The Effects of Methionine-Enkephalin on the Amygdala Nucleus as

Measured by Feeding Response in the Laboratory Rat,

Rattus norvigicus

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Abstract

The mass of food eaten by male rats (<u>Rattus</u> <u>norvigicus</u>) after injection of met-enkephalin into the amygdala nuclei via chronically implanted cannula is studied. Two nuclei within the amygdala, the baso-lateral and the medial, were chosen for comparison based on previous evidence of their relation to feeding behavior and known anatomical connections with hypothalamic nuclei associated with feeding. Met-enkephalin has been shown to be an inhibitory neurotransmitter in previous studies in other laboratories. Based on this evidence, it was hypothesized that met-enkephalin placed in the baso-lateral amygdala should increase eating, and decrease feeding if placed in the medial amygdala. Rats injected in the baso-lateral nucleus with met-enkephalin ate an average of 27.38 g daily and 27.10 g daily when injected with physiological saline. Rats injected in the medial nucleus of the amygdala with met-enkephalin ate an average of 26.06 g daily and 26.65 g daily when injected with physiological saline. No statistical significance was found between daily food eaten during injections of met-enkephalin and during injections of placebo (physiological saline).

Introduction

As early as the 1930's the hypothalamus was shown to play a role in controlling the feeding behavior of the laboratory rat (Brobeck 1946). Two nuclei within the hypothalamus were shown to have the greatest effects upon the feeding behavior of the animal. A lesioning of the ventromedial nucleus of the hypothalamus (VHM) was shown to cause a great increase in appetite as well as obesity in the animal. A lesioning of the lateral hypothalamus (LH) was found to cause a decrease in the animal's appetite and body weight (Netter 1962). Fiber tracts originating in the VHM and terminating in the LH have been discovered using terminal degeneration techniques. It has been proposed that the VHM inhibits the LH, which dominates in the control of the animal's feeding (Arees and Mayer 1967).

The amygdala nucleus has also been shown to influence feeding in lesioning studies similar to those used in studying the hypothalamus. A lesioning of the baso-lateral nucleus of the amygdala (ABL) was found to increase the animal's feeding, while a lesioning of the medial nucleus of the amygdala (AME) was found to decrease the animal's feeding. However, the effects on feeding resulting from lesions in the amygdala were not as great as those seen as a result of lesions in the hypothalamus (Kaada 1971). The amygdala nuclei were found to influence the activity of the VHM via the stria terminalis, which originates in the amygdala nuclei and terminates in the area surrounding the VHM (Dreifuss 1971). A summary of the connections and relationships of the amygdala nuclei

and hypothalamic nuclei is presented in Appendix A. The amygdala's role in the control of feeding behavior is particularly interesting because of the nuclei's association with emotions.

Since their discovery, the endogenous opioids have been shown to influence a great number of body functions and behaviors such as feeding behavior. One endogenous opioid, methionine-enkephalin, has been found to have numerous implications in the control of feeding behavior. Levels of met-enkephalin have been shown to increase in the hypothalamus during the dark cycle when the rat consumes the majority of its food (Baile et al 1986). Injections of enkephalin analogues into various locations in the brain have also been shown to increase feeding (Stanley et al 1988). The amygdala nucleus is particularly rich in neuropeptides, including met-enkephalin (Bloom 1988). Stanley, Lanthier and Leibowitz (1988) injected the enkephalin analogue DALA (D-ala2-met5-enkephalinamide) and found an increase in feeding. They did not, however, localize their cannula placement within the amygdala. Comparing their coordinates with the atlas of Pellegrino, Pellegrino and Cushman (1979), it appears their placement was in the region between the medial nucleus of the amygdala and the stria terminalis. No other studies have been found that examine the effects of met-enkephalin on the amygdala as determined by changes in feeding behavior.

