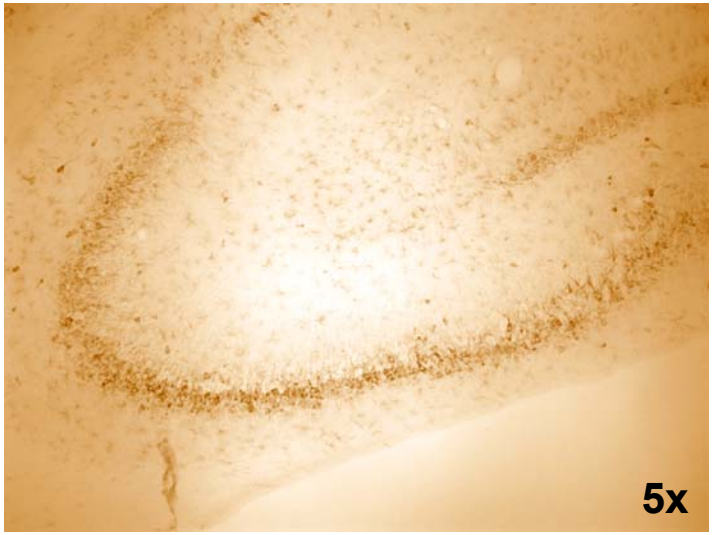




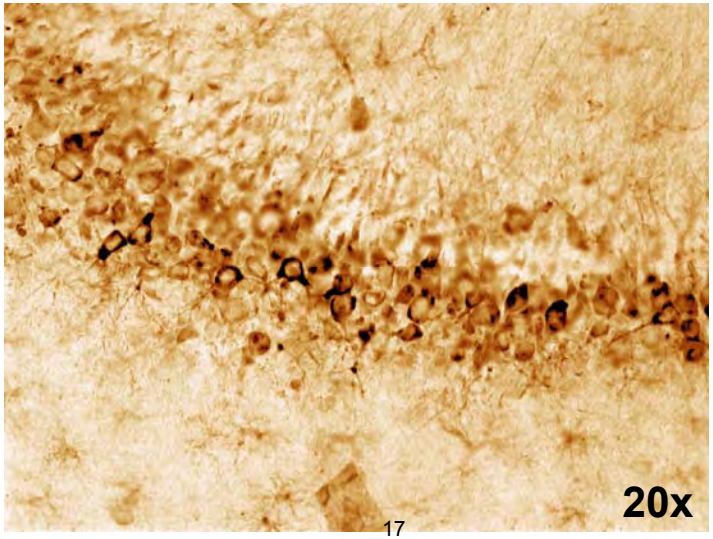
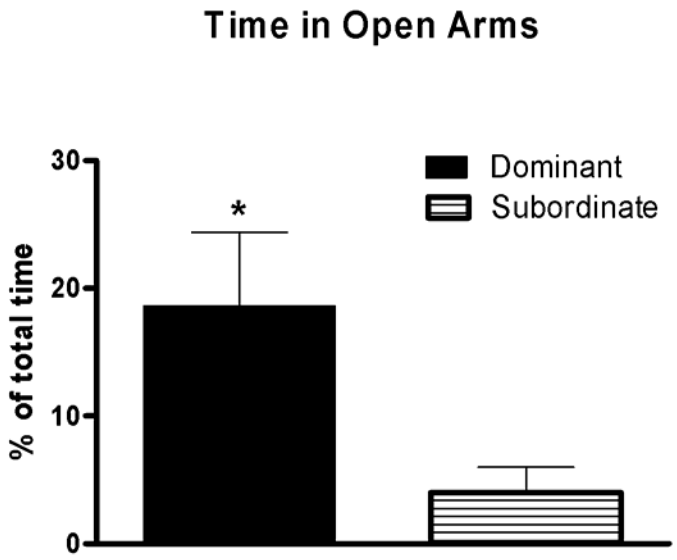


Figure 10.

Benoit, Stephen C., Ph.D.



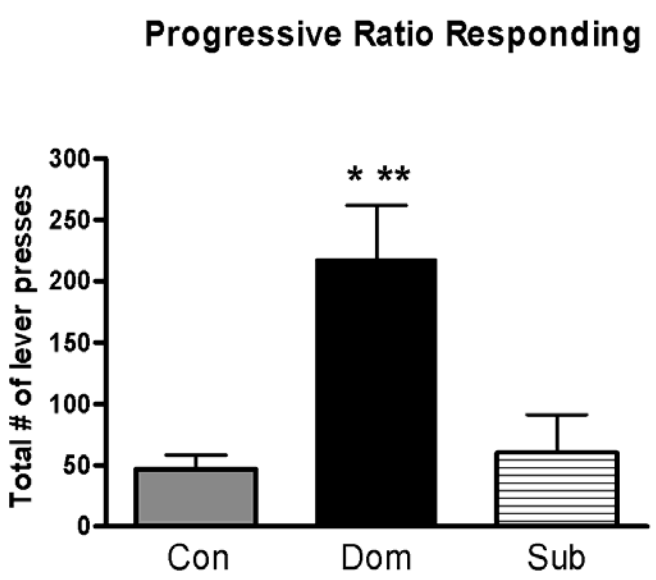
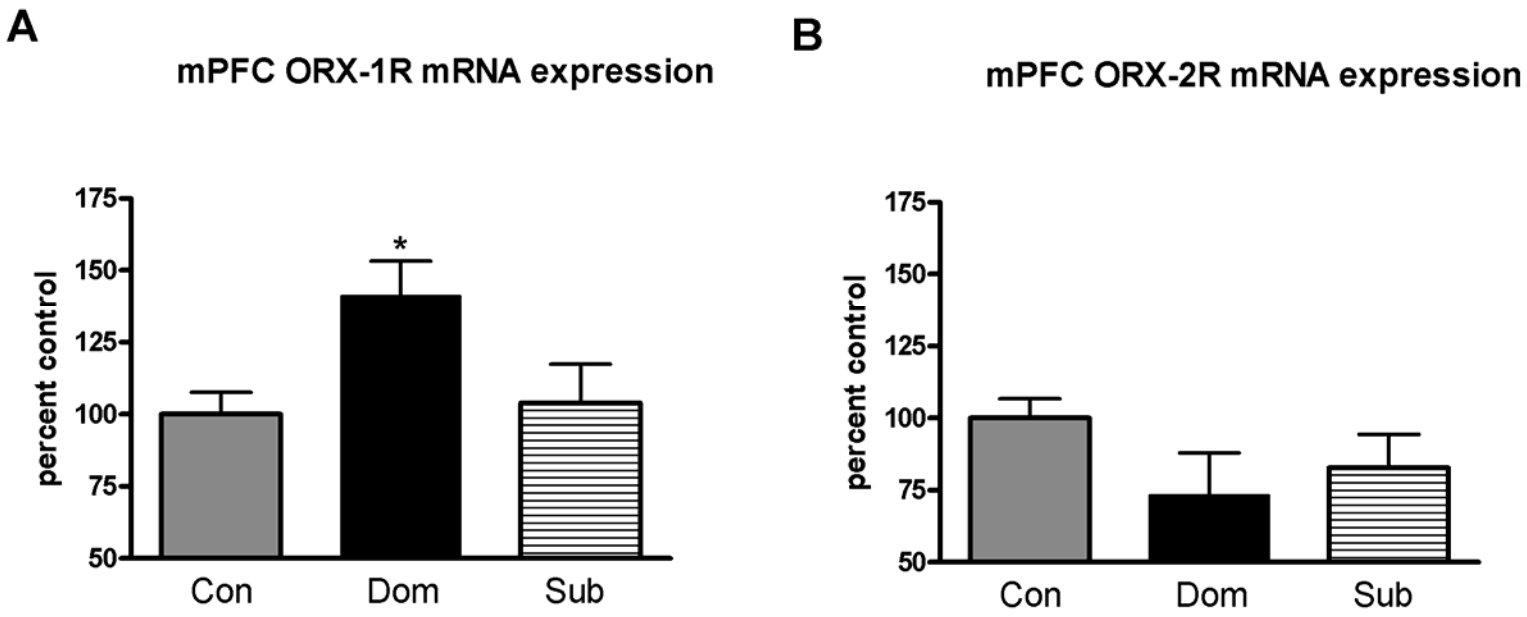


Figure 11.

Benoit, Stephen C., Ph.D.



Introduction:

The incidence of obesity is escalating to epidemic proportions in all segments of society. Even among the military, with much higher levels of fitness than civilian populations, are experiencing a rapid increase in obesity and metabolic disease. Recent research has suggested an important connection between arousal/stress physiology and metabolism. One important basis for this connection may be the brain orexin-A system, which is also the principal target of the anti-fatigue drug modafinil. However, the specific molecular machinery remains unidentified, as do the behavioral effects of manipulating this system. **Objective/Hypotheses:** We have hypothesized that modafinil and other anti-fatigue drugs may act by modulating metabolic pathways in the central nervous system. We also hypothesize that chronic stress and disruption of arousal-sleep system leads to impaired metabolic function and increased susceptibility to obesity. Finally, we hypothesized that central metabolic pathways can be activated by foods and nutritional interventions, in lieu of pharmacological manipulation, with less risk of long-term metabolic complications. To assess these hypotheses, we are conducting several studies in rat subjects. The primary study deigns include administration of modafinil and assessment of the molecular and genetic effects (Project 1) as well as behavioral consequences (Project 2). During the Fourth year of funding we have also pursued a suspected underlying metabolic pathway that might mediate the effects of nutrients on energy regulation as well as behavior. The mammalian target of Rapamycin (mTOR) kinase is a key regulator of several cellular functions, including cell growth and differentiation.

Body:

During the Final year of funding, we have made significant progress toward the stated aims and objectives. All proposed studies for Year 4 have been completed and we detail the results and conclusions here. For each study, we list the statement of work task, specific objectives, methods employed, and results obtained. Figures referenced are presented in an appendix at the end of the report.

Project 1 Year 4 Tasks:

Task 3: Develop key dietary components for maximal metabolic activation (37-48months)

- Test the ability of specific diets to alter orexin neuronal activity.
- Test the ability of specific diets to ameliorate the metabolic effects of modafinil, chronic stress and/or circadian disruption.

Experiment Series 1.4 Methods

Adult, male, Long-Evans rats were used in all of these experiments. The choice of diets for these experiments was critical. In this case, we uses a diet that is 40% fat (by calories) with the predominant source of fat coming from saturated fat or standard chow plus ad-libitum Ensure (1.5 kcal per ml). We have a great deal of experience with this particular diet and it is much closer to a typical human diet than is standard rodent chow which is inconsistent in its ingredients and only 8% fat. Finally, we also assess the effects of a very low carbohydrate diet (ketogenic).

Stress protocol. Rats were placed in a standard restraint tube for 1 hour prior to behavioral experiments or sacrifice. Rats were sacrificed 1 hourf ollowing the restraint by overdose of sodium pentobarbital and perfused with 4% para formaldehyde. For behavioral assays, rats were placed in a

novel test chamber that predicted the delivery of a palatable food reward. We assessed activation of orexin neurons as well as activation of orexin target regions by dual-labeled immunohistochemistry.

Experiment Series 1.4 Results

Diets high in fat elicited increased level of gucocorticoids but blunted the restraint stress response. We observed no effect of the ketogenic diet on either stress response. Additionally, we observed activation of both orexin cells and orexin target projections in the novel context, independent of diet. These data suggest that the orexin system is activated by expectation, independent of maintenance diets. (See Figures 1-2, see Choi et al., 2010 for details).

Project 2 Year 4 Tasks:

Task 4: Identify and measure effects of chronic dietary manipulation (months 36-48).

- Assess long-term effects on cognitive performance and behavior (months 36-42).
    - Potential protective effects for stress.
- Measure potential negative consequences of dietary modifications (months 36-42).
- Assess dietary protection from pharmacological toxicity (months 36-48).

Experiment Series 2.4 Methods

Intraventricular Cannulation. Animals were shaved and surgically prepped. A 2-cm midsaggital skin incision was made to expose the skull. Holes for anchoring screws and the cannula were drilled. A stainless steel (22 gauge, Plastics One) guide cannula extending into the third ventricle was permanently affixed to the skull by means of metal bone screws and quickly-drying dental acrylic. A removable 18 gauge obdurator sealed the guide cannula when not in use. All skull openings are sealed with dental acrylic. Gelfoam or bone wax followed by skin closure with suture. By manipulating the placement of the cannula, we can also put the cannula into specific brain regions for more local injection of substances.

Working / Reference Memory Assessment. In this task, 4 of the 8 arms in an 8-arm radial arm maze are baited with food (see diagram), all arms are open for the animal to enter and remain open for the entire trial. All arms are identical, requiring the animal to utilize spatial cues external to the maze to identify and recall the location of the food. The animals were placed in the center of the maze and allowed to explore freely until all 4 food pellets have been consumed. Rats received daily ICV infusions of 10 ug glucose or 17 ug leucine dissolved in saline.

Peripheral leucine/glucose supplemtation. Long-Evans rats (N = 24) were weight matched into control (water) or 1.5% Leucine in water (n=6 per group) or equicaloric glucose. BW control group on day 0 was 287.31g ; BW Leucine group was 287.23g. They were maintained on water or 1.5% Leucine for 21 days. Blood was only taken at sac via heart stick. Prior to sacrifice all rats underwent Morris water mazet training and testing. Animals were then sacrificed with a fatal plus injection (sac occurred 5hrs into the light phase). Tissues collected were liver, lung, kidney, muscle, white fat, brown fat, and brain.

Experiment Series 2.4 Results

We found that ICV leucine and glucose increased reference memory function, but not working memory. We also measured memory performance (Morris water maze) in rats maintained on peripheral glucose or leucine supplementation. However, we found no significant differences between experimental groups and controls. Following memory testing in both experiments, we again confirmed that nutrients increased immunostaining of pS6 in hippocampus. These data suggest that while acute central administration of the nutrients can enhance cognitive performance, peripheral supplementation (at least using this protocol) was without effect. (See Figures 3-5).

Key Research Accomplishments:

- Central but not peripheral leucine improves cognitive performance in rats
- Inhibition of mTOR signaling blunts cognitive performance in rats and mice
- Anticipation of rewarding food activates orexin neurons (targets of modafinil)
- Anticipation of psychostimulant drugs activates orexin neurons

Reportable Outcomes:

1. Portions of the Year 3 and 4 data were presented at the 2009 annual meeting of the Society for Neuroscience in Chicago, IL.

2. Portions of the Year 4 data were presented at the 2010 annual Winter Conference on Brain Research, Snowmass, UT.

3. No patents or cells lines have been developed.

4. An animal model (mouse) of chronic variable stress based on the hypotheses generated here is currently under development in collaboration with Dr. James Herman (University of Cincinnati).

5. The data collected in Years 3 and 4 are contained in another manuscript that is being prepared for submission.

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1. Woods, S.C., Seeley, R.J., Rushing, P.A., D'Alessio, D.A., and Tso, P. 2003. A controlled high-fat diet induces an obese syndrome in rats. *Journal of Nutrition* 133:1081-1087.

2. Davis JF, Krause EG, Melhorn SJ, Sakai RR, Benoit SC (2009). Dominant rats display increased risk taking and augmented operant responding. Neuroscience, 162, 23-30.

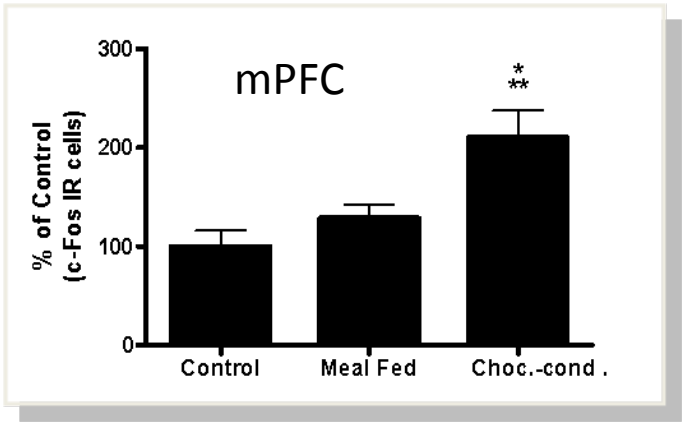
3. Choi DL, Davis JD, Fitzgerald ME, Benoit SC (2010). The Role of Orexin-A in Food Motivation, Reward-Based Feeding Behavior and Food-Induced Neuronal Activation in Rats. Neuroscience, 167, 11-20.

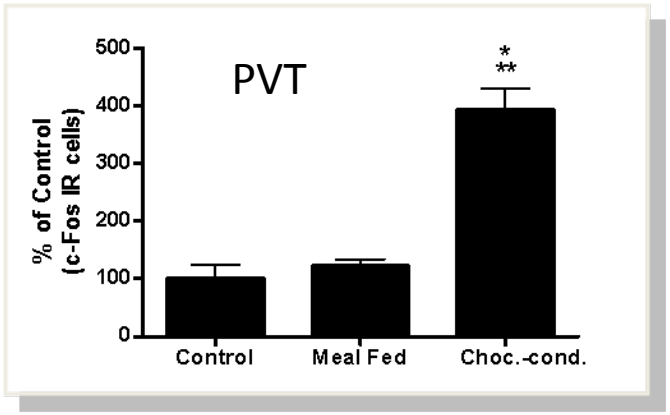
4. Davidson, Chan K, Jarrard LE, Kanoski SE, Clegg DJ, Benoit SC (2009). Contributions of the hippocampus and medial prefrontal cortex to energy and body weight regulation. Hippocampus, 19, 235-252.

Appendices:

1. Supporting Data:

See attached


mPFC
% of Control
(c-Fos IR cells)
300
200
100
0
*
**
Control
Meal Fed
Choc.-cond .


PVT
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(c-Fos IR cells)
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400
300
200
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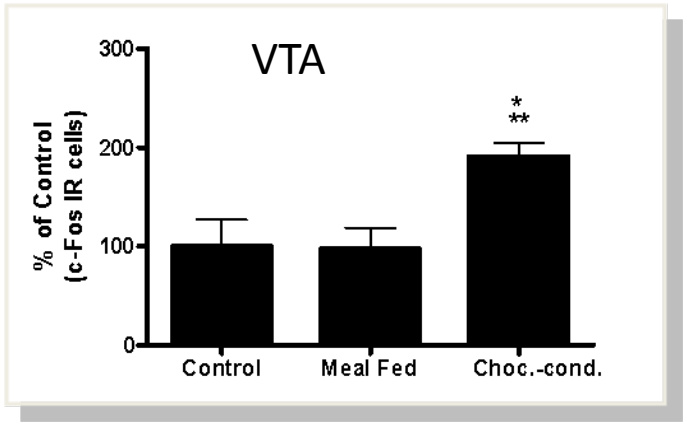
Meal Fed
Choc.-cond.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Meal Fed
Choc.-cond.

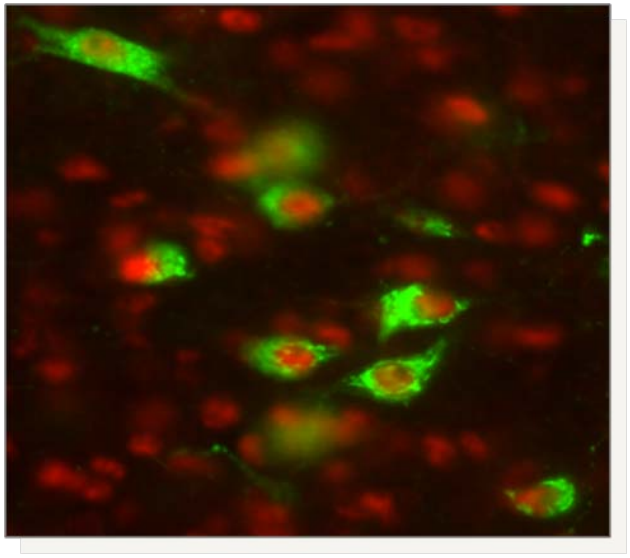
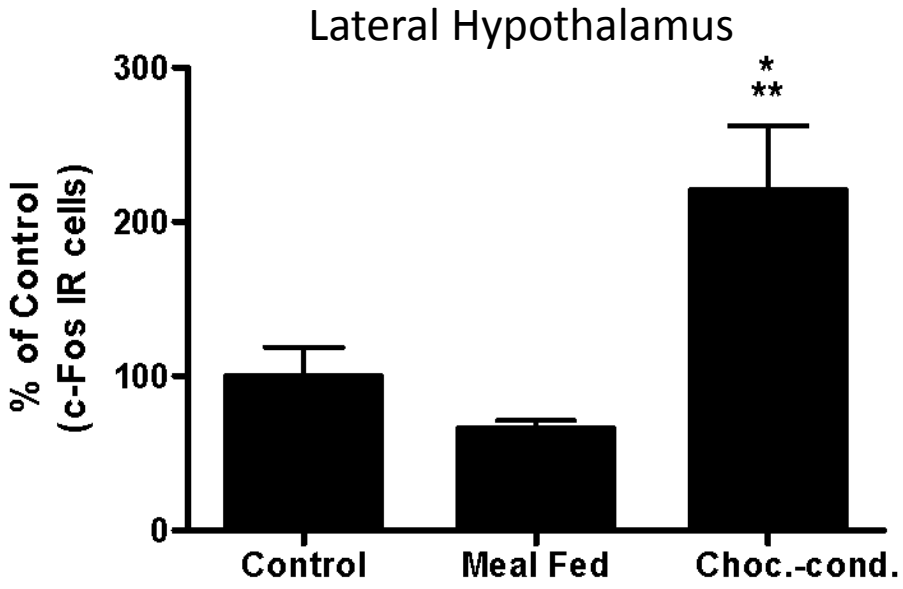
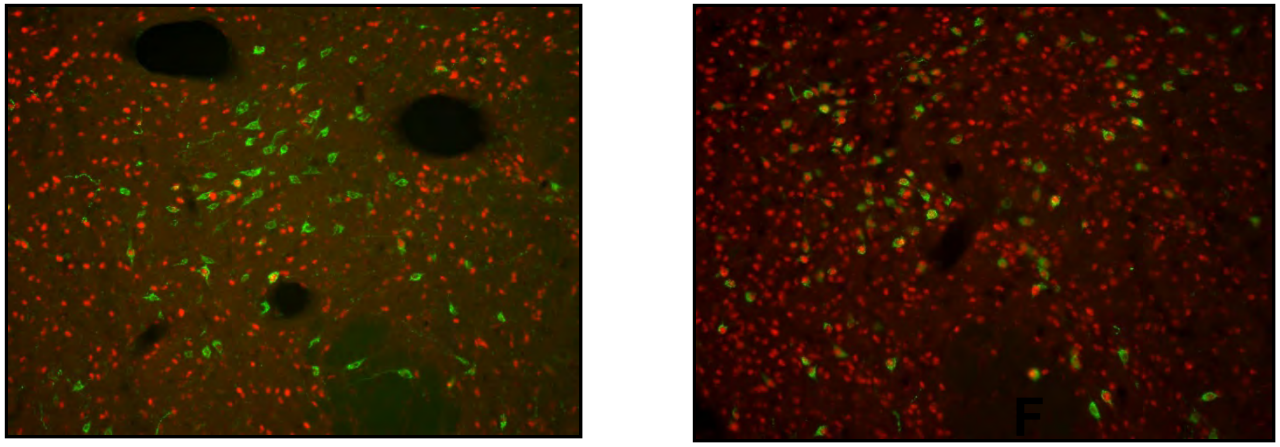


Figure 1.

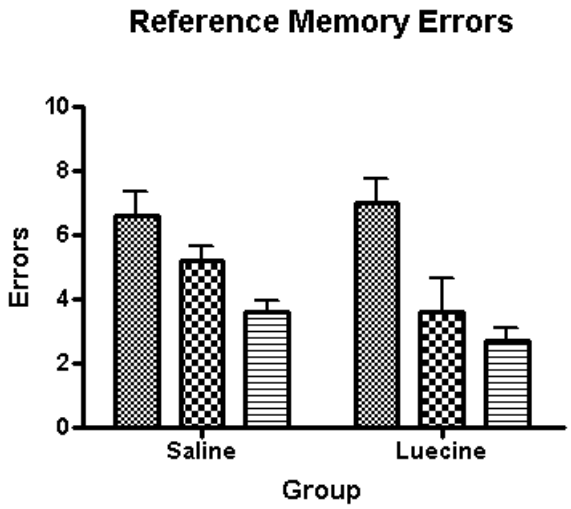




Control

Choc Conditioned

Figure 2.


Reference Memory Errors
Errors
10
8
6
4
2
0
Saline
Luecine
Group

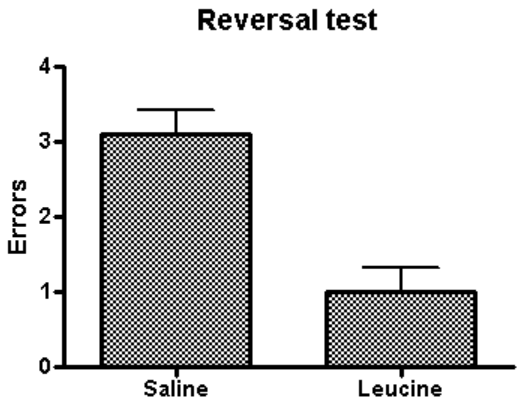
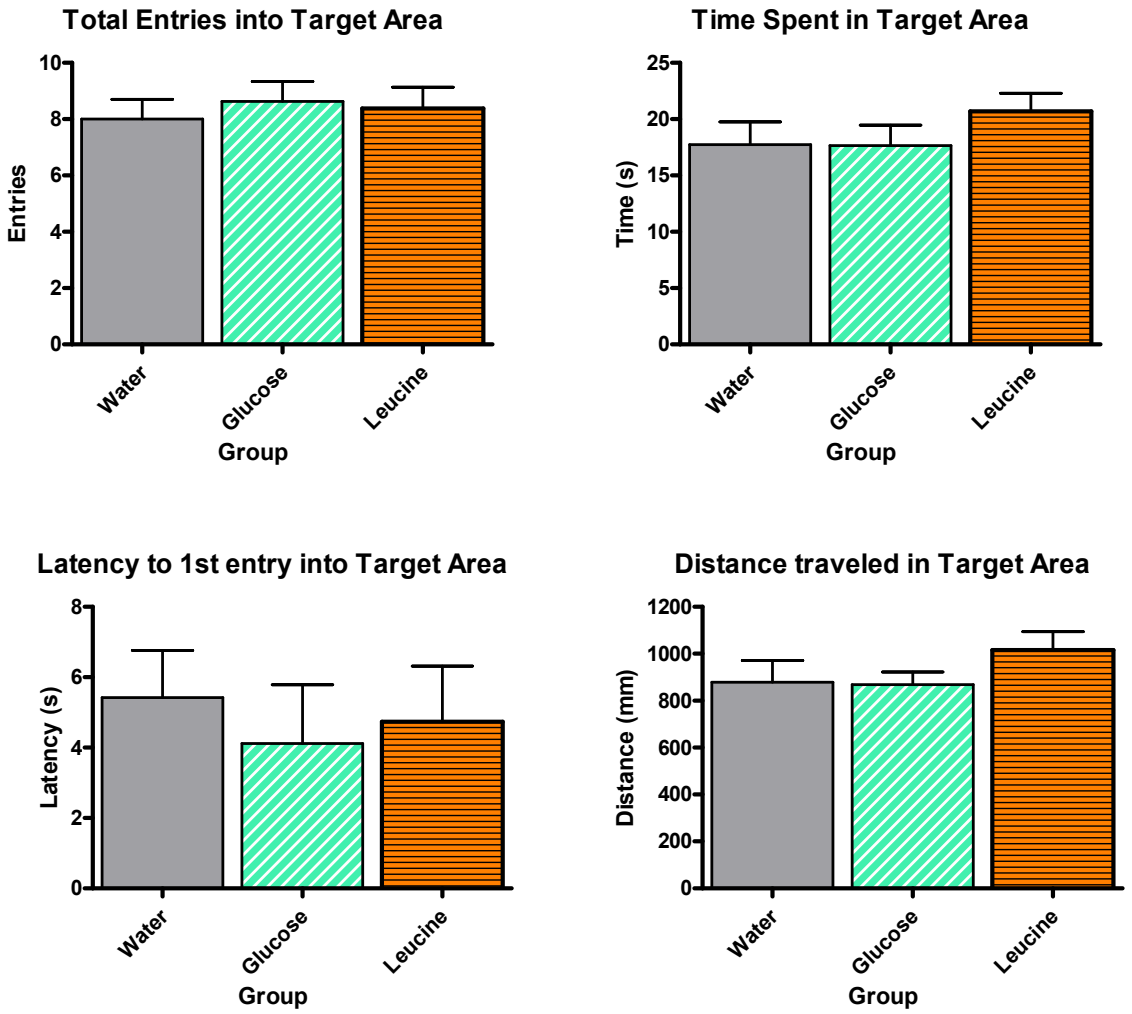

Reversal test
Errors
4
3
2
1
0
Saline
Leucine

Figure 3.


Total Entries into Target Area
Entries
10
8
6
4
2
0
Water
Glucose
Leucine
Group
Time Spent in Target Area
Time (s)
25
20
15
10
5
0
Water
Glucose
Leucine
Group
Latency to 1st entry into Target Area
Latency (s)
8
6
4
2
0
Water
Glucose
Leucine
Group
Distance traveled in Target Area
Distance (mm)
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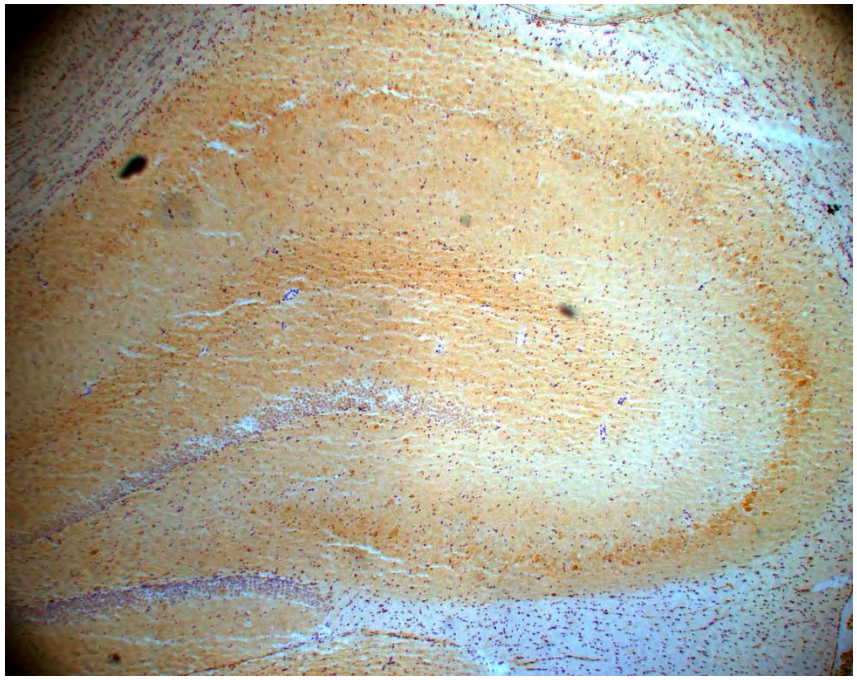
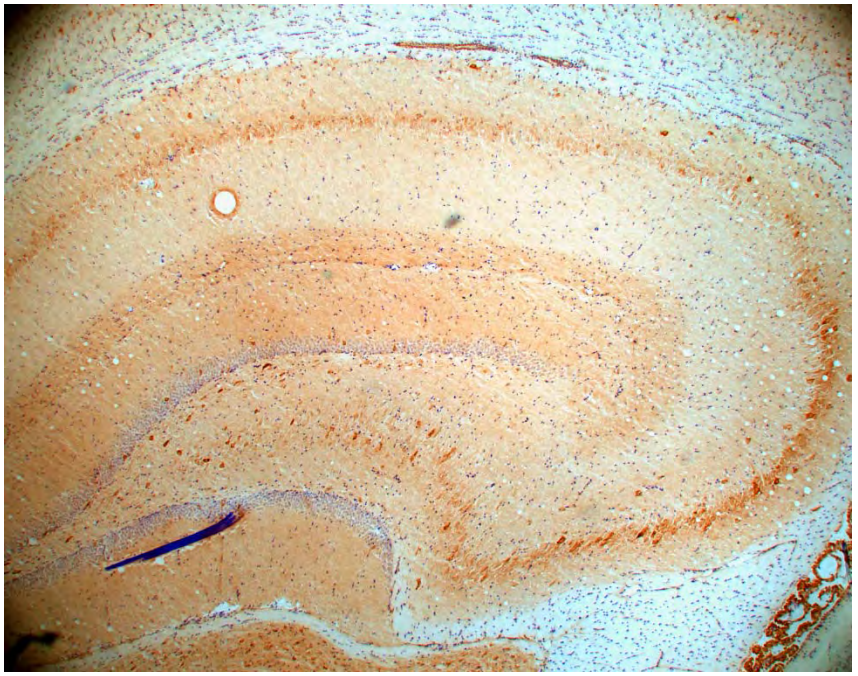
1000
800
600
400
200
0
Water
Glucose
Leucine
Group

Figure 4.



Leucine H2O PS6 Immuno –Hippocampus
Bregma -3.80 mm



H2O PS6 Immuno –Hippocampus
Bregma -3.60 mm

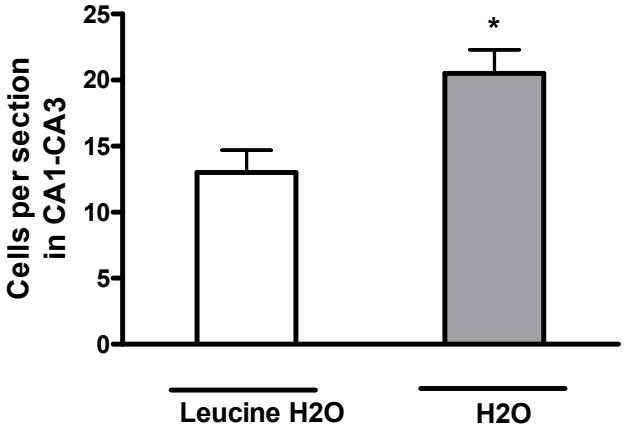


Figure 5.

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Contributions of the Hippocampus and Medial Prefrontal Cortex to Energy and Body Weight Regulation

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Abstract

The effects of selective ibotenate lesions of the complete hippocampus (CHip), the hippocampal ventral pole (VP), or the medial prefrontal cortex (mPFC) in male rats were assessed on several measures related to energy regulation (i.e., body weight gain, food intake, body adiposity, metabolic activity general behavioral activity, conditioned appetitive responding). The testing conditions were designed to minimize the nonspecific debilitating effects of these surgeries on intake and body weight. Rats with CHip and VP lesions exhibited significantly greater weight gain and food intake compared to controls. Furthermore, CHip-lesioned rats, but not rats with VP lesions, showed elevated metabolic activity, general activity in the dark phase of the light-dark cycle, and greater conditioned appetitive behavior, compared to control rats without these brain lesions. In contrast, rats with mPFC lesions were not different from controls on any of these measures. These results indicate that hippocampal damage interferes with energy and body weight regulation, perhaps by disrupting higher-order learning and memory processes that contribute to the control of appetitive and consummatory behavior.

Much research on the causes of overeating and excessive weight gain has been directed at identifying the brain regions where metabolic and hormonal signals that stimulate or suppress intake are detected and utilized (Benoit et al., 2004; Cummings and Overduin, 2007; Leibowitz and Wortley, 2004; Seeley et al., 2004). Although specification of these physiological substrates will be central to any comprehensive account of food intake regulation, it is now clear that such accounts must also consider the role of learning and memory in the control of eating and appetitive behavior (Davidson et al., 2005; Higgs, 2005; Petrovich and Gallagher, 2007; Sclafani, 1997; Woods and Ramsay, 2000). In recent years, the hippocampus, a brain structure long considered critical to the performance of a number of learning and memory functions (Eichenbaum, 2006; Squire, 2004), has received increasing attention related to its potential involvement in energy regulation.

This increased interest in possible involvement of hippocampus in energy regulation is based, in part, on findings that neurohormonal signals involved with meal termination (e.g., cholecystokinin), meal initiation, (e.g., ghrelin) and signaling the status of bodily energy stores

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(e.g., leptin, insulin) have receptors in the hippocampus (Lathe, 2001) and also appear to modulate the operation of hippocampal-dependent learning and memory processes (Diano et al., 2006; Harvey et al., 2006; Matsushita et al., 2003; Zhao et al., 2004). Other data also indicate that the hippocampus is part of the neural circuitry involved with energy regulation. For example, functional magnetic resonance imagery (fMRI) identified the hippocampus and prefrontal cortex as the sites of greatest activation in obese people, following gastric stimulation known to have effects on intake, stomach distention, hormonal and vagal activity similar to those produced by eating a large meal (Wang et al., 2006). Another fMRI study showed that after consuming a liquid meal to satiation, obese and formerly obese people exhibited decreased hippocampal blood flow relative to lean people (DelParigi et al., 2004). Anatomically, direct neural projections from the ventral pole of the hippocampal CA1 cell field to the lateral hypothalamus, along with disynaptic connections from the CA1 field (e.g., via the subiculum) to other hypothalamic loci known to be involved with the control of feeding, have been identified (Cenquizca and Swanson, 2006; Cenquizca and Swanson, 2007).

Furthermore, densely amnesic humans with brain damage that includes the hippocampus have been reported to show reduced sensitivity to interoceptive signals of hunger and satiety (Hebben et al., 1985; Rozin et al., 1998), an effect that has also been observed in rats with highly selective lesions that are confined to the hippocampus (Davidson and Jarrard, 1993; Hirsh, 1974; Hock and Bunsey, 1998). In addition, relative to intact controls, rats with selective lesions of the hippocampus exhibit increased appetitive responding for food (Clifton et al., 1998; Davidson and Jarrard, 1993; Schmelzeis and Mittleman, 1996)), including reduced ability to inhibit responding elicited by food associated stimuli when those responses are no longer reinforced (Chan et al., 2001; Tracy et al., 2001) and reduced ability to use energy state cues as inhibitory signals (Davidson and Jarrard, 1993; Davidson et al., 2005).

The above findings suggest that (a) the hippocampus is sensitive to signals involved with energy regulation; (b) some of these signals induce changes in hippocampal activity that are thought to facilitate learning and memory; (c) information provided by satiety signals may be transmitted via neural pathways from the gut to the hippocampus and from the hippocampus to forebrain circuits involved with energy regulation; (d) hippocampal responses to these signals appear to be altered for people who have a history of obesity.

Surprisingly, only a limited number of studies have attempted to assess the effects of hippocampal damage on body weight. For example, King et al (King et al., 1993) reported that rats with hippocampal lesions ate significantly more but did not gain more weight compared to intact control rats. In an earlier study by Forloni et al., (1986), hippocampal lesions were accompanied by increased food intake and body weight gain when measured over a much longer period, but this effect was found only with female rats. Unfortunately, both of these studies used nonselective lesion techniques that produced damage to extrahippocampal structures and to fibers of passage. In the present experiment, we attempted to avoid this complication by using a highly selective ibotenate lesioning technique to produce hippocampal damage (Jarrard, 1989).

Furthermore, previous studies have typically not assessed or accounted for the nonspecific behavioral suppressive effects of surgery per se, when evaluating the effects of hippocampal damage on food intake and body weight. Specifically, many types of surgeries, including hippocampal surgery, are accompanied by reductions in food intake and body weight during the post-operative recovery period. Indeed, in our experience, body weight of lesioned rats may stay below that of intact controls for several weeks. Ideally, food intake and body weight gain should be compared among lesioned and non- or sham-lesioned controls only after lesioned animals have completely recovered from such nonspecific after-effects of surgery. To decrease the likelihood that the specific effects of hippocampal lesions on intake and body

weight gain would be confounded with any nonspecific effects of the surgical procedure involved with producing those lesions, the present study defined post-operative recovery as complete when lesioned rats achieved a level of body weight that matched the level of a group of ad lib fed control rats that had not undergone surgery.

The present study also employed an additional control condition. Unlike previous studies, we used a pair-feeding procedure to insure that control rats (half sham-lesioned and half unoperated) experienced reductions in food intake and body weight similar to those experienced by lesioned rats in the aftermath of surgery. Thus, these pair-fed control and lesioned rats were equated with respect to intake and body weight during the period prior to achieving the criterion for post-operative recovery mentioned above. With this procedure, it would be difficult to attribute any effects of lesions on energy intake and body weight gain after post-operative recovery to any residual effects of the effects of reduced eating and body weight loss during the pre-recovery period.