Enkephalins have been shown to act as inhibitory neurotransmitters (Hughes et al 1978). It is therefore assumed that injections of met-enkephalin into a nucleus of the brain would have an effect the same as lesioning, only temporary. Therefore,

injections of met-enkephalin into the ABL should increase feeding in the rat, and injections of met-enkephalin into the AME should decrease feeding. However, Baile, McLaughlin and Della-Fera (1986) observe that met-enkephalin increases feeding in the rat regardless of where it is placed. This observation was not in response to studies examining injections of met-enkephalin into the amygdala, but into the hypothalamic areas and cerebral ventricles. It seems possible that this conclusion may not hold for injections of met-enkephalin into the amygdala because this nucleus is known to be particularly rich in neuropeptides and may contain greater numbers of opioid receptors. It is the purpose of this study to determine if injections of met-enkephalin into the ABL will increase feeding and if injections into the AME will decrease feeding.

Methods and Materials

Male Wistar rats (Rattus norvigicus) were obtained at a body mass of 220-250 g. The rats were placed in individual cages equipped with a catch on the inside of the cage to divert crumbs and grindings produced when the rat fed into a small cup placed under the front of the cage. Purina standard laboratory chow was placed in the food cup attached externally to the front of the cage. The internal crumb catch was constructed out of thin gauge aluminum sheets, and measured approximately 2.5" in height from the floor of the cage. This height allowed the rats to feed in a natural posture in order not to greatly disturb their normal feeding habits.

Two to five days after the rats arrived and were placed in their cages, collection of data on the rats' feeding habits, in the form of mass of food eaten nightly, was begun. The rats food was removed at approximately 7:30 to 8:00 AM. That evening the rats were administered a pre-weighed amount of food at approximately 7:30 to 8:00 PM. The following morning, again at 7:30 to 8:00 AM, the food was collected, bagged and labeled for later weighing.

After five days of data were collected on a rat, the rat was unilaterally implanted with a 26 gauge guide cannula obtained from Plastic Products, Inc. Rats were anesthetized with intraperitoneal injections of Nembutal (.1 cc/100 g body weight). A Stoelting stereotaxic instrument was used to position the guide cannula to 1 millimeter dorsal of either the AME or ABL. Coordinates were determined using the atlas of Pellegrino, Pellegrino and Cushman (1979). Implants into the AME were positioned at 5.6 (A/P), -2.2 (V/D) and 3.6 (lateral). Implants into the ABL were positioned at 4.8 (A/P), -1.5 (V/D) and 5.2 (lateral). Guide cannulae were fixed in place to the skull using modeling screws and dental cement. Immediately after the guide cannulae were implanted, a dummy cannula which extended 1mm past the tip of the guide cannula was inserted into the guide cannula. The dummy cannula remained in place at all times except during injections in order to prevent the guide from becoming clogged. Seven animals were given implantations into the AME and six animals were given implants into the ABL.

The animals were allowed to recover for 7 days before injections were begun. Animals were injected once a day prior to

the onset of the dark cycle between 6:00 and 8:00 PM and administered food immediately afterward. Animal body mass data was also collected at the time of injection. Injections were made using a 28 gauge injection cannula attached to a Hamilton 25 microliter syringe with a 1.5 inch piece of plastic tubing. The injection cannula extended 1mm beyond the tip of the guide cannula similar to the dummy cannula. Each animal was given four 5-day sequences of injections. Each five day sequence was separated by a two day recovery period in order to ensure that all of the injected chemicals were properly metabolized. During two of the four 5-day sequences the animals were injected with 6.23 nanomoles of met-enkephalin obtained from Sigma contained in 0.5 microliters of solution made with physiological saline. During the other two five day sequences the animals were injected with the same volume of physiological saline solution, or placebo. Two orders of five day sequences were used. Some animals in each nucleus group were given the sequences in the order met-enkephalin, placebo, placebo, met-enkephalin, and the remaining animals in the group were given the sequences in the order placebo, met-enkephalin, met-enkephalin, placebo. The two orders were used to show that results were not dependant on a specific ordering.

Animals were perfused after completing the injections and their brains removed. The brains were imbedded in gelatin and sectioned using a freezing section microtome. Sections were mounted and stained using a modified Kluver and Barrera procedure shown in Appendix B (Luna 1968). Sections were then examined under a dissecting microscope and the placements of the guide verified

with respect to the target nucleus. Sample verification data are presented in Appendix C.