In addition to examining the effects of destruction of the complete hippocampus (CHip) on energy and body weight regulation, the present experiment also assessed the effects of damage limited, respectively, to the hippocampal ventral pole (VP) and the medial prefrontal cortex (mPFC). Recent research has identified direct neuroanatomical projections from the ventral pole of the hippocampal CA1 cell field (Cenquizca and Swanson, 2006), which comprises approximately 10% of the cells of the entire CA1 region, to a number of hypothalamic nuclei (e.g., ventromedial and lateral hypothalamic nuclei) that have long been implicated in the regulation of food intake and body weight (Grill, 2006; King et al., 1994; Stellar, 1994). This suggests that mechanisms related to memory and energy regulation might be integrated within a ventral hippocampal pole-hypothalamic circuit. Accordingly, lesions confined to the hippocampal ventral pole could impair the control of food intake and body weight by disrupting the operation of this circuit.

Neuroanatomical studies also show that the ventral CA1 cell field projects strongly to the medial prefrontal cortex which has, in turn, dense projections to the lateral hypothalamus (Cenquizca and Swanson, 2006; Swanson, 1981). Functionally, rats with medial prefrontal cortex lesions are like rats with the hippocampus removed in that they exhibit normal acquisition of simple discriminative contingencies but are impaired in inhibiting previously reinforced responses when the discriminative contingencies are reversed (Salazar et al., 2004). Another recent study found that rats maintained for 90 days on a diet high in saturated fat showed impaired reversal learning and reduced levels of brain-derived neurotrophic factor (BDNF) in both the ventral (but not dorsal) hippocampus and the medial prefrontal cortex (Kanoski et al., 2007). Several reports have linked reductions in BDNF and/or exposure to high-fat diets to interference with hippocampal learning and memory processes (e.g., Liu et al., 2004; Molteni et al., 2002; Monteggia et al., 2004; Wu et al., 2003; Yamada and Nabeshima, 2003). The present experiment included rats with lesions confined to the medial prefrontal cortex to assess the possibility that damage to the medial prefrontal cortex might have effects on food intake and body weight that are similar to those produced by hippocampal lesions in chow-fed rats.

We also assessed the effects of each of these lesions on learning and performance of appetitive conditioned responses. As noted above, previous research indicates that, compared to controls, rats with hippocampus removed exhibit increased conditioned appetitive behavior to cues that have history of excitatory and inhibitory training (Davidson and Jarrard, 1993; Schmelzeis and Mittleman, 1996). However, the effects of lesions limited to the hippocampal ventral pole are not known. Furthermore, the performance of conditioned appetitive responses across a wide variety of training conditions is known to depend on level of food deprivation. Although some research with rats indicates that lesions of the medial prefrontal cortex can alter the effects of

satiation on the performance of food-reinforced conditioned responses (Petrovich and Gallagher, 2007), the effects of complete hippocampal or hippocampal ventral pole lesions on the sensitivity of appetitive conditioned performance to deprivation/satiation manipulations are largely unexplored. Also important, the effects of CHip, VP, and mPFC lesions on the sensitivity of body weight to deprivation/satiation manipulations have not been reported. The present experiment attempted to help fill each of these gaps in knowledge. Finally, the effects of each type of lesion on energy expenditure, general behavioral activity, and on ability to regulate body weight in response to variations in level of food deprivation were also assessed.

In summary, previous findings suggest that extra-hypothalamic circuits involving the hippocampus and medial prefrontal cortex may contribute to the regulation of food intake and body weight. Recent anatomical studies demonstrate that the ventral pole of the hippocampus projects directly and indirectly, via the mPFC, to the LH. Furthermore, dietary factors which also promote obesity, have similar effects on neurotrophic activity in the hippocampal ventral pole and mPFC. The present studies further assessed mPFC, CHip, and VP lesions on energy balance and on the performance of learned appetitive responses.

Methods

Subjects

Subjects were adult, male, Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 275 – 325 g at the outset of the study. Animals were housed individually in hanging wire cages with laboratory chow (Lab Diets 5001) and water available ad lib except during testing as described below. The colony room was maintained on a 12:12 light:dark cycle (lights off at 1600 hrs), with temperature maintained at 21 -23 C. All procedures for the care and treatment of the rats during this experiment were approved by the Purdue Animal Care and Use Committee.

Apparatus

All appetitive training and testing procedures were conducted in eight identical conditioning chambers constructed of aluminum end walls and clear Plexiglas side walls, measuring 21.6 × 21.6 × 27.9 cm. The floors of each conditioning chamber consisted of stainless steel bars spaced 1.9 cm apart, measuring 0.48 cm in diameter. A recessed food magazine was located in the center of one end wall of each chamber. A computer-controlled infrared monitoring system was used to record food magazine approaches and entries. One infrared photo transmitter and one receiver were located on each side wall of the recessed food magazine, situated so that a rat could not gain access to sucrose pellet reinforcers without interrupting the photobeam.

Procedures

Surgical and histological procedures—The rats were assigned to seven groups matched on pre-operative body weight calculated two days prior to the beginning of surgery. Rats in the complete hippocampus (CHip), hippocampal ventral pole (VP), and medial prefrontal cortex (mPFC) groups were lesioned at these sites by the use of multiple, focal injections of small amounts of the axon-sparing neurotoxin, ibotenic acid (IBO: Biosearch Technologies). The IBO was dissolved in phosphate buffered saline (pH 7.4) at a concentration of 10 mg/ml. The rats were anesthetized with intraperitoneal injections of a combination of sodium pentobarbital and chloral hydrate, and were placed in a Kopf stereotaxic apparatus with the skull level. Following the procedure described in detail by Jarrard (1989), an incision was made in the scalp, and the bone overlying the area to be damaged was removed. Injections of IBO were made with a 5-μl Hamilton syringe mounted on the stereotaxic frame and held in a Kopf microinjector unit (Model 5000). A small diameter glass micropipette was glued onto the end of the needle of the syringe in order to minimize damage to the cortex overlying the area to be

lesioned. Injections were made over ~ 1 min at each site and the pipette was left in place for ~ 1 min to prevent spread of the neurotoxin up the tract.

Rats in the VP Group (n = 12) received injections of IBO (0.05 μl) at two sites in each hemisphere using the following stereotaxic coordinates: anteriorposterior (AP) - 3.8 mm; mediolateral (ML), +/- 4.3 mm; dorsoventral (DV) − 7.1 mm (taken from dura at the site of injection); and AP -4.3 mm, ML +/- 4.8 mm, DV -6.5 mm (taken from dura as above).

Each rat in the mPFC Group (n = 8) received a total of 12 injections (0.05 μl per site) of IBO at the following coordinates: AP + 2.7 mm, ML +/- 0.8 mm, DV − 3.0 and -1.5 mm from dura; AP + 3.5 mm, ML +/- 0.8 mm, DV − 3.0 and -1.5 mm; and AP + 4.2 mm, ML +/- 0.8 mm, and DV − 3.0 and -1.5 mm. Injections of IBO at 30 sites (0.05 to 0.12 μl per site; for stereotaxic coordinates see (Jarrard, 1989; Jarrard, 2002) were made in each rat in the CHip Group (n = 8).

Each of these lesioned groups had their own corresponding pair-fed control group (n = 8 each). Food rationing was used to match these control groups with respect to the amount of weight lost by the lesioned groups at the outset of the postoperative recovery period. Half of the rats in each control group received sham-lesions, which were produced using the same procedures as described for their respective lesion group with the exception that no IBO was administered. The remaining rats in each pair-fed control group were unoperated controls. The final group of rats (n = 6) were ad lib fed controls. This group received neither food deprivation nor food rationing during the study. All surgeries were conducted over a three-day period. The number of rats that received surgery on each day was equated for each surgical and corresponding pair-fed control condition.

Following testing, all rats were administered an overdose of the anesthetic and were perfused transcardially with a mixture of buffered physiological saline followed by 10% formaldehyde solution. The brains were removed, embedded in egg yolk, cryoprotected in a 30% solution of sucrose-formalin, and subsequently cut on a cryostat into 40-μm sections. Every fifth section from rats in the CHip and mPFC Groups was saved for histology, while every second section from VP rats was saved and stained. A cresyl violet stain was used to determine cell loss and gliosis resulting from the lesions.

Food intake and body weight—The rats were weighed daily beginning on the first day of surgery until each rat in each lesioned group had returned to their respective pre-operative body weighs. Food intake was also monitored daily for each rat during this period. Each rat in each lesioned group had a corresponding weight-matched, pair-fed control. Food rationing was used to maintain each pair-fed control at the same body weight as its corresponding lesioned rat until the body weights of lesioned rats recovered to their preoperative level. When this recovery was achieved the rats were given ad libitum lab chow. Body weight and amount of food consumed was recorded every 48 hrs for each rat throughout the remainder of the study. Amount consumed was calculated by weighing the amount of food given at the beginning of each measurement period and subtracting from that number the amount of food remaining in the food hopper plus crumbs collected from papers placed beneath each cage.

Analysis of conditioned appetitive behavior—The acquisition and extinction of appetitive conditioned responses and potential differences in behavioral sensitivity to low and relatively high levels of food deprivation was assessed by using a type of latent discrimination procedure (e.g., Davidson et al, 1992). With this technique, rats are exposed to a small numbers of acquisition trials followed immediately by extinction training, though learning is not evident until the extinction phase.

Prior to the beginning of training, the rats in each lesion group and their pair-fed controls (ad libitum-fed controls were not included) were assigned to squads and conditioning chambers such that equal numbers of rats from each surgical condition were trained in each of the 8 chambers, with one exception. Because there were 12 rats with VP lesions, eight of the rats in this group were assigned to each of the 8 conditioning chambers and the remaining four rats were randomly assigned to four of these chambers.

Adapting procedures used in longer-term studies of similar design (Davidson et al., 2005a; Kanoski et al., 2007b), the rats in the present study received single daily training sessions under alternating levels of 24-hr and 0-hr food deprivation. On each session the rats were placed into the conditioning chambers for 4 minutes, without discreet stimuli. When the rats were placed in the conditioning chambers after they had been food deprived for 24 hrs, this four-minute trial period terminated with the delivery of five sucrose pellets (45 mg sucrose pellets, P.J. Noyes Company, Inc. Lancaster, NH). When rats were placed into the conditioning chambers after they had been given ad libitum lab chow in the home cage for approximately 24 hrs, (i.e., under 0-hr food deprivation, meaning that the rats were not food deprived), the feeder mechanisms operated, but no pellets were delivered, Thus, during training for all rats, trials that took place under 24-hr food deprivation were reinforced whereas trials that took place under 0-hr food deprivation were not reinforced. Throughout the experiment, each 4-min trial was divided into twenty-four 10-s bins, the last of which terminated with the operation of the feeder mechanism. The percentage of these 10-s periods in which the photobeam inside the recessed food cup was interrupted served as the index of conditioned appetitive behavior. On both 24-hr and 0-hr deprived trials, the rats remained in the conditioning chambers for an additional two minutes before being returned to their home cages.

The rats received six training sessions under 24-hr food deprivation and 6 under 0-hr food deprivation. The rats received only one such four-min trial on each training session and they received no more than one training session per day. Training sessions were always held at the same time of day (1430 hrs). Although food deprivation levels alternated each day between 0- and 24-hrs, sessions did not occur every day to prevent the pellets from being delivered according to a single-alternating schedule. The schedule of training sessions conducted under 24- and 0-hr food deprivation was: 24, 24, 0, 24, 0, 0, 24, 24, 0, 0, 24, 0.

When six trials under each level of food deprivation had been completed, pellet deliveries were suspended and all rats were tested in extinction for 10 additional sessions, five under 0-hr and five under 24-hr food deprivation, according to the following sequence: 0, 24, 0, 0, 24, 24, 0, 24, 24, 0. The procedures for extinction testing were identical to those used during training except that no sucrose pellets were delivered on any trial. The rats were weighed immediately prior to each training and extinction session.

Indirect calorimetry—Indirect calorimetry was performed using the Columbus Instruments Oxymax 5.41 system to measure oxygen (O2) consumption. Rats were placed in individual metabolic chambers 2 hours prior to the onset of the dark cycle. They remained in the chambers for 24 hours with ad libitum access to chow and water. Samples were collected approximately every 15 minutes. For each time point, the samples for each group were averaged. Average kcal/h values were compared during the light and dark cycles for all groups. The flow rate to the individual chambers was set at 0.6 liters per minute, with room air as the reference. The system measuring time was set for 60 seconds and represents the amount of time during which the indirect calorimeter monitors the gas concentrations. The indirect calorimeter takes as many readings as possible during this interval and derives a mean value. The system settling time was set at 120 seconds and represents the amount of time between the opening of the intake valve and the onset of the measuring time. This time allows for the complete purging of any residual gas in the system.

Body composition analysis—Body composition was analyzed with a custom-designed rodent quantitative NMR apparatus (Echo MRI Whole Body Composition Analyzer; Echo Medical Systems). Briefly, each rat was transported into a room that housed the NMR and placed (without anesthesia) in a Plexiglas tube that was then inserted into the NMR for body composition analysis. Body fat and lean tissue were measured during the test, which took less than 60 seconds per rat to perform.

Homecage behavioral activity analysis—Behavioral activity was determined using the SmartFrame stainless steel cage rack frame (Hamilton-Kinder Scientific Company, Poway, CA) which was placed around each animal's shoebox homecage. Infrared photobeam interruption sensors (7X and 15Y) mounted in the frame detected movement which was recorded and analyzed using the HMM100 MotorMonitor software. Vertical and horizontal activity within the homecage was recorded for 48 hours, and the events were collapsed into 60 min bins. The data were analyzed as the average number of beam interruptions per group per hour. Additional analyses were conducted to analyze the location of the rat (measured by beam interruptions) across time to produce percent time spent in the half of the cage containing the food hopper and percent time in the opposite half of the cage.

Data analysis—Analysis of variance (ANOVA) with Groups (i.e., surgical condition) as between-subjects factors and Days or Sessions as within-subjects factors were used to evaluate the effect of lesions on body weight, food intake, and appetitive behavior. Analysis of simple main effects and post-hoc Newman-Keuls tests were used to identify specific patterns of differences on which findings of significant main effects and interactions were based. ANOVAs with only Groups as a (between-subjects) factor were also used to assess the effects of lesions on metabolic and general behavioral activity and body composition. Dunnett tests were used to assess the basis of significant main effects in these analyses. Alpha level was set at 0.05 for all statistical tests. In addition, separate analyses of the type described above were used to compare each of the respective control groups that were used for the CHip-, VP-, and mPFC-lesioned groups. When these analyses failed to reveal significant main effects or interactions involving the different control conditions, the controls were combined for comparison with the lesioned groups.

Results

Histology

The nature and extent of the brain damage resulting from the surgical procedures is described in the following paragraphs. Microphotographs of sections from rats with VP and mPFC lesions are shown in Figures 1 and 2, respectively.

Complete Hippocampus (CHip) lesion—All animals in the CHip lesion Group had extensive loss of the cells that comprise the hippocampus including the CA1-CA3 pyramidal, dentate granule cells, and the cells within the hilar region of the dentate gyrus. Further, there was minimal involvement of adjacent structures. The brain damage was generally similar to that reported in a number of other studies where focal injections of IBO have been employed as a lesion procedure (Jarrard, 1989; Jarrard, 2002; Jarrard and Davidson, 1990; Jarrard et al., 2004). Since microphotographs of the resulting lesions are shown in these and other published papers, photographs of the complete hippocampus lesion will not be included here.

Atrophy of the hippocampus was present in all rats in the CHip Group, and as a result the ventricles appeared enlarged with slight distortion of the remaining adjacent structures. The only sparing of hippocampus was some remaining, normal looking cells in a limited unilateral area of dorsal CA1 in 2 rats and in ventral CA3 and CA1 in several other rats; however, these

small 'islands' of normal looking cells were few in number and were generally unilateral. While it is not known if these remaining neurons were functional, given the isolation of the cells and the limited number, one would guess they were nonfunctional.

Hippocampal ventral pole (VP) lesion—The CA1 and CA3 cells in the ventral hippocampus that comprise the ventral pole are shown in the coronal section in Figure 1A for an unoperated rat. The area of interest is the area of the hippocampus within the square. The images in Figure 1B are from a rat with similar bilateral injections and a 3-day survival. The cell loss shown in the figure is representative of that in most rats included in the ventral pole Group. As can be seen in Figure 1B, there was an extensive loss of CA3 and CA1 cells in the area of interest together with a proliferation of glial cells.

In several rats the cell loss included adjacent neurons in the subiculum but this extra damage was not great in amount and was unilateral. Of special concern was possible loss of cells in the amygdala but careful examination of the brains did not indicate that this structure was damaged.

Medial prefrontal (mPFC) lesion—There was clear, bilateral loss of neurons in the prelimbic, medial orbital cortex, and infralimbic cortical areas in 6 of the 8 rats in the mPFC Group. The cell loss in the 2 other rats was similar to that of the 6 rats in one hemisphere but there was less damage to the area of interest on the contralateral side. Statistical analyses of results for the mPFC group did not differ whether or not these two rats were included. Therefore, the data for these 2 rats were included in the overall analyses. As shown in Figure 2 (B) at three A-P levels, the mPFC lesion (identified with arrows) included considerable bilateral damage to the three main divisions of the medial prefrontal cortical area.

Body weight gain

Pre-surgical baseline—Rats were assigned to groups matched on mean ad libitum body weight (i.e. baseline) calculated two days prior to the beginning of surgery. Mean baseline body weights (gms) for each group were: CHip 318.56g, SEM = 3.38; CHip pair-fed controls 319.63g, SEM = 3.31; VP 319.21g, SEM = 2.17; VP pair-fed controls 319.00g, SEM = 2.63; mPFC 318.75g, SEM = 2.90; mPFC pair-fed controls, 318.94g, SEM = 2.94; Ad-lib fed controls 317.58g, 4.73. None of these differences were statistically significance ($F(6,51) < 1$).

Weight changes during post-operative recovery—Figure 3 shows mean body weight gain for each group compared to baseline on the day of surgery (Day 0) and at the end of each two-day period post-surgery until the lesioned groups achieved the same mean body weight (+/- 1 g) as the ad-lib fed controls—the point at which we considered post-operative recovery to be complete. As can be seen in that figure, the CHip,(left panel) VP (center panel) and mPFC (right panel) groups, and their pair fed controls, all exhibited weight loss on postoperative Day 2, with the largest weight loss being observed for the rats with CHip lesions and the smallest weight loss exhibited by the VP-lesioned group. An ANOVA evaluating the decrease in body weight for each of the lesioned groups from the day of surgery (Day 0) to post-operative Day 2, obtained significant main effects of Group, Day, and a significant Group × Day interaction smallest $F(2,25) = 16.68$, $p < .01$ for the main effect of Group). Analyses of simple main effects found no significant differences among the groups on Day 0 ($F(2,25) < 1$); However, the main effect of Group was highly significant on post-operative Day 2 ($F(2,25) = 23.12$, $p < .01$). Post-hoc Newman-Keuls test confirmed that significantly greater weight loss was sustained on Day 2 by the CHip-lesioned compared to both the mPFC- and VP lesioned groups (ps < .01), whereas the mPFC-lesioned group also showed greater weight loss than the VP-lesioned group on Day 2 ($p < .05$).

Figure 3 shows that the lesioned groups differed not only with respect to the amount of weight loss after surgery, but also with respect to the number of days that were needed for each group to complete post-operative recovery as defined by achieving a mean body weight that matched (+/- 1 g) the Ad lib-fed controls. The criterion was achieved on (postoperative) Day 20 for Group CHip, on Day 16 for Group VP and on Day 30 for the mPFC-lesioned group. Furthermore, pair-fed groups were returned to free access to food when the post-operative body weights of their corresponding lesioned rats returned to or exceeded the presurgical body weight recorded on Day 0. Based on this criterion, all of the Pair-fed controls for CHip group were given ad lib food beginning on post-operative Day 8, whereas ad lib feeding began on postoperative Day 4 and postoperative Day 6, for the pair-fed controls of the VP- and mPFC-lesioned, respectively. Because of these differences in the apparent nonspecific effects of lesions on post-operative body weight loss and recovery, subsequent analyses focused on comparing body weight changes for each lesion group with its Pair-fed and Ad lib Fed controls after post-operative recovery was deemed complete for each lesioned group.