Results

Mean food mass consumed for individual rats during pre-operative testing, placebo and met-enkephalin injections is presented in Table 1. Grand means for each of these three treatments are presented in Table 2. Animal body mass data were not treated in this study.

Table 1. Mean food mass consumed during pre-operative testing, placebo injections and met-enkephalin injections for individual rats.

AME Implanted Rats:

Rat	Pre-Operative (g)	Met-Enkephalin (g)	Placebo (g)
1	22.94	25.57	24.35
2	27.40	28.54	27.56
3	28.12	23.56	27.11
8	22.94	23.74	24.11
18	22.88	28.21	27.37
19	24.34	25.05	26.68
20	26.10	27.53	29.40

Table 1. (continued)

ABL Implanted Rats:

Rat	Pre-Operative (g)	Met-Enkephalin (g)	Placebo (g)
12	24.26	27.85	27.55
13	29.56	27.09	26.89
15	31.34	28.06	23.57
16	27.74	28.90	27.88
22	25.02	24.48	29.67
23	25.26	27.88	27.02

Table 2. Grand means of masses of food consumed by rats with implants in the AME or ABL during pre-operative testing, placebo injections and met-enkephalin injections.

Nucleus:	AME	ABL
Pre-Operative (g):	24.96	27.20
Met-Enkephalin (g):	26.03	27.38
Placebo (g):	26.65	27.10

A Student's t-test was used to compare pre-operative data for each nucleus. No significant difference was found to exist between these two groups, $t(12)=1.96$, $p \geq .05$. A two-way ANOVA was used to compare the data between nuclei and treatments. No significance was found to exist between the treatments, or the interaction $F(1,36)=0.241816$, $p \geq .05$. A significant difference was found to exist between the two nuclei, $F(1,36)=6.48615$, $p \geq .05$. Student's t-tests were used to make further comparisons. Comparisons were made between the treatments within each nuclei individually, and between the nuclei for each individual treatment. No significant differences were found in any of these comparisons (AME:

$\underline{t}$(12)=0.593504, $\underline{p}\geq$.05; ABL $\underline{t}$(12)=-0.272540, $\underline{p}\geq$.05; met-enkephalin AME vs. ABL: $\underline{t}$(11)=-1.31136, $\underline{p}\geq$.05; placebo AME vs ABL: $\underline{t}$(11)= -0.412922, $\underline{p}\geq$.05).

Discussion

Results of both the ANOVA and Student's t-tests indicate that the injection of met-enkephalin into either the medial nucleus of the amygdala or the baso-lateral nucleus of the amygdala does not significantly affect the feeding behavior of the animal. While not statistically significant, it is perhaps worthy to note that the differences in mean eating behavior were consistent with the original hypothesis. The hypothesis stated that injections of met-enkephalin into the baso-lateral nucleus of the amygdala would increase the feeding of the animal. The grand mean for all the injections of met-enkephalin into the ABL is seen to increase to 27.38 g from the grand mean during injections of the placebo in the same animals of 27.10 g. The hypothesis further states that injections of met-enkephalin into the AME should decrease the feeding of the animal if the opioid does act as an inhibitory neurotransmitter as it is described by Hughes, Snyder and Bloom (1978). The grand mean for injections of met-enkephalin into the AME was seen to decrease from the grand mean value during injections of the placebo of 26.65 g to 26.03 g.

The significant difference between the nuclei determined by the ANOVA test may support the hypothesis in showing that different effects should be seen in each nucleus. However, this significance

was not confirmed in the specific comparisons of the nuclei for each treatment using t-tests. Another possible explanation for this result may be that the animals placed in the ABL group naturally ate more than the rats placed in the AME group due to natural differences in individual metabolisms or other factors which would effect their mean daily food intake. Although there was found to be no significance between the pre-operative daily food intake data for the rats in the ABL group and the rats in the AME group, the values were somewhat different. The rats in the ABL group had a mean value of 27.20 g, while the rats in the AME group had a mean value of 24.96 g, a difference of 2.24 g. The means during the pre-operative phase were based on 5 days of eating behavior, while the means during the testing phase were based on 20 days of eating behavior. The greater stability of the testing means would enhance the likelihood of finding significance.