An ANOVA comparing pre–recovery weight gain for the CHip-lesioned rats with that of their Pair-fed and the Ad lib-fed controls on Days 2-20 following surgery yielded significant main effects of Group ($F(2,19) = 18.4$, $p < .01$), Day ($F(10, 190) = 263.84$, $p < .01$) as well as a significant Group × Day interaction ($F(20,190) = 12.21$, $p < .01$). Post hoc analyses comparing the CHip-lesioned with their Pair-fed control groups during the same period obtained a significant main effect of Day ($F(9.126) = 233.72$, $p < .01$), indicating that both groups exhibited significant weight gain and also a significant Group × Days interaction ($F(9,126) = 1.96$, $p < .05$). Analyses of simple main effects showed that these two groups differed significantly only on post-operative Day 2, with weight loss greater for the lesioned group. The same type of analysis comparing Group CHip with the Ad lib-fed controls found that the main effects of Group ($F(1,12) = 24.10$, $p < .01$) Day ($F(9,12) = 199.28$, $p < .01$) and the Group × Day interaction ($F(9,108) = 25.70$, $p < .01$) were significant. Analyses of simple main effects revealed significant differences in mean body weight between the CHip group and the Ad-lib fed control group on post-operative Days 2-14 (largest $F(1,12) = 5.49$, $p < .05$ on Day 14) whereas these differences were not significant on post-operative Days 16-20.

ANOVA comparing VP-lesioned rats and controls from post-operative Days 2 -16 obtained significant main effects of Group and Day (smallest $F(2, 23) = 8.25$, $p < .01$ for Group) and a significant interaction between these two factors ($F(14,161) = 8.47$, $p < .01$). Analysis of simple main effects revealed that mean body weight gain for VP-lesioned rats was significantly higher than their Pair-fed controls on post-operative Days 12-16 (smallest $F(1,18) = 4.55$, $p < .05$ on Day 12). No differences between these groups on other days were significant. The same type of analysis showed that VP-lesioned rats weighed significantly less than Ad lib-fed controls on Days 2-8 (smallest $F(1,16) = 4.85$, $p < .05$ on Day 8), with no significance differences on postoperative Days 10-16.

Statistical evaluation of differences between mPFC-lesioned rats and controls obtained a significant effect of Day ($F(14,266) = 238.29$, $p < .01$) and a significant Group × Day interaction ($F(28,266) = 4.20$, $p < .01$), but no significant main effect of Group ($F(2,19) = 2.15$, $p < .01$). Subsequent analysis comparing only the mPFC group with their Pair-fed controls failed to yield either a significant main effect of Group ($F(1,14) < 1$) or a significant Group × Day interaction ($F(14,196) < 1$). In contrast, when this analysis compared only the mPFC and Ad lib fed groups, the main effect of Days ($F(14,168) = 132.78$, $p < .01$) and the Group × Day interaction ($F(14,168) = 5.54$, $p < .01$) were significant. Analyses of simple main effects showed that mPFC groups weighed significantly less than the Ad lib-fed control on post-operative Days 2-14 (smallest $F(1,12) = 4.95$, $p < .05$, Day 14). Differences on Days 16-30 were not significant.

Weight gain following post-operative recovery—Weight gain for the CHip, VP, and mPFC lesion groups were compared to their controls during the 20-day period beginning, for each group, immediately after their postoperative recovery was deemed complete (i.e., after the post-surgical body weight of the group matched that of the ad lib fed control). Figure 4 shows weight gain for each lesion group, relative to their pre-operative baseline, on the day that postoperative recovery was deemed complete (PR) and for the 20-days thereafter. Within each lesion condition, there were no significant differences between the body weights of the pair-fed and ad lib fed controls at the end of the recovery period. In, addition, ANOVA revealed no significant main effects of Group or Group × Day interactions for any comparison of pair-fed and ad lib-fed controls groups during the 20 days after recovery from surgery. Therefore, these data for the control groups were combined for the remaining analyses of body weight gain.

The left panel of Figure 4 indicates that rats with CHip lesions exhibited a faster rate of weight gain following post-operative recovery compared to their combined controls. Although the difference in mean weight gain for the two groups over all of the 20-day post recovery period did not achieve significance (main effect of Group, $F(1,20) < 1$), a significant main effect of Day showed that both groups exhibited significant weight gain during the post-recovery period and a significant Group × Day interaction ($F(10,200) = 2.58$, $p < .01$) confirmed that the rate of weight gain across days was significantly higher for Group CHip than for controls.

The middle panel of Figure 4 shows that rats with VP lesions gained more weight and gained weight faster than their combined controls. ANOVA confirmed these impressions by obtaining significant main effects of Group ($F(1,24) = 10.68$, $p < .01$) and Day $F(10,240) = 188.80$, $p< .01$) and also a significant Group × Day interaction ($F(10,240) = 7.57$, $p < .01$).

Little difference in either rate or amount of weight gain was observed when Group mPFC was compared with their combined controls (see right panel of Figure 4). ANOVA yielded a significant main effect of Day ($F(10,200) = 176.87$, $p < .01$) confirming that both groups gained weight across the 20-day period following recovery from surgery. However, the failure to obtain a significant main effect of Group ($F(1,20) < 1$) or a significant Group × Days interaction ($F(10,200) <1$) provides no evidence that mPFC lesions had effects on body weight that were different from the control treatments.

Food Intake

Amount eaten (in kcals) was recorded at the end of each 2-day block during the 20 days following post-operative recovery for each group. Comparisons of the Pair-fed and Ad lib-fed controls for each respective surgical treatment yielded no significant main effects of Group or Group × Day interactions. Thus, the control groups for each respective lesion group were combined for the analyses of the effects of CHip, VP, and mPFC lesions on food intake.

Figure 5 shows mean amount of food consumed by the lesioned groups and the combined control group on each 2-day block that took place over the first 20-days following post-operative recovery. Both CHip and VP lesioned rats ate more than their respective controls. Little difference was observed for mPFC lesioned rats relative to their controls. An ANOVA comparing CHip rats with their controls yielded a significant main effect of Group ($F(1,20) = 16.54$, $p < .01$), whereas the Group × Block interaction was not significant ($F(10,200) = 1.11$, $p >.35$). Similarly, a significant main effect of Group ($F(1,23) = 6.76$, $p < .01$) but no Group × Block interaction ($F(10,230) <1$) was found when the VP-lesioned group was compared to their controls. In contrast, when the mPFC-lesioned rats and their controls were compared, neither the main effect of Group nor the Group × Block interaction achieved significance (Fs < 1).

Conditioned Appetitive Behavior

ANOVA comparing the previously pair-fed control groups for each lesion condition obtained no significant main effects or interactions involving Group. Thus, these controls were combined for subsequent comparison with the lesioned groups.

The results depicted in the left portion of each panel in Figure 6 show that conditioned appetitive responding was elevated at the outset of training under 0-hr and throughout training under 24-hr food deprivation for rats with CHip lesions compared to controls and to rats with VP and mPFC lesions. In addition, only CHip lesioned rats responded more under 24-hr than under 0-hr food deprivation by the end of reinforced training. ANOVA of these data obtained a significant main effect of Group ($F(3,48) = 7.9$, $p < .01$) and significant Group × Deprivation level ($F(3,48) = 4.3$, $p < .01$) and Group × Deprivation level × Sessions ($F(15,240) = 2.6$, $p < .01$) interactions. Newman-Keuls tests found that rats in Group CHip showed significantly more conditioned appetitive responding overall (all ps < .01) and significantly more responding under both the 0-hr (all ps < .05) and the 24-hr (all ps < .05) food deprivation levels compared to each of the groups VP, mPFC, and controls and that none of these latter three groups differed significantly from one another. Post-hoc analysis of discriminative responding revealed that on the first two sessions of training under each deprivation level Groups VP, mPFC, and controls responded significantly more on nonreinforced trials under 0-hr food deprivation than on reinforced trials under 24-hr food deprivation, whereas this pattern of responding was obtained for Group CHip only when the first session under each deprivation level was compared. Responding more on nonreinforced compared to reinforced trials is common at the outset of discrimination training when overall response strength is increasing and rats have not differentiated among the discriminative stimuli. In contrast, significantly greater responding on reinforced (24-hr food deprivation) compared to nonreinforced (0-hr food deprivation) trials emerged for Group CHip (ps < .05 for sessions 3, 5 and 6).

The purpose of extinction was to assess whether tendencies to respond more under 24- compared to 0-hr food deprivation, that were not apparent (i.e., were latent) at the conclusion of reinforced training would emerge on nonreinforced trials during extinction. The right portion of each panel in Figure 6 shows that response strength decreased for all groups under each deprivation level across extinction trials, an effect that was confirmed statistically by a significant main effect of sessions ($F(4,192) = 37.50$, $p < .01$). Furthermore, the higher level of responding exhibited by Group CHip on trials under 24-hr compared to 0-hr food deprivation at the end of training, was largely maintained across extinction testing. Each of the remaining groups that did not respond differentially based on deprivation level at the end of training, did exhibit some tendency for latent discrimination, by coming to respond more on sessions under the previously reinforced 24-hr food deprivation level, compared to sessions under 0-hr deprivation.

ANOVA for the extinction test data obtained a significant Group × Deprivation level interaction ($F(3,48) = 6.82$, $p < .01$). Newman-Keuls tests showed that Groups CHip and mPFC responded significantly more during extinction under 24-hr compared to 0-hre food deprivation (ps <.05), whereas this difference was not significant for either Group VP or the controls. However, separate ANOVAs comparing each lesioned group with controls yielded a significant Group × Deprivation level interaction only for the comparison of Group CHip with controls ($F(1,30) = 16.41$, $p< .01$). This indicates that the magnitude of the difference between responding on 24-hr versus 0-hr deprivation was significantly larger compared to controls only for CHip lesioned rats. Although Group CHip showed significant differential responding during extinction, this can't be termed latent discrimination as it was also apparent at the end of training.

Body weight under 0-hr and 24-hr food deprivation

During appetitive conditioning, the body weights of the rats in each group were recorded immediately prior to the beginning of each training (sessions 1-6) and extinction (sessions 7-11) session under 0- and 24-hr food deprivation. Although the rats received 0- and 24-hr deprivation sessions in a irregular order. Figure 7 segregates mean body weights recorded for each group on 0-hr deprivation sessions (left panel) from those recorded on 24-hr food deprivation sessions (right panel).The left panel of Figure 7 shows that mean body weight prior to sessions under 0-hr food deprivation remained relatively stable for rats in the CHip group, but decreased across session for the rats in the VP, mPFC, and control conditions. The rate of decrease in body weight appeared to be largest for rats in the control group. On the last 0-hr session, mean weight was highest for the rats with CHip lesions, followed respectively by the VP, the mPFC, and Control groups.

ANOVA obtained significant main effects of Group ($F(3,48) = 3.05$, $p < .01$), Session ($F(10,480) = 71.64$, $p < .01$) and a significant Group × Sessions interaction ($F(30,480) = 6.83$, $p < .01$). Subsequent analyses comparing CHip-lesioned rats with controls obtained a significant Group × Sessions interaction ($F(1,30) = 24.24$, $p < .01$.). Analyses of simple main effects found that mean body weight for CHip-lesioned rats did not differ from controls on the first session under 0-hr food deprivation ($F(1,30) = 1.21$, $p > .28$), but that this difference was highly significant on the last 0-hr food deprivation session ($F(1,30) = 14.26$, $p < .01$). In contrast, the same comparison between VP-lesioned and control rats yielded a significant main effect of Group ($F(1,34) = 5.90$, $p < .05$), but lacked a significant Group × Sessions interaction indicating that the magnitude of the this difference was about the same across sessions. Neither the main effect of Group nor the Group × Session interaction achieved significance when the mPFC and control rats were compared across the first and last 0-hr food deprivation session.

The right panel of Figure 7 shows mean body weight for each group prior to each session under 24-hr food deprivation. Rats in Group VP weighed more than each of the other groups, which did not differ, on the first session under 24-hr food deprivation. Although all groups lost weight with repeated 24-hr deprivation sessions, the amount of weight reduction appeared to be less for the rats with CHip lesions, than for the rats in the VP, mPFC, and Control conditions, such that by the last of these sessions, the highest mean body weight was shown by the CHip group. An overall ANOVA obtained a significant main effect of Sessions ($F(10,480) = 141.32$, $p < .01$) and a significant Group × Sessions interaction ($F(30,480) = 5.34$, $p < .01$). A separate analysis comparing the CHip and control rats also yielded a significant Group × Sessions interaction ($F(10,300) = 12.05$, $p < .01$). Simple main effects analysis showed that mean weight for these groups did not differ on the first session of 24-hr food deprivation ($F(1,30) < 1$), but differed significantly on the last session under 24-hr food deprivation ($F(1,30) = 4.18$, $p < .05$). The same analysis comparing the VP lesioned group with controls obtained significant main effects of Group and Session (smallest $F(1,34) = 5.32$, $p < .05$, for Group), and no significant interaction. An ANOVA comparing the mPFC and control groups obtained only a significant main effect of sessions ($F(10,300) = 89.98$, $p < .01$).

Indirect calorimetery

We assessed differences in energy expenditure in the CHip-, VP-, mPFC-lesioned and controls. Because of equipment limitations, the control group for this and all remaining analyses were composed of two rats from the Pair-fed control of each lesion group and two rats from the Ad lib-fed control condition. The two rats selected from each control group were the rats that were closest to the mean body weight of all rats in each respective control condition.

We found that CHip-lesioned rats exhibited significantly increased rates of energy expenditure (as measured by O2 consumption) relative to other groups (See Figure 8). The left panel of

Figure 9 shows cumulative energy expenditure during the 12 hour dark period. The right panel depicts cumulative energy expenditure across the 24-hr light/dark cycle. Specifically, rats in Group CHip had higher levels of O2 consumption during the dark phase of the diurnal cycle, when rats are most active. On dark-phase energy expenditure, Dunnett's individual comparisons yielded a significant difference between CHip and CON groups ($q = 2.57$, $p < .05$). Neither VP nor PF rats were significantly different than CON rats. There were no significant differences during light-phase for total energy expenditure.

Body composition analysis

Figure 9 shows the final body weight (left panel) and final total body adiposity (right panel) for each group. This weight was recorded approximately 150 days following surgery. As seen in that figure, rats in Groups VP and CHip had higher body weights relative to rats in the mPFC and control groups. One-way ANOVA on final body weight confirmed a significant main effect of Group ($F(3,35) = 3.46$, $p < .05$) and follow-up Dunnett's tests revealed that both VP and CHip lesioned rats weighed significantly more than control rats. Additionally, there was a trend for increased body adiposity in each of these two lesioned groups, relative to control rats. However, these differences were not confirmed statistically by ANOVA or by individual comparisons (ps > .05).

Behavioral activity in the homecage

Rats with CHip lesions exhibited increased levels of home cage activity, relative to all other groups. The left panel of Figure 10 depicts mean cumulative (24-hr) photobeam breaks. As seen in that figure, rats in group CHip exhibited an approximate doubling of locomotor activity relative to groups CON, VP and PF. These difference were confirmed statistically by one-way ANOVA ($F(3,35) = 8.16$, $p < .05$). Dunnett's individual comparisons showed that activity levels in group CHip were significantly greater than the mPFC, VP, or controls.

We have previously hypothesized that increased activity levels in CHip-lesioned rats may be due in part to conditioned responses based on the availability of food (Benoit et al., 1999; Davidson and Jarrard, 2004). It is well-known that rats exhibit increased activity levels in the presence of discrete and contextual stimuli that predict the availability of food. It is also well-known that general activity levels follow a predictable circadian rhythm that can be entrained based on the delivery of food to food-deprived rats. We predicted that at least part of the increased activity levels would be based on contextual cues that predict the availability of food and, further, that hippocampal rats would be impaired at inhibiting such cue-driven conditioned responses. Consistent with this we observed that rats in Group CHip spent significantly more time near the food cup than controls. The right panel of Figure 10 depicts the amount of time (number of beam breaks across time) rats spent in the front half of the cage containing the food hopper. While rats in all surgical groups exhibited increased percent time in the food-hopper containing side of the cage, this difference was statistically significant only when Group Chip and controls ($t = 2.27$, $p < .05$, one-tailed) were compared.

Discussion

Recent accounts propose that (a) environmental food cues will tend to evoke eating until that behavior is inhibited by biological control mechanisms and (b) obesity may be more prevalent because these biological control mechanisms are failing (Berthoud, 2004b; Prentice, 2005)). What these control mechanisms might be, and why they fail are two questions fundamental to understanding, and ultimately controlling, continuing trends toward increased body weight and obesity in the human population. Much previous work aimed at addressing these questions has focused on hypothalamic control mechanisms and on identifying direct effects of changes in regulatory neuropeptides (e.g., leptin, CCK, ghrelin, etc.) and their receptors. By showing that

damage to the hippocampus, a brain structure considered to be an important substrate for learning and memory, interferes with the control of food intake and body weight, the present findings encourage us to think about energy dysregulation, not solely as a deficit in some type of hypothalamic signaling system but, at least in part, as a disorder of higher-order learning and memory functioning (Davidson et al., 2007; Davidson et al., 2005).

There is wide agreement that learned cues can exert strong control over appetitive and consummatory behavior. This control depends, in part, on the formation of simple associations between food-related conditioned stimuli (CSs) and highly salient appetitive unconditioned stimuli (USs) that are produced as a consequence of eating (Berthoud, 2004a; Davidson et al., 2005; Davidson and Swithers, 2004; Holland and Petrovich, 2005; Sclafani, 1997; Woods and Ramsay, 2000). A food-related CS comes to promote the performance of appetitive and consummatory responses by exciting or activating a representation of its appetitive US in memory (Bouton and Moody, 2004). This type of simple association formation does not appear to depend on the hippocampus as animals with the complete hippocampus removed are not impaired at learning that discrete CSs signal delivery of appetitive USs, or at solving simple discriminations where an event always signals reinforcement and another event is always nonreinforced (Benoit et al., 1999; Han et al., 1995; Squire, 1992).

Although not necessary for the formation of simple associations, the hippocampus appears to be involved with the performance of certain higher-order learning and memory operations. Morrris (2006) noted that several modern accounts converge on the idea that one function of the hippocampus is to solve problems that involve “predictable ambiguity”. These problems often require animals to learn that the relationship between an event and a particular outcome varies depending upon the presence or absence of other events or conditions. For example, animals with the hippocampus removed often show deficits in appetitive problems (e.g., extinction, discrimination-reversal, feature-negative discrimination, working memory) where performance depends on learning to refrain from responding to cues that are, under some conditions, reliable signals for reinforcement (Berger and Orr, 1983; Chan et al., 2003; Holland and Bouton, 1999; Jarrard et al., 2004). In these cases, it may be that hippocampal damage reduces the ability of animals to inhibit their appetitive behavior by impairing their ability to learn or remember when events will not be followed by reinforcing outcomes.

In the present experiment, damage to the complete hippocampus was not only accompanied by greater food intake and body weight gain, but also by increased appetitive responding in the conditioning apparatus, especially on trials under 24-hr food deprivation, and elevated behavioral activity in the home cage, especially in the vicinity of the food magazine, when the rats were fed ad libitum. Although heightened appetitive responding could be indicative of stronger simple excitatory appetitive conditioning, elevated appetitive performance can also be a consequence of impaired inhibitory learning. As discussed elsewhere (e.g., Benoit et al., 1999) in Pavlovian conditioning, apparatus cues are both reinforced at the time of US delivery and nonreinforced during periods prior to presentation of the US. This could make apparatus cues ambiguous predictors of reinforcement. Intact rats could use handling or temporal cues to determine when to respond and to inhibit their responding to the apparatus cues. However, if removing the hippocampus interferes with the ability to use such contextual cues as signals that predict the nonreinforcement of apparatus cues, then weaker inhibition of responding to the apparatus cues would be expected. Furthermore, consistent with the training contingencies, if the expectation of receiving the sucrose pellet US was greater under 24-compared to 0-hr food deprivation, the effects of impaired inhibition would be more obvious when the rats were under the higher level of food deprivation—the outcome obtained in the present experiment.

Similarly, background cues in the home cage were presumably associated with a strong appetitive postingestive US when rats were hungry (e.g., prior to a meal) but not when they

were food sated (e.g., after eating). Under these circumstances, the rats could use interoceptive cues produced by satiety to signal when food and food-related cues in the apparatus will not be followed by postingestive reinforcement. Increased activity on the part of CHip-lesioned rats, relative to controls, is consistent with the hypothesis that CHip lesions reduced the ability of satiety cues to signal the nonreinforcement of food cues, and thus to inhibit behaviors evoked by those cues. The finding that our CHip-lesioned rats spent significantly more time than controls on the side of the apparatus where food was delivered, indicates that some, if not all, of the increased homecage activity exhibited by CHip-lesioned rats was attributable to heightened appetitive behavior (Tracy et al., 2001).