The lack of a statistically significant effect on feeding behavior from the injection of met-enkephalin may have resulted because of the short duration of met-enkephalin's activity. According to Baile, McLaughlin and Della-Fera (1986), no increases in food intake resulting from injections of met-enkephalins have been reported. However, numerous studies such as one conducted by Stanley, Lanthier and Leibowitz (1988) have shown increases in food intake resulting from the injection of enkephalin analogues such as DALA (D-ala2-met5-enkephalinamide), which is described as "long acting". Further, the half-life of met-enkephalin has been found to be from approximately 11 to 22 minutes in various tissues of the Albino Rabbit (Lee 1986). This indicates that any excess met-

enkephalin will be broken down very early in the animals' normal daily feeding period. Therefore, if met-enkephalin molecules bound to receptor proteins are quickly destroyed by enzymes as in the case of other neurotransmitters, there will not be more met-enkephalin present to replace the broken down molecules.

Effects may also have not been seen because of some uncertainty about the exact quantity of met-enkephalin being injected. The quantity of chemical being injected was contained in 0.5 microliters of solution. Although the injection syringe was graduated in 0.5 microliter increments, the accuracy and repeatability of this measurement is somewhat uncertain because of its extremely small magnitude. This may have resulted in reduced doses. Also, the rate of decomposition of the met-enkephalin in solution is not known. The solutions were stored in temperatures of 0-15°C as suggested by the manufacturer. It may be possible that decomposition decreased the concentration of the chemical in the solution or completely destroyed it.

Although no statistically significant results were obtained, the observations made concerning the increase and decrease of the daily food intake grand means provide some impetus for future work. It seems possible from these observations that a study similar to this one substituting one of the long acting enkephalin analogues such as DALA or DALE (D-ala2-leu5-enkephalin) may find statistical significance in changes in feeding behavior. It would also be interesting to use an opioid antagonist such as naloxone in order to help show that any effects resulted from the opioid. This could be accomplished by adding additional sequences where naloxone was

injected prior to the met-enkephalin. This would prevent the met-enkephalin from having its normal physiological effect on the neurons in the nuclei studied and therefore from causing any changes in the animals feeding.
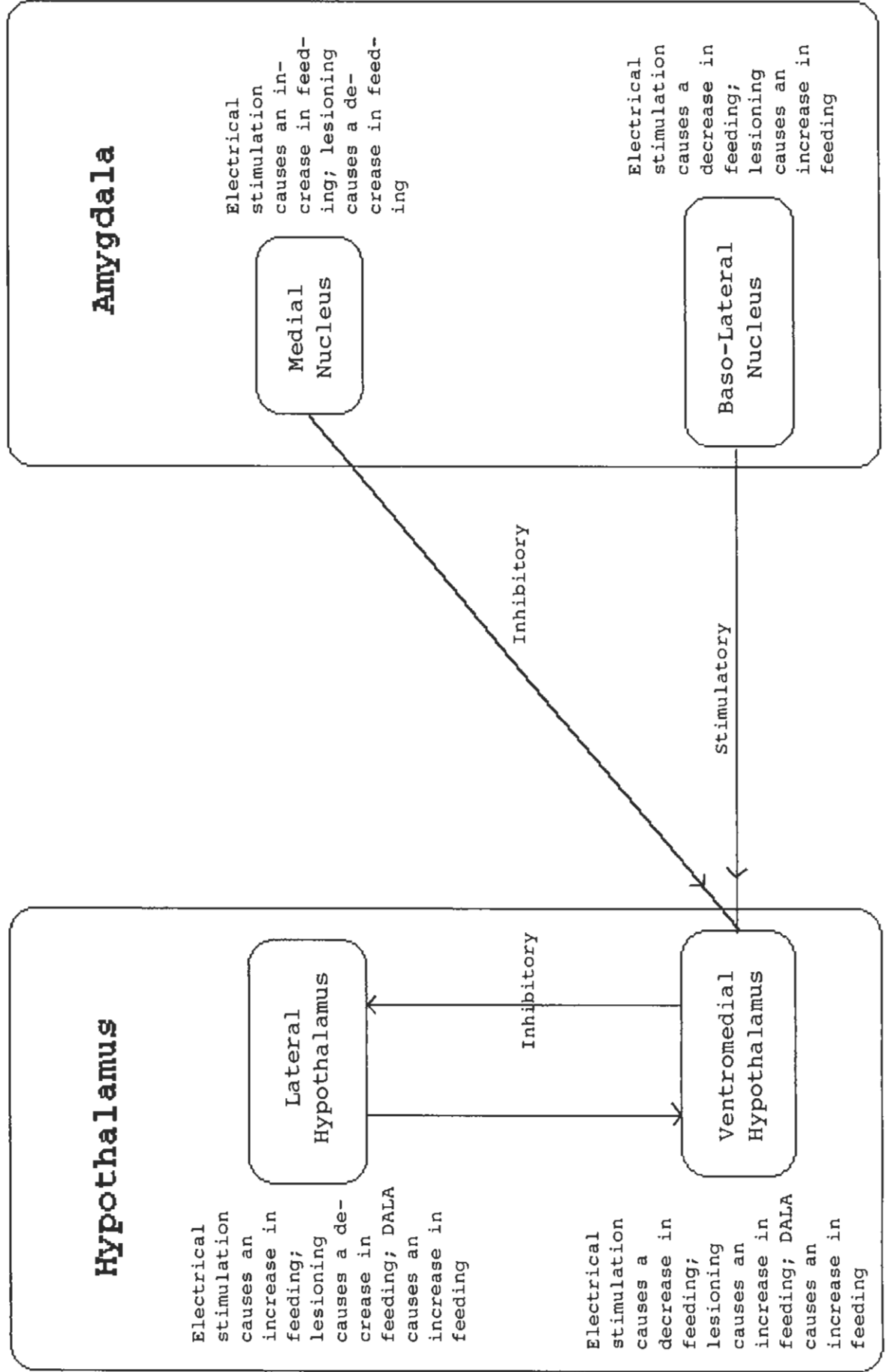
Literature Cited and Consulted