A general feature of this analysis is the assumption that the decision to eat or refrain from eating may involve higher-order or conditional learning processes that would help animals predict when food CSs are followed by an appetitive (pleasant or satisfying) postingestive US and when they are not (Davidson et al., 2007; Davidson et al., 2005). Given that survival depends on efficiently performing many behaviors (e.g., reproduction, defense, driving in rush hour traffic) in addition to procuring and consuming food, it would be highly adaptive if the ability of food CSs to excite memories of appetitive outcomes which promote food-seeking and eating responses was inhibited during times of food satiation. The present analysis is consistent with the idea that the performance of this adaptive function could depend on the hippocampus.

Could hippocampal dysfunction contribute to current global trends toward increased obesity in humans? Compared to the well-known and dramatic increases in food intake and body weight that accompany other types of experimental manipulations, such as lesioning the hypothalamus or genetic mutations (King, 2006; Lindstrom, 2007; Tschop and Heiman, 2001), the effects of hippocampal lesions on food intake and weight gain that are reported here may seem modest. However, very few humans show dramatic increases in food intake and body weight like those shown by hypothalamic-lesioned or genetically-altered rodents. One could argue that the gradual increase in body weight seen in our rats makes them more similar to the current U.S. human population, which has exhibited about a 10% increase in body weight over the past 10 years (Lewis et al., 2000).

Clear links between the function of the hypothalamus and recent increases in the incidence of obesity in the general population have not yet been identified. For example, there are relatively few cases of overweight or obese humans that can be attributed causally to hypothalamic pathologies or genetic mutations in hypothalamic signaling systems (Eikelis et al., 2007; Pinkney et al., 2002). Thus, although surgical, genetic, and other manipulations of the hypothalamus may have profound effects on energy regulation in laboratory settings, it is not yet clear how these manipulations are related to the reduced regulatory control that is occurring outside of the laboratory.

A potential link between the hippocampus and energy dysregulation in humans is suggested by evidence that dietary manipulations known to promote excessive food intake and body weight also disrupt hippocampal-dependent learning and memory processes. For example, Molteni et al., (Molteni et al., 2002) reported that rats maintained for 60 days on a diet high in saturated fat and sucrose, showed impaired hippocampal-dependent spatial memory in a Morris water maze compared to rats maintained on normal (low-fat, high-carbohydrate) lab chow. Similarly, Kanoski et al (Kanoski et al., 2007) found that giving rats 90-day ad libitum access to a diet high in saturated fat and dextrose had long-term detrimental effects on performance in Pavlovian conditioning tasks (reversal learning and extinction) that depend on the hippocampus or prefrontal cortex. These same rats did not exhibit performance deficits on a simple discrimination task that does not require an intact hippocampus or prefrontal cortex. Consistent with this general analysis, deficits in performance on hippocampal-dependent

spatial learning problems are also observed in rat models of obesity (Matsushita et al., 2003; Nomoto et al., 1999; Winocur et al., 2005).

Furthermore, Molteni (Molteni et al., 2002) reported that spatial memory deficits by rats maintained on the high-fat diets, were accompanied by reduced levels of hippocampal brain-derived neurotrophic factor (BDNF). Kanoski et al (Kanoski et al., 2007)) also found that BDNF was significantly reduced in the ventral hippocampus and medial prefrontal cortex, but not in the dorsal hippocampus, in rats that showed deficits in nonspatial reversal and extinction performance following maintenance on the high-fat diet. BDNF contributes to the survival, growth, and maintenance of many types of neurons (Allen and Dawbarn, 2006; Nottebohm, 2004) and is thought to contribute to hippocampal long-term potentiation (LTP) and neurogenesis ((Bramham and Messaoudi, 2005; Lee et al., 2002; Rossi et al., 2006; Wibrand et al., 2006). Both of these processes have been described as important mechanisms for hippocampal-dependent forms of learning and memory (Dalla et al., 2007; Gruart et al., 2006; Kitabatake et al., 2007; Whitlock et al., 2006). It may be that the ability of high-fat diets to promote increased food intake and body weight gain occurs as a consequence of interfering with the same hippocampal-dependent mechanisms that were disrupted by hippocampal lesions in our present experiment.

Obviously, hippocampal damage could also influence behavior by interfering with processes that do not involve learning and memory. In the present study, indirect calorimetry revealed that metabolic activity during the dark phase of the light-dark cycle was elevated for rats with CHip lesions compared to controls. It may be that this increased energy expenditure was a byproduct, at least in part, of the increased appetitive behavioral activity exhibited by rats with CHip lesions. However, heightened metabolism might have also been induced, in part, by the increased food intake on the part of the CHip-lesioned rats. Several studies have shown that metabolism increases, perhaps as a counterregulatory response, when animals are forced to consume calories in excess of their metabolic needs ((Balkan et al., 1993; Harris et al., 2006; Shibata and Bukowiecki, 1987; Weyer et al., 2001). It may be that increased metabolism is an effect of excess caloric intake that was difficult for rats with CHip lesions to control. However, in the present study increased metabolism was not enough to abolish weight gain on by rats with CHip lesions.

In addition, a relatively unexplored possibility is that the disturbances in energy regulation reported here involve a reduction in direct sensing by the hippocampus of nutrients or peripheral factors that regulate energy balance. As mentioned previously, the hippocampus expresses many of the same receptors (e.g., insulin, leptin, ghrelin and CCK) that are thought to be important for energy balance in the hypothalamus and brainstem. Thus, in our rats with hippocampal lesions, the sensing or relaying of this information may have been damaged contributing to increased food intake and/or body weight gain. Similarly, it is conceivable that intake of diets high in saturated fat could also interfere with this type of hippocampal functioning. An intriguing possibility is that selective genetic deletion of hippocampal nutrient or hormonal receptors might result in changes in energy balance as well. Consistent with this idea, Irani et al., (2007) reported that intake of a high-fat diet is associated with reduced insulin binding in the hippocampal CA1 cell field of rats.

Rats with lesions confined to the hippocampal ventral pole also ate significantly more and gained significantly more weight relative to their controls. However, unlike rats with the complete hippocampus removed, VP lesions were not associated with significant increases in appetitive behavior, general activity, or metabolism. Furthermore, compared to rats with CHip lesions, weight gain for rats with VP lesions appeared to increase faster, relative to their controls, during the post-surgical recovery period and during the first 20 days after complete recovery from surgery. Despite exhibiting greater initial weight gain, the magnitude of the

increase in food intake for VP-lesioned rats relative to controls appeared smaller than that observed for rats with CHip lesions. The finding that rats with VP lesions recovered from surgery more rapidly than CHip-lesioned rats could reflect that the debilitating effects VP surgery subsided more rapidly compared to the debilitation produced by much more extensive lesions of the complete hippocampus. These differences in recovery may have allowed the facilitating effects of VP lesions on intake and body weight gain to emerge more quickly compared to CHip lesions.

In the present study the damage produced by the CHip lesion encompassed all of the hippocampus including the ventral pole. The ventral pole lesion was relatively small by comparison (injection of IBO at 30 sites for the CHip lesion compared to 4 sites for the VP lesion). While the intent with the VP lesion was to remove all of the cells that comprise the ventral pole, it is possible that there was some sparing of the relevant cells in this group compared to the damage found in the CHip lesioned rats. Thus, differences in the effects of the two types of lesions on energy and body weight regulation can not be attributed solely to a common disruption of direct connections between the VP and the lateral hypothalamus. Further, It may be that the greater effect of the CHip lesion reflects interference with learned behavioral control processes in addition to those mediated by the hippocampal ventral pole-lateral hypothalamic circuit

On the other hand, it is possible that VP lesions interfered with the same learning and memory mechanisms as did CHip-lesions, but that the magnitude of this interference was smaller for VP-lesioned rats. It is difficult to evaluate the above possibilities since the effects of lesions confined to the VP on learning and memory, including occasion setting and similar hippocampal-dependent processes, have not yet been thoroughly studied.

It is also the case that rats with neurotoxic lesions of the medial prefrontal cortex did not differ from their controls with respect to any of the measures (e.g., intake, appetitive behavior, body weight gain, etc.) that were recorded in the present experiment. These rats required more time than rats with either CHip- or VP-lesions to achieve the criterion for post-operative recovery. However, it is not clear whether this effect was a consequence of greater general behavioral debilitation produced by mPFC lesions or weaker facilitation of eating and appetitive behavior, compared to CHip and VP lesions. The latter possibility seems likely based on the finding that during the post-recovery period, neither mean food intake nor weight gain for mPFC rats differed significantly relative to their controls.

Our findings that mPFC lesions had no significant effects on intake or weight gain is noteworthy for several reasons: first, the area of the mPFC that was lesioned in this experiment was the same area that showed reduced levels of BDNF following exposure to a maintenance diet high in saturated fat and dextrose (Kanoski et al., 2007). Given that destruction of this area had little impact on energy regulation in the present study, this suggests that the excess intake and weight gain exhibited by rats maintained on the high fat + dextrose diet used in the study by Kanoski et al were not based on the effects of that diet on functions performed by the medial prefrontal cortex or by neural circuits that include this area of the brain. Second, the lack of effects of mPFC lesions on intake and body weight gain that we observed is consistent with another report that rats with lesions of the medial prefrontal cortex, albeit at a site slightly (but perhaps importantly-see below) ventral to the site of the mPFC lesions used in the present experiment, showed no differences in home cage food intake or in body weight relative to controls ((Petrovich and Gallagher, 2007).

However, previous studies have shown that rats with lesions that include the ventral mPFC exhibited less “conditioned stimulus potentiated eating” when either discrete CSs or contextual cues that were trained to predict food when the rats were hungry, are presented when the rats

are subsequently food sated (Petrovich and Gallagher, 2007; Petrovich et al., 2007). In the present study, rats with mPFC lesions did not differ significantly from controls with respect to their appetitive responding to contextual cues in the training apparatus, under either food deprived or nondeprived conditions. However, in addition to differences in exact location of medial prefrontal cortex damage, the present experiment also employed different food deprivation manipulations and training procedures compared to the earlier studies. It is possible that the different lesion effects reported in these experiments might be reconciled if rats were tested under more similar lesion or training conditions. In any event, the results of the present study provide no compelling evidence that energy and body weight regulation depends on the structural integrity of the medial prefrontal cortex or on any hippocampal-prefrontocortical neural pathway.

Conclusions

Previous research shows that the hypothalamus, especially the arcuate nucleus, contains receptors that are involved with the detection of a variety of neurohormonal hunger, satiety, and adiposity signals. The identification of these signals and their receptor sites has contributed much to our understanding of the control of food intake and body weight regulation. However, the question of how the detection of these cues is translated into adaptive behavioral outcomes has often been addressed by little more than an arrow in a diagram (e.g.,Berthoud, 2003; Woods and Seeley, 2000). The results of the present study suggest that to more fully understand the mechanisms that underlie energy and body weight regulation it may be necessary to describe how the operation of neurohormonal signaling systems that depend on the hypothalamus are integrated with higher-order learning and memory processes that depend on the hippocampus.

In the present study we found that destruction of the complete hippocampus in the rat is accompanied by increased food intake, body weight gain, appetitive behavior, metabolic, and general behavioral activity, whereas the effects of damaging the hippocampal ventral pole were limited to increased food intake and body weight gain. We suggested that the operation of higher-order, hippocampal-dependent learning and memory processes may underlie the ability of interoceptive satiety signals and perhaps other types of conditional cues to inhibit appetitive and consummatory responding evoked by food and food-related environmental stimuli. Within this model, damage to the hippocampus could therefore interfere with the inhibition of appetitive and eating behaviors. Thus, the question of “how” physiological satiety signals inhibit food intake and reduce body weight gain may be addressed, in part, with reference to learning and memory mechanisms that depend on the hippocampus. As others have suggested, improved understanding of the functional links between the neural controls of food and drug intake and the operation of higher-order learning and memory processes may be key to developing effective therapeutic interventions that can combat obesity (Berthoud, 2002; Moran and Gao, 2006).

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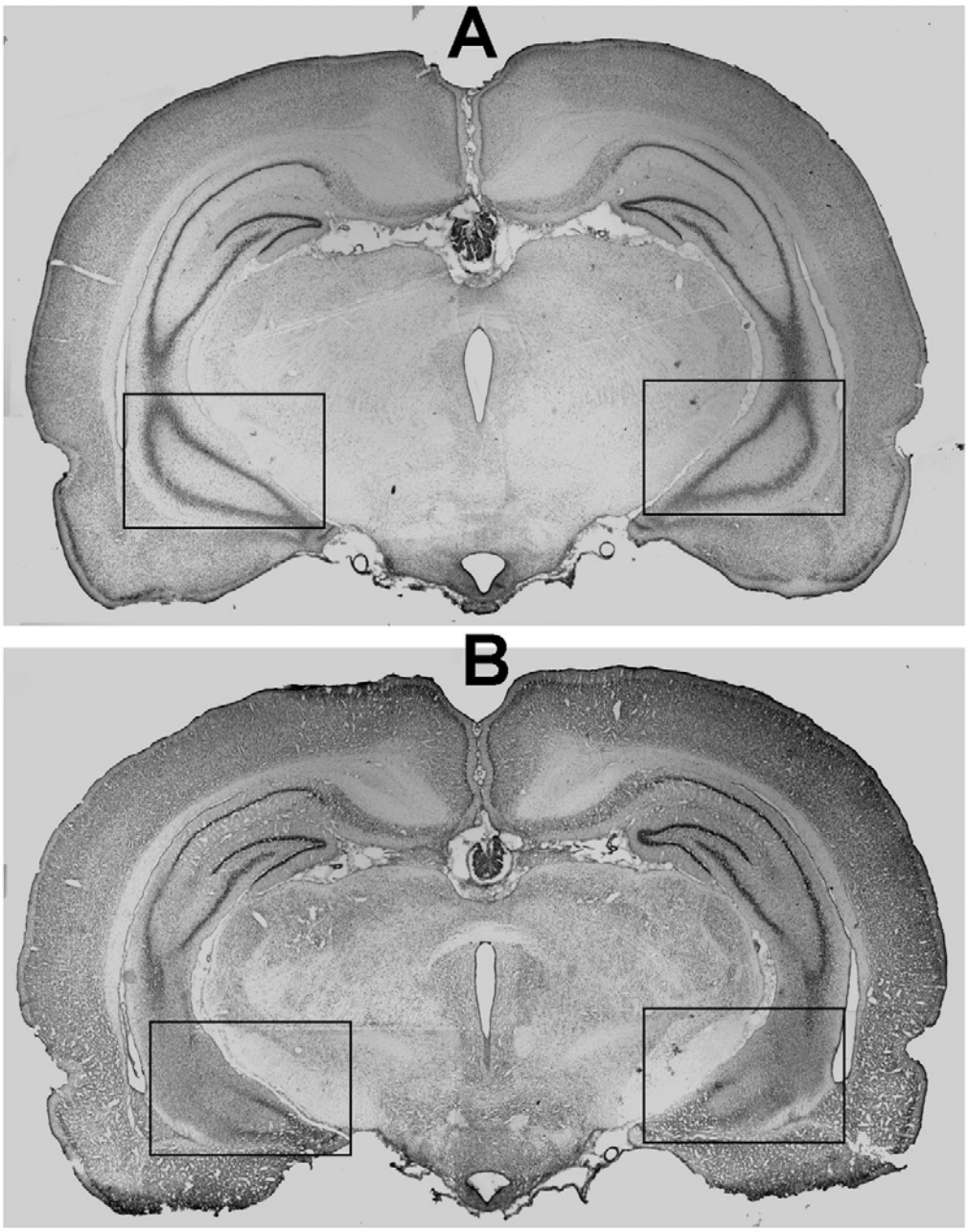

Figure 1.
Photomicrographs of a coronal section from an unoperated rat (A), and the cell loss in a representative rat that received the bilateral ventral pole (VP) hippocampal lesion with a 3-day survival (B). The hippocampal ventral pole is the area outlined with boxes.

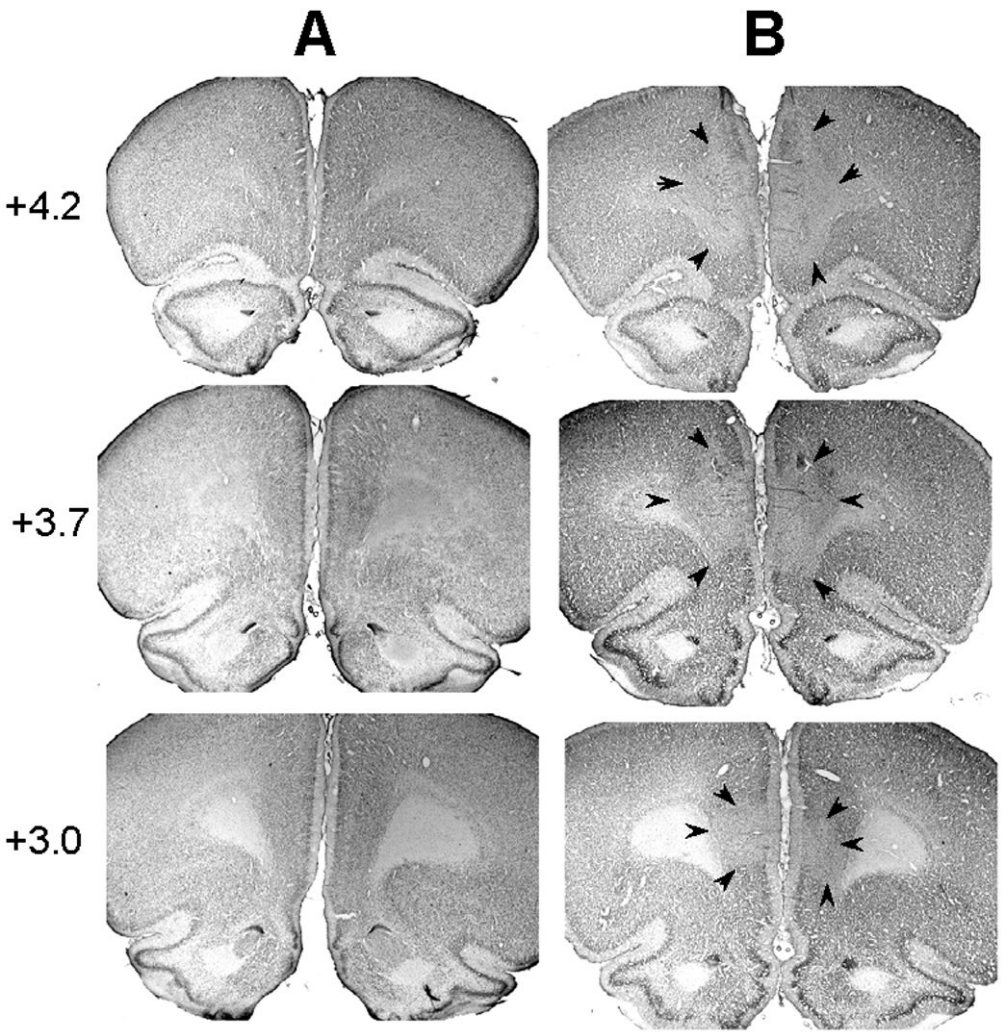

Figure 2.
Photomicrographs showing the medial prefrontal cortex (mPFC) at three anterior-posterior levels from an unoperated rat (A) and a lesioned rat (B). The mPFC and the area of cell loss is identified with arrows in B.

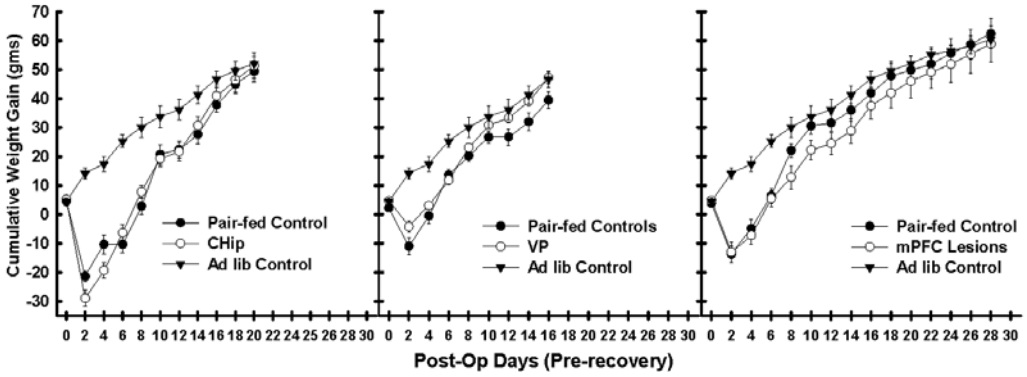

Figure 3.