Arees, E.A.; Mayer, J. Anatomical connections between medial and lateral regions of the hypothalamus concerned with food intake. Science 157: 1574-1575, 1967.

Baile, C.A.; McLaughlin, C.L.; Della-Fera, M.A. Role of cholecystokinin and opioid peptides in control of food intake. Physiological Reviews 66 (1): 173-234, 1986.

Bloom, F.E. Neuropeptides. Readings from Scientific American: The Biology of the Brain: from Neurons to Networks. Llinás, R.R., Ed. W.H. Freeman and Company: New York, 1988.

Brobeck, J.R. Mechanism of the development of obesity in animals with hypothalamic lesions. Physiological Review 26: 541-559; 1946.

Eager, R.P.; Chi, C.C.; Wolf, G. Lateral hypothalamic projections to the hypothalamic ventromedial nucleus in the albino rat: demonstration by means of a simplified ammoniacal silver degredation method. Brain Research 29: 128-132, 1971.

Eckert, R.; Randall, D.; Augustine, G. Animal Physiology: Mechanisms and Adaptations. W.H. Freeman and Company: New York, 1988.

Heimer, L.; Nauta, W.J.H. The hypothalamic distribution of the stria termanilis in the rat. Brain Research, 13: 284-297; 1969.

Holloway, M. Prophile: Solomon H. Snyder, the rewards of ideas that are wrong. Scientific American, August 1991.

Hughes, J.; Snyder, S.H.; Bloom, F.E. Endorphins and Enkephalins. Neurosciences Research Program Bulletin 16 (2): 231-237, 1978.

Kaada, B.R. Stimulation and regional ablation of the amygdaloid complex with reference to functional representations. The Neurobiology of the Amygdala: The Proceedings of a Symposium on the Neurobiology of the Amygdala, Bar Harbor, Maine, June 6-17, 1971. Plenum Press: New York, 1972.

Lammers, H.J. The neural connections of the amygdaloid complex in mammals. The Neurobiology of the Amygdala: The Proceedings of a Symposium on the Neurobiology of the Amygdala, Bar Harbor, Maine, June 6-17, 1971. Plenum Press: New York, 1972.