Mean weight gain by rats with CHip, VP, and mPFC lesions and their pair-fed and adlib controls during pre-recovery period beginning immediately after surgery and ending when mean body weight for the lesioned groups achieved the level of their ad lib controls.

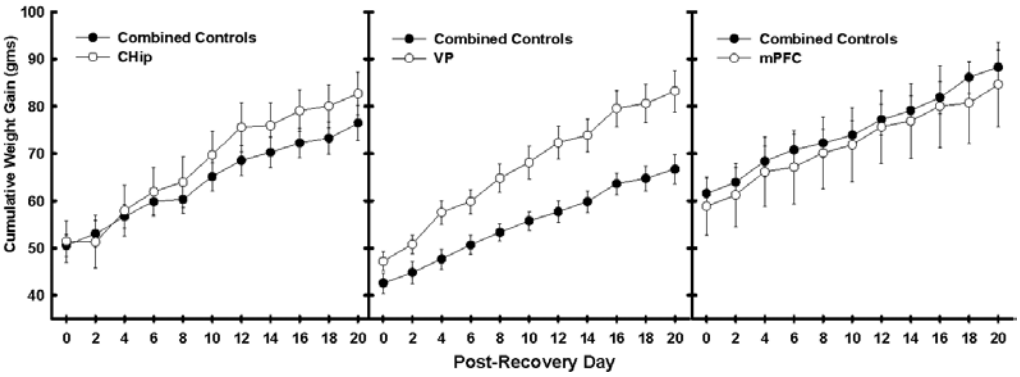

Figure 4.

Mean weight gain by rats with CHip, VP, and mPFC lesions and their combined controls (pair-fed and adlib) during post-recovery period which began after mean body weight for the lesioned groups achieved the level of their ad lib controls.

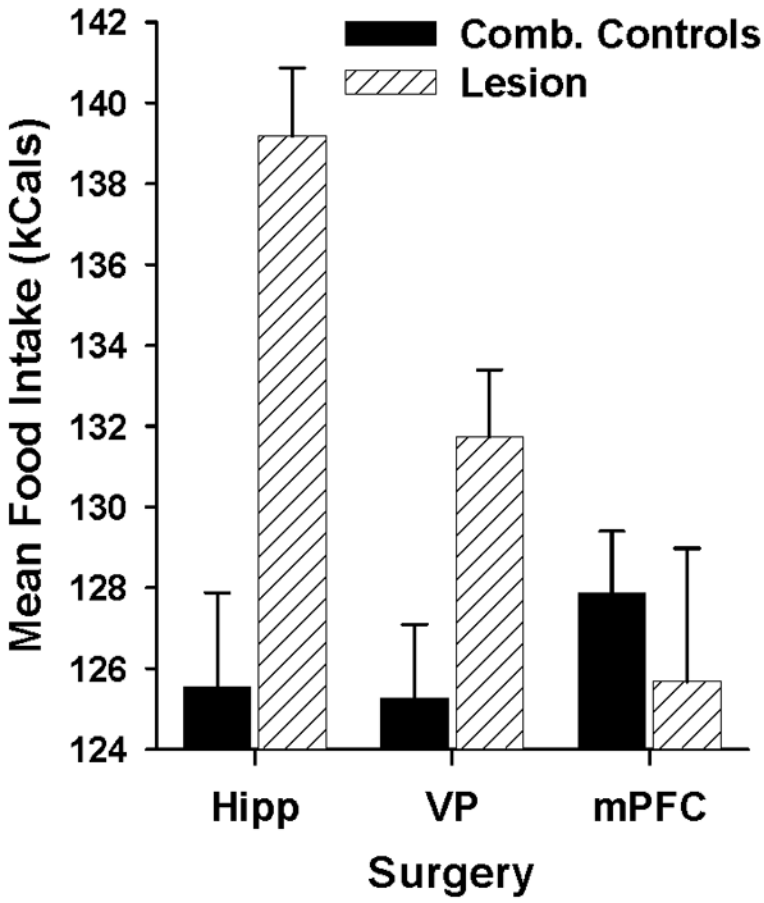

Figure 5.
Mean food intake (in kcals) by rats with CHip, VP, and mPFC lesions and their combined controls (pair-fed and adlib) during post-recovery period which began after mean body weight for the lesioned groups achieved the level of their ad lib controls.

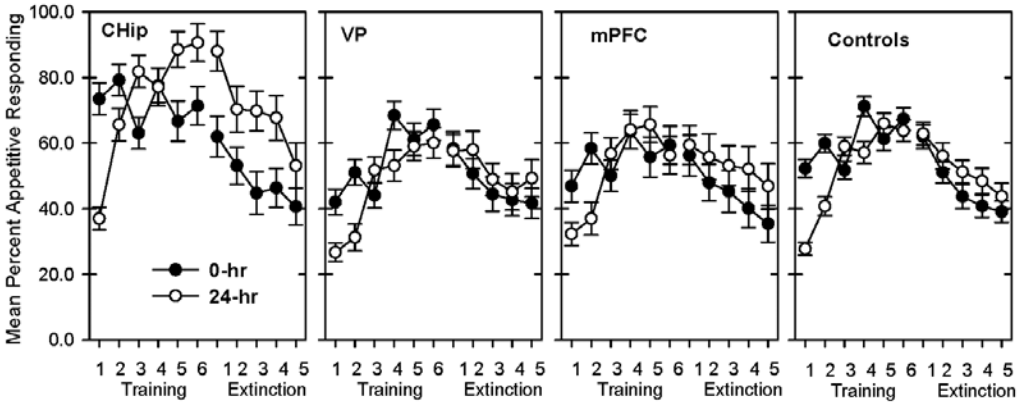

Figure 6.

Mean conditioned appetitive responding during training (data on left of each panel) and extinction (data on the right of each panel) on under 24-hr (reinforced during training and nonreinforced during extinction) and 0-hr (nonreinforced during training and extinction) food deprivation by rats with CHip (leftmost panel), VP (left-center panel), and mPFC (right-center panel) lesions and their combined (pair-fed controls (rightmost panel).

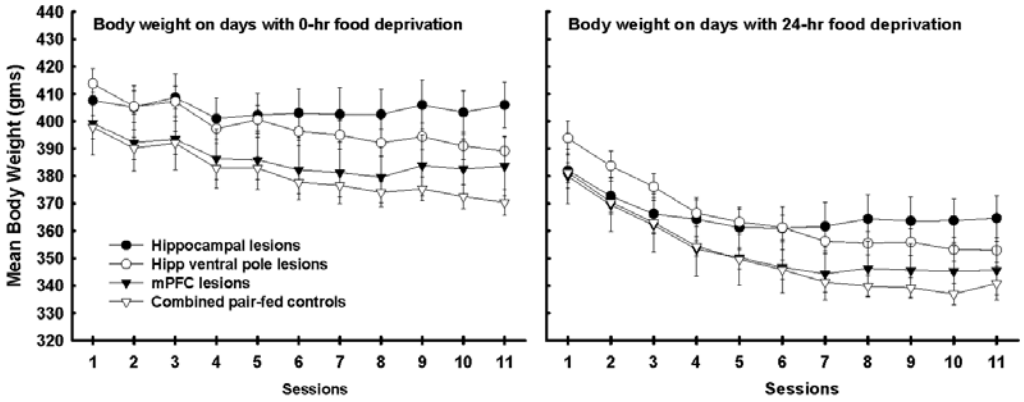

Figure 7.

Mean body weight prior to training (sessions 1-6) and extinction (sessions 7-11) sessions under 0-hr and 24-hr food deprivation for rats with CHip, VP, and mPFC lesions and their combined (pair-fed) controls. Although the rats received 0- and 24-hr deprivation sessions in a irregular order, in the figure mean body weights recorded for each group on 0-hr deprivation sessions (left panel) are segregated from those recorded on 24-hr food deprivation sessions (right panel).

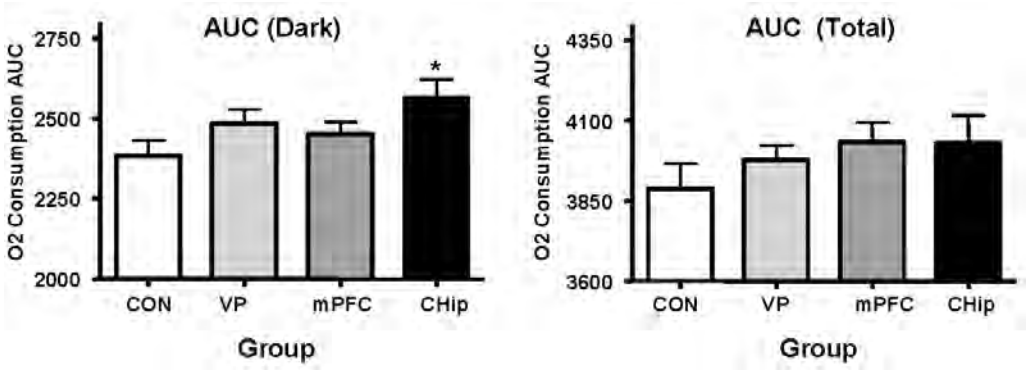

Figure 8.

Mean energy expenditure (O2 consumption, area under the curve (AUC) during the dark phase of the light/dark cycle (left panel) and in total (right panel) by rats with CHip, VP, and mPFC lesions and controls.

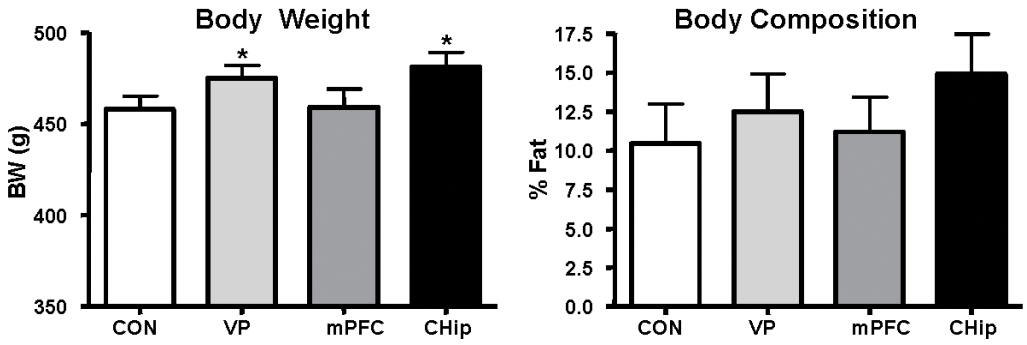

Figure 9.
Mean body weight (left panel) and mean body adiposity (right panel) approximately 150 days post-surgery by rats with CHip, VP, and mPFC lesions and controls.

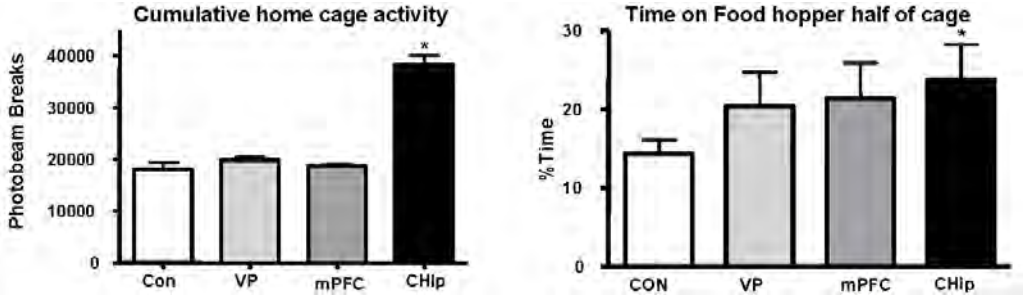

Figure 10.

Mean behavioral activity (left panel) and mean percent time spent on the half of the chamber where the food hopper was located (right panel) during the dark phase of the light/dark cycle by rats with CHip, VP, and mPFC lesions and controls.

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THE ROLE OF OREXIN-A IN FOOD MOTIVATION, REWARD-BASED FEEDING BEHAVIOR AND FOOD-INDUCED NEURONAL ACTIVATION IN RATS

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Abstract—Consumption beyond homeostatic needs, referred to here as reward-based feeding behavior, is a central contributor to the current obesity epidemic worldwide. Importantly, reward-based feeding can be driven by palatability, the taste and texture of the food, as well as cues associated with the consumption of palatable foods. The hypothalamic orexin system regulates both diet preference and anticipation of food rewards making it a likely target to modulate reward-based feeding behavior. In the current manuscript we hypothesized that orexin signaling mediates food-motivated behaviors and reward-based feeding behavior. We further hypothesized that orexin neurons and targets of the orexin system become activated in response to cues associated with the consumption of palatable food. Data from these studies suggest that orexin signaling promotes progressive ratio responding for palatable foods while blockade of orexin signaling attenuates reward-based feeding of a high fat diet. In addition, cues linked to the consumption of chocolate, or the receipt of a daily meal, activate the orexin system and its target regions differentially. Collectively, these data suggest that orexin signaling mediates reward-based feeding behavior and, within specific target regions, may regulate cue-induced overconsumption of palatable foods.

Key words: orexin, food reward, food anticipation, progressive ratio, non-homeostatic feeding, high fat overfeeding.

Consumption beyond homeostatic needs, referred to here as "reward-based feeding behavior," is a central contributor to the current obesity epidemic worldwide (Berthoud, 2004). Over the past decade it has become increasingly clear that reward-based feeding is influenced by cognitive emotional processing within brain reward circuits (Kelley and Berridge, 2002; Saper et al., 2002; Cota et al., 2006; Zheng and Berthoud, 2007). Importantly, reward-based feeding can be driven by palatability, like the taste and texture of the food, as well as cues associated with the consumption of palatable foods. The observation that metabolic hypothalamic circuits interface with the midbrain dopamine system to regulate feeding (DiLeone et al., 2003; Lutter and Nestler, 2009) suggests an anatomical means by which the regulation of such feeding might occur. The hypothalamic orexin system has the capacity to respond to metabolic signals (Sakurai, 2007) and regulate feeding behavior through its effects on mesolimbic reward circuitry (Zheng et al., 2007) making it a likely candidate to modulate reward-based feeding behavior.

In particular, orexin neurons in the lateral hypothalamus (LH) are activated in response to hypoglycemia (Cai et al., 1999; Moriguchi et al., 1999), caloric restriction (Sakurai et al., 1998) and systemic leptin administration (Lopez et al., 2000) suggesting that this system is responsive to internal homeostatic signals in place to maintain energy homeostasis. Interestingly, LH orexin neurons are also activated in anticipation of palatable food rewards (Harris et al., 2005) illustrating the ability of the orexin system to respond to external environmental cues linked to the cognitive aspects of feeding. Environmental cues are potent inducers of feeding in both humans and rodents (Weingarten, 1983; Cornell et al., 1989; Rogers and Hill, 1989; Petrovich et al., 2005, 2007) even when energy levels are satisfied. Thus, anticipation of feeding represents a critical point of regulation in regards to reward-based feeding behavior. Taken together, these data suggest that orexin neurons become activated by circulating factors and cues which induce feeding.

In addition to its ability to regulate anticipation of feeding, the hypothalamic orexin system also regulates feeding and food reward behaviors. Central administration of orexin-A induces feeding behavior and preferential consumption of high fat diet (Sakurai et al., 1998; Clegg et al., 2002). Receptors for orexin peptides are present in both hypothalamic and mesolimbic regions which regulate food hedonics (Trivedi et al., 1998; Hervieu et al., 2001; Marcus et al., 2001) suggesting that orexins may regulate food intake and diet selection by signaling within mesolimbic regions. In support of this notion, direct application of orexin into the ventral tegmental area (VTA) induces dopamine release in the nucleus accumbens (NAcc) (Narita et al., 2006). Moreover, blockade of orexin signaling within the VTA attenuates opioid-driven feeding behavior (Zheng et al., 2007) and psychostimulant-induced neuroadaptations within VTA neurons (Borgland et al., 2006). Collectively, these data suggest that the orexin signaling promotes feeding behavior through modulation of brain reward circuits.

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Abbreviations: ANOVA, analysis of variance; HFD, high fat diet; IR, immunoreactivity; LH, lateral hypothalamus; mPFC, medial prefrontal cortex; NAcc, nucleus accumbens; OX1R, orexin-1 receptor; PFA–LH, perifornical area and lateral hypothalamus; PR, progressive ratio; PVT, paraventricular thalamus; RT, room temperature; VTA, ventral tegmental area.

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In the current manuscript we hypothesized that orexin signaling mediates food-motivated and reward-based feeding behaviors. In particular we predicted that pharmacological manipulation of orexin signaling would modulate dopamine-dependent operant responding and high fat feeding in sated animals. We further hypothesized that orexin neurons and their target regions would become activated in response to cues associated with the consumption of palatable food. Data from these studies suggest that central orexin application increases progressive ratio (PR) responding for sucrose while blockade of orexin signaling attenuates PR responding. In addition, blockade of orexin signaling attenuated consumption of a high fat diet in a model of reward-based feeding in sated rats. Moreover, environmental cues linked to the consumption of chocolate increased activation specifically within orexin-1 receptor (OX1R)-containing neurons in the paraventricular thalamus (PVT) while expectation of both chocolate and a daily meal increased activation within orexin neurons.

EXPERIMENTAL PROCEDURES

Animals

Male Long–Evans rats (Harlan; Indianapolis, IN, USA) weighing 300–350 g were housed individually in a vivarium on a 12-hr light–dark cycle schedule. Room temperature was maintained at 25 °C. All animals were given *ad libitum* access to pelleted standard rodent chow (3.41, 0.51 kcal/g from fat; Harlan Teklad) and water unless noted. Behavioral tests were all conducted during the first half of the rats' dark phase unless noted.

Apparatus

Operant responding was conducted in four identical chambers constructed of aluminum end walls and clear plexiglas sides and measuring $21.6\times21.6\times27.9$ cm^3. A grid of stainless steel bars served as the floor of each chamber. A food cup was located on an end wall of each chamber inside a recessed opening. Two levers were located to the left and right of the food cup. Only the right lever was active and corresponded with food pellet availability during this experiment. All experimental events were controlled and recorded by computers located in an adjoining room running ABET software (Lafayette Instruments; Lafayette, IN, USA). Conditioning for context-conditioned expectation of chocolate was conducted in standard rat plastic shoebox cages containing scented kitty litter (one cage per rat; Cats Pride; Chicago, IL, USA).

Cannulation surgery

After a 1 week habituation period, all animals in experiment 1 were deeply anesthetized with a 1 ml/kg dose of (0.22 g Ketamine/0.03 g Xylazine) and placed into a stereotaxic apparatus. Subsequently, an indwelling cannula was lowered into the third ventricle using the following coordinates, AP=−2.2, ML=0, DV=−7.0. When utilizing this procedure, care was taken to avoid puncturing the central sinus by displacing the sinus using a 23 gauge beveled needle while the cannula was lowered. All animals were allowed to recover for 1 week during which time they regained their presurgical body weight.

Central orexin-A effects on food intake

Food intake experiments were conducted in the middle of the light phase to eliminate the contribution of circadian factors known to initiate feeding during the animals' active phase. To determine the effects of orexin-A on food intake, four separate groups of rats (n=4 or 5/group) were given third ventricle (i3vt) injections of 1, 2.5 or 5 nmol of orexin-A (Phoenix Pharmaceuticals; Burlingame, CA, USA) or vehicle (saline) in 2 μl. Food hoppers were returned 1 h after injection and reweighed 2 h later.

Central orexin-A effects on progressive ratio responding

Operant training was carried out over 15 consecutive days for 1 h per day (n=5). The reinforcer was a 45 mg sucrose pellet (TestDiet; Richmond, IN, USA). Rats were trained up to lever-press for sucrose under a PR schedule of reinforcement, in which the animals had to work progressively harder to obtain each subsequent reinforcer. The response requirements of the PR schedule increased through the following series: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, 328, 402, 492, 693, 737, 901. PR sessions were terminated for an individual animal when 20 min had elapsed without earning a reinforcer. The last completed bar press requirement was termed the breakpoint (Davis et al., 2008; Tracy et al., 2008). On the first day of testing, all rats received 2 μl i3vt injections of saline approximately 30 min before being placed in operant chambers. On the second day of testing, all rats received a 2 μl i3vt injection of 1 nmol orexin-A 30 min before being placed in operant chambers.

Effects of OX1R antagonist on progressive ratio responding

To test the hypothesis that antagonism of the orexin system decreases operant responding, two groups of rats (n=4 and 5) were trained to stably respond under a PR schedule of reinforcement identical to that used in Experiment 4. In this experiment, the reinforcer was a 45 mg sweet high fat pellet (38% calories from fat; TestDiet; Richmond, IN, USA). On the day of testing, each rat received i.p. injections of either vehicle (10% DMSO in saline) or 10 mg/kg OX1R antagonist (SB-334867; Tocris Bioscience; Ellisville, MO, USA) approximately 1 h before being placed in operant chambers.

Effects of OX1R antagonist on reward-based feeding behavior

In order to determine the effects of orexin receptor blockade on reward-based feeding behavior we developed a feeding paradigm in which sated rats voluntarily overconsume a palatable test diet. To do this, three groups of rats (n=4–6/group) were subjected to a daily meal feeding schedule in which each rat was given 3 h of *ad libitum* access to the standard chow diet. Prior to the initiation of the meal feeding regimen, each rat was fed a small amount of high fat diet (HFD; 4.41 kcal/g, 1.71 kcal/g from fat; Research Diets; New Brunswick, NJ, USA) to prevent neophobia. Rats were meal-fed for 10 consecutive days prior to testing. On the day of testing, chow food hoppers were weighed, placed on each cage and subsequently reweighed each hour for 2 h. Each rat received i.p. injections of vehicle (10% DMSO in saline), 10 or 20 mg/kg OX1R antagonist (SB-334867) after the first hour of chow access. Following the second hour of chow access, a separate set of food hoppers containing the HFD were weighed and placed on each cage beside the previously placed chow hoppers. Both sets of food hoppers were reweighed after 1 h.

Context-conditioned neuronal activation

To observe the effects of contextual conditioning on orexin neuronal activation, two groups of rats (n=4/group) were subjected to daily 1 h conditioning sessions for 15 consecutive days. Contex-

tual conditioning was conducted in separate rat shoebox cages in which kitty litter was used in place of standard bedding to create a novel test environment. Single pieces of Hershey's chocolate (The Hershey Company, Hershey, PA, USA; 3.58 g; 54% calories from fat) were given to the rats 5 min after being placed in the novel environment. Control rats were exposed to the novel environment for 1 h without any chocolate. A third group of rats ($n=5$), meal-conditioned for 20 consecutive days, were given 4 h of daily chow access in a novel test environment. This group required additional conditioning to ensure that the meal feeding regimen had been acquired, as defined by post-conditioning intake equating pre-conditioned levels. One day after the last conditioning session, all rats were transcardially perfused 70 min after being placed into the conditioning environment without any food.

Immunohistochemistry

Animals were injected with an overdose of pentobarbital and transcardially perfused with saline for 1 min followed by 4% paraformaldehyde for 20 min. The brains were post-fixed overnight and then stored in 30% sucrose with 0.01% glycerol for a minimum of 24 h. Brains were frozen on dry ice and sectioned at 35 mm intervals and collected (in series of one-in-four sections) in 20% glycerol.

One complete series of sections was processed for c-Fos immunohistochemisty using nickel-enhanced 3,3′-diaminobenzidine tetrahydrochloride (DAB-Ni). Sections were pretreated in 1% hydrogen peroxide, 1% sodium borohydride and blocked with 0.1% bovine serum albumin (BSA) with 0.4% Triton X-100. Rabbit anti-c-Fos antibody (1:5000; Santa Cruz Biotechnology; Santa Cruz, CA, USA) was applied to sections for overnight incubation at room temperature (RT). The secondary antibody, biotinylated goat anti-rabbit IgG (1:200; Jackson Immuno Research; West Grove, PA, USA), was applied to the sections at RT followed by incubation in avidin–biotin complex (1:500; Vectastain ABC Elite Standard; Vector Laboratories, Burlingame, CA, USA). The sections were stained using a DAB-Ni reaction and mounted onto slides for visualization. All immunolabeling was viewed and images collected on a Zeiss Axioplan 2 light/fluorescence microscope with an AxioCam camera using Axiovision software (Carl Zeiss Microimaging; Thornwood, NY, USA).

Sections containing lateral hypothalamus were double-labeled for c-Fos and orexin-A. After washes in 1% sodium hydroxide with 1% hydrogen peroxide, 0.3% glycine, and 0.03% sodium dodecyl sulfate, sections were blocked in 4% horse serum with 0.4% Triton X-100. Rabbit anti-c-Fos antibody (1:500) was applied to the sections for overnight incubation at RT. The secondary antibody, biotinylated goat anti-rabbit IgG (1:300; Vector Laboratories, Burlingame, CA, USA), was applied to the sections at RT followed with incubation in avidin–biotin complex (1:500; Vectastain ABC Elite Standard; Vector Laboratories; Burlingame, CA, USA) and an incubation in biotinyl tyramide signal amplification solution (1:250; PerkinElmer LAS; Boston, MA, USA). Cyanine 3 (Cy3; 1:200; Jackson Immuno Research; West Grove, PA, USA) was then applied to the sections. The sections were reincubated in blocking solution, and mouse anti-orexin-A antibody (1:250; Santa Cruz Biotechnology; Santa Cruz, CA, USA) was applied overnight at RT. The secondary antibody, Alexxa 488-conjugated goat anti-mouse IgG (1:200; Invitrogen; Carlsbad, CA, USA) was applied to the sections and they were mounted onto slides for immunofluorescent visualization.

A full series of sections was double-labeled for c-Fos and OX1R immunohistochemistry. A wash in 1% hydrogen peroxide was followed by incubation in a blocking solution of 10% goat serum, 0.1% BSA and 0.25% Triton X-100. Rabbit anti-OX1R antibody (1:600; Alpha Diagnostic International; San Antonio, TX, USA) was applied to the sections overnight at RT. A secondary antibody, goat anti-rabbit IgG (1:250) diluted in blocking solution was applied. The sections were then incubated in avidin–biotin complex (1:500) and then in biotinyl tyramide signal amplification solution (1:250). DyLight 549 (1:200; Jackson Immuno Research; West Grove, PA, USA) was then applied to the sections. The sections were reincubated in 4% goat serum with 0.4% Triton X-100 blocking solution and rabbit anti-c-Fos antibody (1:500) was applied to the sections for overnight incubation at RT. The secondary antibody, Alexxa 488-conjugated goat anti-rabbit IgG (1:200; Invitrogen, Carlsbad, CA, USA), was applied to the sections and subsequently mounted onto slides for immunofluorescent visualization.

Statistical analyses

Data were analyzed using STATISTICA 6.0 for Windows (StatSoft; Tulsa, OK, USA). Data for orexin-A effects on food intake, OX1R antagonist effects on operant responding and all immunohistochemistry experiments were analyzed between subject groups using analysis of variance (ANOVA). Least significant differences post hoc comparisons were used to assess the source of significant main effects. Data for orexin-A effects on operant responding and OX1R antagonist effects on high fat overconsumption were analyzed using 2- and 1-tailed *t*-tests (respectively). Significance was set at $P<0.05$.

RESULTS

Central orexin-A increases food intake

To establish a dose range, the effects of ICV-administered orexin-A were assessed on food intake in four separate groups of *ad libitum* fed rats ($F(3,16)=9.06$, $P<0.05$). 1 and 2.5 nmole doses of orexin-A did not significantly affect 2 h food intake ($P>0.05$). In comparison to the vehicle, 1 and 2.5 nmol doses, 5 nmol orexin-A increased chow intake at 2 h post injection ($P<0.05$, Fig. 1A).

Orexin promotes progressive ratio responding

The effects of ICV-administered orexin-A were then tested on operant responding for food reward pellets in unrestricted rats responding under a PR schedule of reinforcement. In this experiment, a sub-threshold dose of orexin-A that did not have effects on feeding behavior (1 nmol; Fig. 1A) was used to isolate the effects of orexin-A on PR responding. Orexin-A significantly increased break point responding for sucrose suggesting that it is sufficient to increase motivation to obtain food rewards ($P<0.05$; Fig. 1B). Subsequently, in a separate set of studies, the effects of OX1R antagonism on PR responding were assessed ($F(1,7)=21.16$, $P<0.05$). Selective antagonism of OX1R, by systemic injection of 10 mg/kg SB-334867, decreased PR responding for high fat pellets when compared to vehicle-treated controls ($P<0.05$; Fig. 2A). These results are consistent with previous reports (Nair et al., 2008) in which animals, food restricted prior to operant testing, displayed attenuated responding under a fixed ratio schedule after OX1R antagonism. In the current study, animals were maintained on an *ad libitum* feeding schedule prior to operant testing to isolate the influence of reward-based feeding on PR responding. Additionally, a reinforcement schedule with a much higher work requirement was used to measure dopamine dependent responding (Salamone, 1992; Zhang et al., 2003).

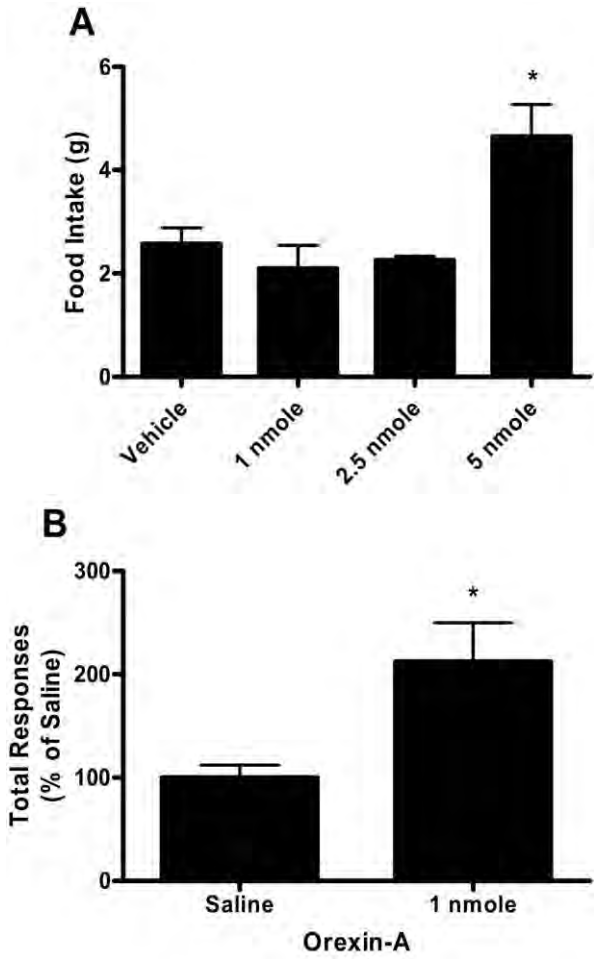

Fig. 1. Intracerebroventricular (ICV) orexin-A increases 2 h food intake and progressive ratio (PR) responding for food rewards. (A) Orexin-A increases 2 h chow intake (g) at a dose of 5 nmol. (B) 1 nmole orexin-A increases the total number of responses in rats responding under a PR schedule for sucrose pellets, expressed as a percent of saline (* $P<0.05$).

OX1R blockade decreases HFD overconsumption in sated rats

Previous studies suggest that the hypothalamic orexin system interfaces with mesolimbic circuitry to drive reward-based feeding behavior. In particular, orexin-A administration promotes feeding from high calorie diets (Clegg et al., 2002) and blockade of orexin signaling within VTA neurons attenuates opioid-induced high fat feeding (Zheng et al., 2007). In order to assess the effects of orexin signaling during a natural bout of reward-based feeding, a non-pharmacological model was used to measure feeding in sated rats. In this model, rats are conditioned to daily 4 h feeding sessions on standard chow diet during which they are able to consume their daily caloric needs. On the day of testing, the third hour of chow access is accompanied by access to another hopper of a palatable HFD. Using this model, results indicate that rats typically consume almost all of their daily chow within the first hour of chow access, as shown by reduced chow intake during the second hour of access (Fig. 2B). Interestingly, when given the opportunity, sated rats will over consume HFD much like humans would with a highly palatable dessert following a filling meal. Here, we tested the effects of OX1R blockade on the overconsumption of HFD using this model. Specifically, when compared to vehicle-treated controls, rats receiving 20 mg/kg SB-334867 consumed significantly less HFD ($P<0.05$). There was a consistent reduction of HFD intake within the group receiving 10 mg/kg SB-334867, however this trend did not reach significance ($P>0.05$, Fig. 2B).

Neuronal activation in context-conditioned expectation of chocolate

To test the hypothesis that the orexin system mediates context dependent activation within circuits that mediate

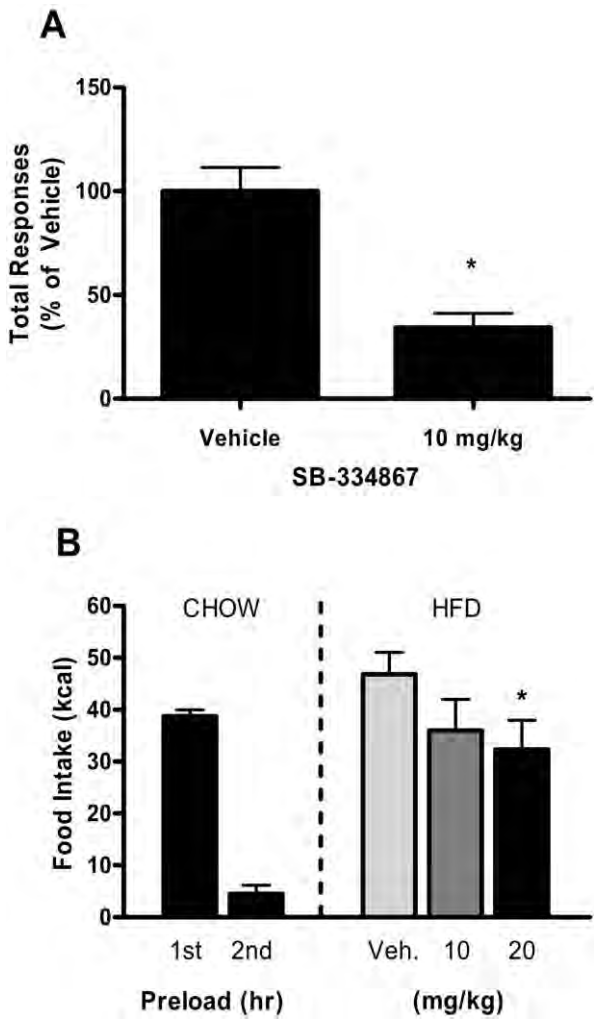

Fig. 2. Systemic orexin-1 receptor (OX1R) antagonism (SB-334867) attenuates progressive ratio (PR) responding for food rewards and high fat diet (HFD) overconsumption in sated rats. (A) 10 mg/kg SB-334867 decreases the total number of responses in rats responding under a PR schedule for sweet high fat pellets, expressed as a percent of vehicle. (B) Rats conditioned to 3 h/d meal-fed chow are sated during the first 2 h of chow preload access. Pretreatment with 20, but not 10, mg/kg SB-334867 significantly decreases subsequent 1 h HFD intake (kcal) when compared to vehicle (* $P<0.05$).

food seeking behavior, c-Fos immunoreactivity within orexin neurons was assessed in rats trained to expect their daily meal of chow or a piece of chocolate. Previous reports have demonstrated that learned cues associated with food activate neurons in the medial prefrontal cortex (mPFC) that also, in turn, activate LH neurons (Harris et al., 2005; Petrovich et al., 2005). Consistent with these findings, context-conditioned expectation of chocolate led to increased neuronal activation in both hypothalamic and forebrain regions (Fig. 3A–D). There was a significant main effect of group on c-Fos-immunoreactivity (IR) in the perifornical area and lateral hypothalamus (PFA–LH; $F(2,10)=11.10$, $P<0.05$). Specifically, rats expecting chocolate displayed increased c-Fos-IR in neurons of the PFA–LH, site of orexin production, when compared to control and to meal-fed groups ($P<0.05$). There was no difference between control and meal-fed groups ($P>0.05$, Fig. 3A). Double-labeling for orexin-A and c-Fos revealed an increase in orexin-A neuronal activation in animals expecting their daily meal of chow or chocolate (Fig. 3D). A significant main effect of group on the proportion of orexin-A neurons positive for c-Fos-IR in the PFA–LH was found when quantifications were analyzed with ANOVA ($F(2,9)=13.86$, $P<0.05$). When compared to control rats, both meal-fed and chocolate-expecting rats displayed a significant increase in the proportion of orexin-A neurons positive for c-Fos-IR in the PFA–LH ($P<0.05$), but no difference between each other ($P>0.05$, Fig. 3B). A closer analysis of the activational profiles in two distinct populations of orexin-A neurons (Harris and Aston-Jones, 2006) revealed a significant main effect of group on the proportion of orexin-A neurons positive for c-Fos-IR specifically in the PFA ($F(2,9)=15.47$, $P<0.05$), but not the LH ($F(2,9)=1.91$, $P>0.05$). Similar to the above PFA–LH analysis (Fig. 3B), when compared to control rats, both meal-fed and chocolate-expecting rats displayed a significant increase in the proportion of orexin-A neurons positive for c-Fos-IR in the PFA ($P<0.05$), and not the LH ($P>0.05$),

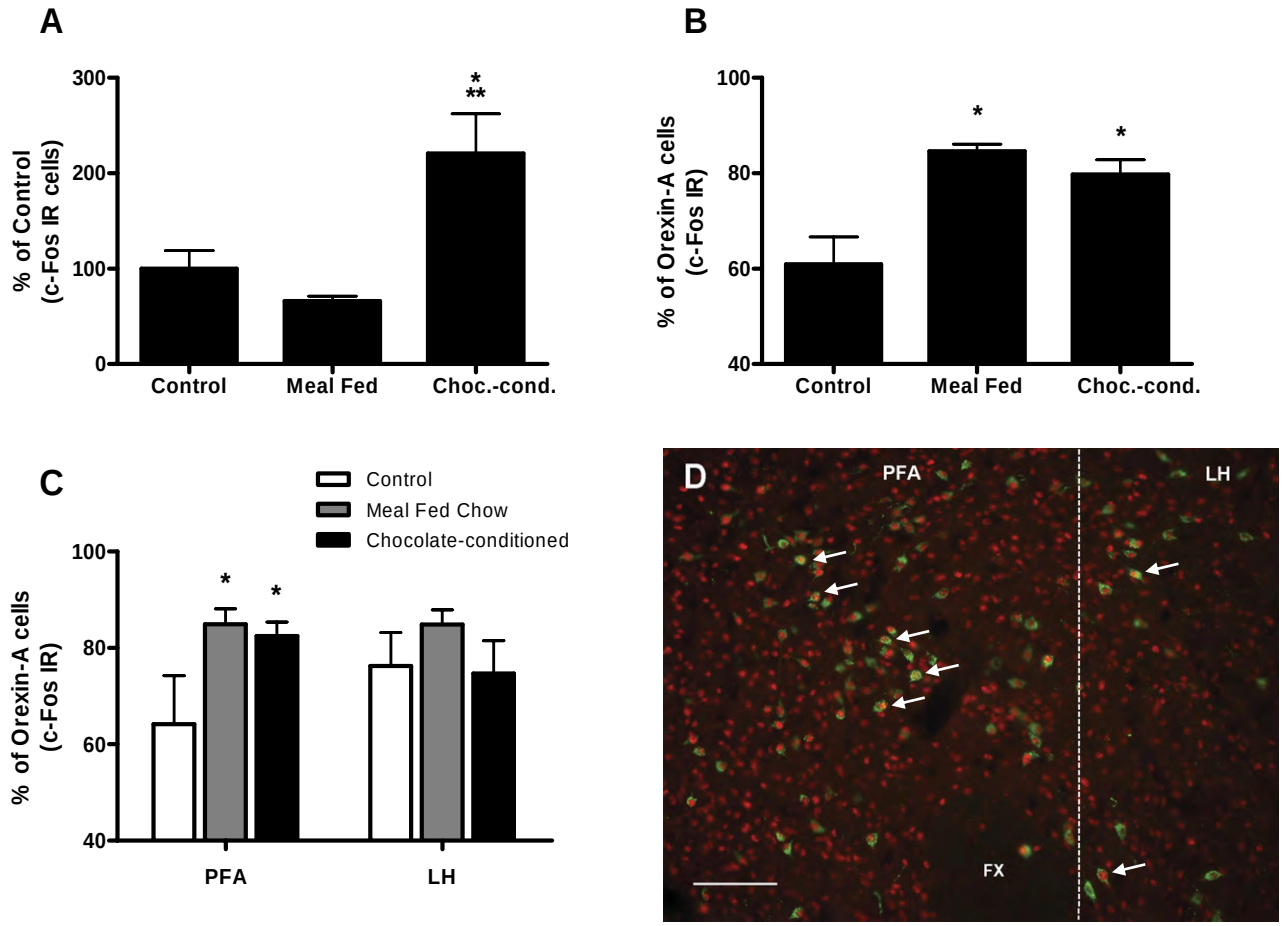

Fig. 3. Context-dependent expectation of chocolate increases c-Fos expression in orexin-A-expressing neurons. (A) Expectation of chocolate increases c-Fos expression in the perifornical area and lateral hypothalamus (PFA-LH) when compared to both control and meal-fed chow groups. (B) Expectation of both chocolate or a daily meal of chow increases c-Fos expression in orexin-A-expressing neurons of the PFA-LH when compared to control group. (C) Expectation of both chocolate or a daily meal of chow increases c-Fos expression in orexin-A-expressing neurons of the PFA, but not the LH, when compared to control group (*, ** $P<0.05$). (D) A representative higher-magnification image from the PFA-LH of a rat expecting chocolate showing orexin-A (green) co-localized with c-Fos (red) indicated by white arrows. The dotted white line indicates the boundary used to distinguish PFA and LH throughout this study. Scale bar: 200 μm. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

but no difference between each other ($P>0.05$, Fig. 3C). Further analysis by ANOVA of single-labeling for c-Fos-IR showed that there were also significant increases in c-Fos-IR in the PVT, VTA and mPFC of rats expecting chocolate, but not rats expecting their daily meal of chow ($P<0.05$ compared to control and meal-fed groups, Fig. 4A–I). In order to further characterize the activation of these extra-lateral-hypothalamic sites, another series of sections was double-labeled for OX1R and c-Fos. Qualitative analyses revealed co localization of OX1R expression with c-Fos-IR on neurons in the PFA–LH and PVT, but very little to none in the VTA (Fig. 5A–C).