Lee, V.H.L. Enzymatic barriers to peptide and protein absorption and the use of penetration enhancers to modify absorption. Delivery Systems for Peptide Drugs. Davis, S.S.; Illum, L., Eds. Plenum Press: New York, 1986.

Literature Cited and Consulted (continued)

Luna, L.G., Ed. Manual of Histological Staining Methods of the
     Armed Forces Institute of Pathology. McGraw-Hill Book
     Company: New York, 1968.

Nauta, W.J.H.; Feirtag, M. The organization of the brain. Readings
     from Scientific American: The Workings of the Brain:
     Development, Memory and Perception. Llinás, R.R., Ed.
     W.H. Freeman and Company: New York, 1990.

Netter, F.H. The CIBA Collection of Medical Illustrations. Volume
     I: Nervous System. CIBA Pharmaceutical Company, 1962.

Pellegrino, L.J.; Pellegrino, A.S.; Cushman, A.J. A Stereotaxic
     Atlas of the Rat Brain. Plenum Press: New York, 1979.

Roberts, G.W.; Woodhams, P.L.; Polak, J.M.; Crow, T.J.
     Distribution of neuropeptides in the limbic system of the rat:
     the amygdaloid complex. Neuroscience 7 (1): 99-131, 1986.

Rossier, J.; Vargo, T.M.; Minick, S.; Ling, N.; Bloom, F.E.;
     Guillemin, R. Regional dissociation of ß-endorphin and
     enkephalin contents in rat brain and pituitary.
     Proclaimations of the National Academy of Science 74 (19):
     5162-5165, 1977.

Sheer, D.E., ed. Electrical Stimulation of the Brain: and
     Interdisciplinary Survey of Neurobehavioral Integrative
     Systems. University of Texas Press: Austin, 1961.

Stanley, B.G.; Lanthier, D.; Leibowitz, S.F. Multiple brain sites
     sensitive to feeding stimulation by opioid antagonists: a
     cannula mapping study. Pharmocology Biochemistry and Behavior
     31(4): 825-832, 1988.

Sutin, J,; Eager, R.P. Fiber degeneration following lesions in the
     hypothalamic ventromedial nucleus. Annals New York Academy of
     Science 157: 610-628; 1968.

Appendix A. Connections and hypothesized relationships between nuclei in the hypothalamus and and amygdala nuclei.

Amygdala

Medial Nucleus

Electrical stimulation causes an increase in feeding; lesioning causes a decrease in feeding

Baso-Lateral Nucleus

Electrical stimulation causes a decrease in feeding; lesioning causes an increase in feeding

Hypothalamus

Lateral Hypothalamus

Electrical stimulation causes an increase in feeding; lesioning causes a decrease in feeding; DALA causes an increase in feeding

Ventromedial Hypothalamus

Electrical stimulation causes a decrease in feeding; lesioning causes an increase in feeding; DALA causes an increase in feeding

Inhibitory

stimulatory

Inhibitory

Appendix B.   Modified Kluver and Barrera staining proceedure used
             for histological verification of guide cannula
             placements.

1.)   fix section to slide with albumin

2.)   70% ethanol for 15 minutes

3.)   Luxol Fast Blue MBS, 01% solution in ethanol, 49°C for
      15 minutes

4.)   rinse in 95% ethanol for 20 seconds

5.)   distilled water

6.)   0.05% lithium carbonate for 5-10 seconds

7.)   70% ethanol

8.)   70% ethanol

9.)   distilled water for 2 minutes

10.) Cresylecht violet with 7 drops glacial acetic acid, 49°C
      for 6 minutes

11.) 95% ethanol

12.) 95% ethanol

13.) absolute ethanol

14.) absolute ethanol

15.) xylene

16.) xylene (until cover slip applied)

17.) fix cover slip with Permount

Appendix C. Sample results of histological verification of placements of guide cannula. Target nuclei are shown in the line-fill pattern; guide cannula tracks are shown in bold outline or solid fill with the number of the corresponding rat. Rats 1-3 show implantations into the AME and rat 13 shows implantation into the ABL.