DISCUSSION

Recent studies indicate that orexin signaling is involved in the anticipation of food rewards (Harris et al., 2005). Importantly, in animals that are calorically replete, food intake can be driven by exposure to environmental cues (Petrovich et al., 2005, 2007) suggesting that anticipation of food may regulate reward-based feeding behavior. Emerging evidence suggests that reward-based feeding when caloric needs are met, referred to as "non-homeostatic feeding," is a major contributor to the current obesity epidemic (Berthoud, 2004; Zheng and Berthoud, 2007). Thus, it is possible that environmental cues associated with palatable foods may induce reward-based feeding and, subsequently, obesity. The central aim of the present manuscript was to test the hypothesis that orexin signaling mediates the motivation to obtain palatable foods and reward-based feeding. This hypothesis was tested by examining the ability of orexin to modulate PR responding for food rewards and reward-based feeding in a model in which sated

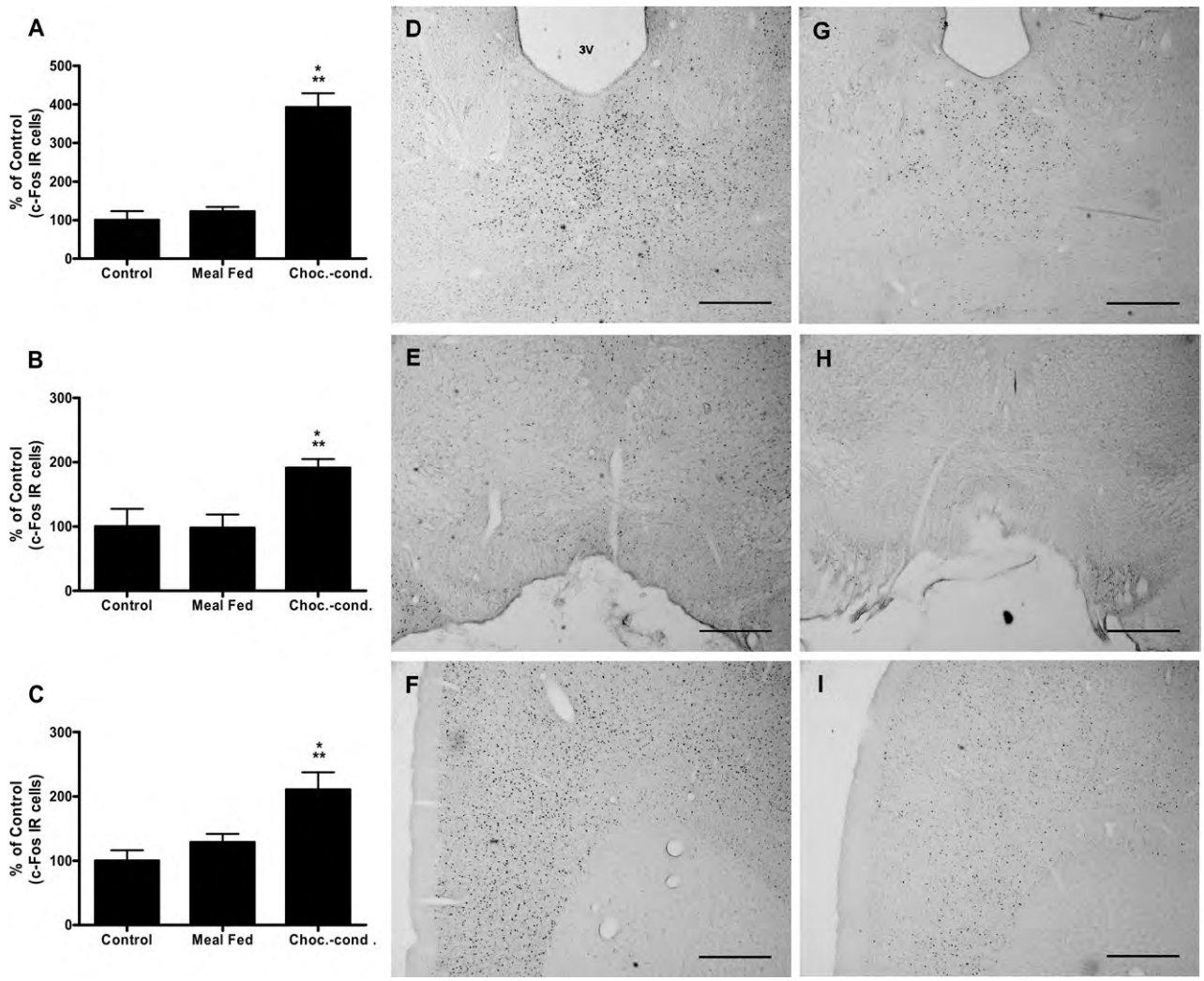

Fig. 4. Context-dependent expectation of chocolate increases c-Fos expression in extra-hypothalamic areas. (A) Expectation of chocolate increases c-Fos expression in the paraventricular nucleus of the thalamus (PVT) when compared to both control and meal-fed chow groups. (B) Expectation of chocolate increases c-Fos expression in the ventral tegmental area (VTA) when compared to both control and meal-fed chow groups. (C) Expectation of chocolate increases c-Fos expression in the medial prefrontal cortex (mPFC) when compared to both control and meal-fed chow groups (*, ** $P<0.05$). (D–F) Representative lower-magnification images of c-Fos immunolabeling in chocolate-expecting rats from the PVT, VTA and mPFC respectively. (G–I) Representative lower-magnification images of c-Fos immunolabeling in control rats from the PVT, VTA and mPFC respectively. Scale bars: 500 μm.

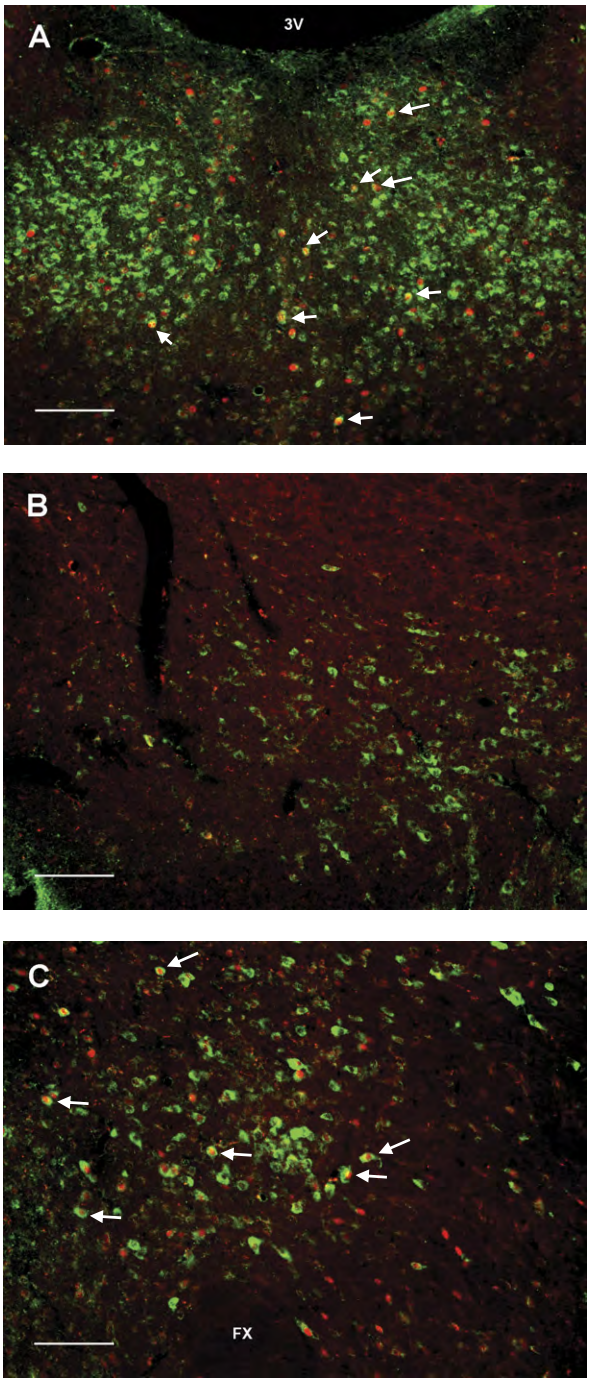

Fig. 5. Immunohistochemical double-labeling of orexin-1 receptor (OX1R) expression (green) and c-Fos (red). Representative higher-magnification images from (A) paraventricular nucleus of the thalamus (PVT), (B) ventral tegmental area (VTA), and (C) perifornical area and lateral hypothalamus (PFA-LH). Co-localization of OX1R and c-Fos are indicated by white arrows. Scale bars: 200 μm. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

rats selectively overfeed on a HFD. In addition, we examined neuronal activation within orexin-containing neurons and their target regions in fed animals trained to expect chocolate or their daily ration of maintenance diet to determine if activation of the orexin system is selective to cues associated with reward-based feeding. From these studies, four significant findings emerged: (1) Central administration of orexin or systemic antagonism of orexin signaling selectively modified PR responding for palatable food rewards, (2) antagonism of orexin signaling attenuated high fat reward-based feeding in sated animals, (3) cues associated with both palatable foods and a chow meal activate orexin neurons, and (4) anticipation of chocolate selectively activates OX1R-expressing neurons within the PVT, a midline thalamic relay center, indicating that this region may represent an important integration site for orexin signaling during anticipation of palatable foods. Collectively, these results suggest that manipulation of orexin signaling is sufficient to alter the motivation to obtain food rewards as well as reward-based feeding behavior. Furthermore, the orexin system targets thalamic and mesolimbic regions during the anticipation of food rewards.

Orexin and progressive ratio responding

To determine if orexin-A is sufficient to alter the motivation to work for food we utilized a model of PR responding, in which animals must work progressively harder to obtain each subsequent reinforcer (Hodos, 1961). A dose which does not increase food intake was chosen to assess orexin-A's effects on PR responding to assess whether orexin's ability to regulate motivation is separate from its effects on food consumption. The results from the current study suggest that central orexin-A is sufficient to increase the motivation to obtain palatable food rewards. These results are consistent with previous reports in which direct application of orexin-A directly into the PFA–LH increased PR responding for food rewards (Thorpe et al., 2005). In a parallel set of experiments, we tested the necessity of orexin signaling for PR responding using an OX1R antagonist (SB-334867). Pharmacological blockade of OX1R signaling attenuated PR responding for high fat pellets. These results are consistent with previous work in which orexin receptor antagonism decreased responding for high fat pellets under both low and high work requirements (Nair et al., 2008; Borgland et al., 2009). Importantly, responding under PR schedules of reinforcement requires intact dopamine processing (Salamone, 1992) and suggests that this effect may involve processing within mesolimbic dopamine circuitry. Interestingly, when administered directly into the VTA orexin increases dopamine flux in the NAcc (Narita et al., 2006). Thus it is possible that the effects reported here on PR responding might be regulated by orexin signaling within mesolimbic regions. However, to test this possibility it will be necessary to determine the effects of intra-VTA orexin as well as SB 334867 on PR responding. Nevertheless when viewed collectively, these results suggest that orexin-A may signal specifically through the OX1R to affect food-motivated behaviors.

Orexin and reward-based feeding

Reward-based feeding behavior is defined as feeding when caloric needs are met or feeding for pleasure (Zheng and Berthoud, 2007), a phenomenon hypothesized to con-

tribute to weight gain and obesity in humans (Berthoud, 2004). In an effort to model reward-based feeding in our laboratory, we developed a paradigm of feeding on a highly palatable food following caloric satiation. In this experiment, rats preloaded on chow selectively overfeed on HFD. This is similar to a human situation in which individuals will over consume (despite being calorically satiated on a main course meal) of a highly palatable dessert, a phenomenon commonly defined as sensory-specific satiety (Rolls et al., 1981). Here, we report that pre-treatment with the OX1R antagonist (SB-334867) attenuates the effect of HFD to stimulate feeding in sated rats, suggesting a role for orexin signaling in the regulation of reward-based feeding. It is important to note here that the effects of orexin antagonism on feeding in our reward-based feeding model do not reflect alterations in conditioned responding as the high fat feeding observed in sated rats was a novel experience for these animals. Additionally, it is possible that orexin antagonism decreased general arousal or increased satiety thresholds which could account for the observed decrease in HFD feeding in our model. However, this finding is in agreement with studies which report that intra-VTA administration of SB-334867 decreases opioid-induced HFD intake (Zheng et al., 2007). In that study, the ability of opioid peptides to stimulate feeding was not a "learned" effect as animals were naive to the treatment (i.e. opioid agonist, DAMGO) prior to testing. Thus, the discrepancy in differences reported using this compound to modify conditioned or unconditioned responding may be best explained by the ability of orexin to regulate learned associations with reward behavior. In either case, the present findings are consistent with the hypothesis that orexin signaling regulates reward-based feeding behaviors.

Orexin and neuronal activation

In the current study, anticipation of a food reward increased neuronal activation within the PFA–LH, PVT, mPFC and VTA confirming previous reports (Schroeder et al., 2001; Harris et al., 2005; Petrovich et al., 2005; Schiltz et al., 2007). Recent reports indicate that the mPFC is a major target of the hypothalamic orexin system and that direct application of orexin-A within the mPFC is capable of enhancing cognitive performance (Huang et al., 2006; Lambe et al., 2005). Thus, the ability for cues associated with palatable foods to activate this region might implicate cortical orexin signaling in the activation of mPFC neurons during the anticipation of palatable foods. Perhaps more important is the observation that both chocolate-conditioned and meal-fed rats displayed increased neuronal activation within orexin neurons suggesting that orexin neurons are excited by cues associated with palatable foods as well as chow availability. These increases in orexin-A neuronal activation in both groups were specific to PFA, but not LH populations. Previous reports indicate that pharmacologically-induced high fat feeding specifically activates medial (PFA) rather than lateral (LH) orexin-A neurons (Zheng et al., 2007). Harris et al. report that drug- and food-associated cues activate LH, but not PFA, orexin-A neurons (Harris et al., 2005). Our present findings appear to be more consistent with the findings from Zheng et al., indicating that not only the initiation of feeding, but also the expectation of food may be linked to arousal as opposed to reward-specific activation according to a proposed dichotomy in orexin function (Harris and Aston-Jones, 2006). The ability of meal-fed rats to consume their daily allotment of calories in a 4 h time period is a learned phenomenon (Drazen et al., 2006; Woods, 1991), and thus being aroused prior to meal delivery may promote this learned association in addition to benefiting the consumption of a meal under such conditions. However, the expectation of a meal of chow increases mesolimbic dopamine (Radhakishun et al., 1988) suggesting that low-palatability chow may acquire increased salience during periods of restricted feeding. In this way, during meal feeding, cues associated with the receipt of the daily meal may engage reward circuitry to facilitate learned responses associated with meal delivery (Schultz, 2007; Fiorillo et al., 2008). Orexin neurons are known effectors of mesolimbic dopamine (Narita et al., 2006), thus it is possible that activation of orexin neurons in anticipation of a daily meal may represent a means by which brain reward circuits can become activated. The observation that orexin neurons become activated in anticipation of food reward is consistent with previous studies (Harris et al., 2005); however the finding that both palatable and non-palatable reinforcers activate orexin may implicate this system as being necessary for adapting to multiple feeding conditions.

Palatable food cues activate orexin target regions

In the context of food "addiction," cue-induced craving is a potent modulator of reward-based feeding. Cues associated with the availability of palatable foods have the capacity to increase both feeding and food-seeking behavior (Cornell et al., 1989; Rogers and Hill, 1989). Functional anatomical studies indicate that cue-induced anticipation of feeding activates mPFC neurons which project to the LH (Petrovich et al., 2005) and that removal of the mPFC within this circuit attenuates cue-induced elevations in feeding (Petrovich et al., 2007). Other recent reports suggest that this circuit is functionally regulated by the orexin system (Huang et al., 2006) and that cue-induced anticipation of food rewards activates hypothalamic orexin neurons (Harris et al., 2005). Collectively, these data suggest that orexin signaling within this mPFC–LH circuit may have the ability to regulate reward-based feeding behavior.

In addition to the activation of orexin-producing neurons of the PFA–LH, OX1R-expressing neurons within the PVT also become activated in anticipation of food rewards. The PVT is a midline thalamic structure that receives both moderate and dense projections from orexin neurons (Peyron et al., 1998; Fadel et al., 2005; Kirouac et al., 2005) and has been hypothesized to mediate cognitive arousal through its connections to the mPFC (Huang et al., 2006). Lesions of the PVT increase food intake and body weight (Bhatnagar and Dallman, 1999) suggesting that signaling within this region has functional consequences on feeding behavior. When applied directly, orexin-A acti-

vates PVT neurons (Ishibashi et al., 2005; Huang et al., 2006). Moreover, electrical stimulation of the PVT is sufficient to increase dopamine concentrations in the nucleus accumbens independent of VTA function (Parsons et al., 2007), and both psychostimulants alone and cues paired with psychostimulant exposure activate the PVT (Brown et al., 1992; Deutch et al., 1998).

In the current study, animals trained to expect chocolate showed the greatest increase in neuronal activation in the PVT suggesting that this region selectively responds to cues associated with food rewards. In support of this contention, the PVT has been proposed to act as a relay between hypothalamic peptide systems implicated in feeding behavior and areas of the mesolimbic system that mediate reward-related feeding (Bassareo and Di Chiara, 1999; Carelli et al., 2000; Parsons et al., 2007). Importantly, dopamine flux is a known to be a sub-second modulator of food seeking in rodents (Roitman et al., 2004; Day et al., 2007). Thus, it is possible that during the anticipation of a palatable meal, orexin neurons in the LH target the PVT to modulate NAcc dopamine release and food-seeking behavior.

CONCLUSION

Our results support the hypothesis that orexin signaling mediates motivation to obtain palatable foods and overconsumption of palatable diets in conditions of satiation. In addition, context-conditioned expectation of palatable food or daily meals activates orexin-producing neurons suggesting that orexin signaling may be involved in adapting to different feeding conditions. Thus, targets of the orexin system that become activated in anticipation of feeding may represent a divergence of feeding for pleasure or calories. In this context, orexin receptor-expressing neurons in the PVT, a critical relay and integration site for feeding and reward, preferentially responded to expectation of palatable foods, but not feeding in general. It has been suggested that feeding can be controlled by both hypothalamic and mesolimbic circuitry, and that the interface between the two may drive overconsumption of palatable foods (Saper et al., 2002; Cota et al., 2006). The PVT and the mesolimbic dopamine system play a critical role in integrating and/or relaying sensory and motivational information to ultimately affect the control of cognition and motivation (Huang et al., 2006; Parsons et al., 2007). Our data are in agreement with this notion and suggests that orexin's ability to signal within the PVT may serve to promote the anticipation of palatable foods and subsequently food-seeking behavior.

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