Title
Control of Feeding by Glutamate and GABA

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CONTROL OF FEEDING BY GLUTAMATE AND GABA IN THE AMYGDALA

By

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A capstone project submitted for
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Abstract

With the widespread prevalence of obesity and other eating related disorders, research to understand the neural mechanisms is urgent. The hypothalamus has been a focus due to its ability to sense circulating nutrients and for its abundance of feeding-related neurotransmitters. Research from our lab has shown that the amino acid neurotransmitter glutamate elicits eating when injected into the lateral hypothalamus (LH), and that the neuropeptide galanin elicits eating when injected into the amygdala. The amygdala directly innervates the LH, suggesting a possible interaction in feeding control. The purpose of my study was to examine a role for glutamate and GABA in feeding by determining whether direct microinjection of the glutamate receptor agonist NMDA and the GABAA receptor antagonist picrotoxin into the central nucleus of the amygdala (CeA) would elicit feeding. Eleven adult male rats with indwelling cannulas targeted towards the CeA were injected with artificial CSF or dose-specific NMDA (1.0, 3.3 & 10 nmol/0.3 µl artificial CSF) and picrotoxin (17, 33, & 133 pmol/0.3 µl artificial CSF) and food intake was measured 1, 2 and 4 hours post-injection.

Acknowledgements

I would like to give thanks to my faculty mentor Dr. Glenn Stanley. Thank you for accepting me at the end of my first year as an undergraduate into your laboratory. Your continuous support and guidance in carrying out this pilot study taught me ownership and responsibility for my work in the laboratory. Thank you also to my graduate student mentor Andy Tseng for his constant aid and availability. You were the backbone of this project and I could not have completed it without you. I want to also thank graduate student Courtney Wood for her collaboration on this pilot study with me during the quarter she rotated through our laboratory, I am honored to have been a member of this laboratory and to have had the opportunity to work with such great people. Lastly, I want to thank graduate student Michelle Calderwood and my fellow undergraduate students Esther Kuan and Qi Chu for their assistance as well.

Table of Contents

List of Figures

Introduction

Obesity is a prevalent and growing issue in the United States. According to the National Health and Nutrition Examination Survey, the rate of obesity in 2017 increased to 39.8% in adults and to 18.5% in youth. Individuals with obesity are at higher risk to develop diseases such as diabetes, hypertension, and coronary artery disease. Eating behavior is regulated by homeostatic mechanisms that promote energy balance and the maintenance of body weight. However, it is evident that the motivation to eat often supersedes what is required based on energy needs alone. Therefore, continued research into the neural circuits involved would aid in understanding the processes that lead to overeating.

The hypothalamus has been intensely studied for its role in the control of appetite due to its ability to sense circulating nutrients and for its wealth of neuropeptides that promote or suppress feeding. Previous studies have shown that the primary amino acid neurotransmitters gamma-Aminobutyric acid (GABA) and glutamate can influence feeding behavior. For example, glutamate or its agonists, or a $GABA_A$ receptor antagonist, were found to elicit feeding when injected into the lateral hypothalamus (LH) of satiated rats (Stanley et al, 1993). Given the widespread distribution of these neurotransmitters in the brain, they likely mediate food intake through multiple brain sites. Specifically, I examined the amygdala, which is part of the limbic system's reward circuitry and is involved in motivation. The amygdala is a central component in the limbic system and feeding behavior is a motivated behavior. Thus, it is likely the amygdala or another component of the limbic system is involved somehow in the neural mechanisms that modulate feeding behavior. With this in mind, it became a challenge of which specific nucleus in the amygdala to test.

Previous research involving our lab unexpectedly found that the neuropeptide galanin stimulated food intake in the amygdala, implicating this brain region in the regulation of feeding (Kyrkouli et al, 1990). The basolateral amygdala (BLA) directly innervates the LH, providing a direct link to feeding circuitry, and this connection is required for the potentiation of eating in the presence of a conditioned cue (Holland et al, 2002). When injected into the BLA, the hypothalamic neuropeptide Orexin-A increased feeding (Avolio et al, 2012). Direct injection of muscimol, a $GABA_A$ receptor agonist, into the central nucleus of the amygdala (CeA) decreased food intake. This was blocked by the $GABA_A$ receptor antagonist bicuculline, confirming the involvement of local $GABA_A$ receptors in this effect (Minano et al, 1992). CeA injection of the NMDA receptor antagonist MK801 was shown to increase feeding in food-deprived rats (Solati et al, 2009). These findings illustrate that distinct subregions of the amygdala are capable of modulating ingestive behavior, including through the involvement of glutamate and GABA. With this known, manipulations of receptors mediating glutamate and receptors mediating GABA to induce feeding behavior was the core of my study and experimental procedure.

The purpose of my study was to further characterize the role of glutamate and GABA in the CeA in the control of feeding. Given the previous finding that a $GABA_A$ agonist suppresses feeding in the CeA, I wanted to determine if injections of a $GABA_A$ receptor antagonist would elicit feeding when injected into the CeA of satiated rats. Because neuronal excitation can similarly be achieved by application of glutamate or its agonists, I also tested whether an NMDA receptor agonist can alter food intake. I predicted that glutamate agonist injections and $GABA_A$ receptor antagonist injections into the CeA will elicit eating in satiated rats.

Notably, the experimental methodology was designed with previous successful work completed in the Stanley laboratory in the past. Specifically, the goal of the study aimed to elicit

feeding as inducing feeding can be attributed more to drug treatment than any other variables. On the contrary, a reduction in feeding behavior can be attributed to many other variables and may be less likely to be due to the drug treatment being tested in the experiment. In addition, NMDA and picrotoxin were chosen as the drugs to be used in the study because they have been used in the laboratory in the past and been to have shown to have effects.

Methods

Subjects and Surgeries

Eleven adult male Sprague-Dawley rats were used for this experiment. Rats were bred in an on-campus vivarium and housed individually in a room with standard 12-hour light/12-hour dark cycle, with food and water available *ad libitum*. Rats under pentobarbital anesthesia were stereotaxically implanted with a stainless steel guide cannula placed 1.0 mm dorsal to the CeA target site and held in place with skull screws and dental acrylic. The stereotaxic coordinates for the CeA are: 6.1 mm anterior to the interaural line, 4.2 mm lateral to the midline, and 7.6 mm ventral to the dorsal surface of the skull. Animals were given one week to recover from surgery, during which they were handled and given mock injections prior to experimental testing.

Drug Treatments

Three days prior to testing, animals were given a mash diet consisting of ground Purina rat chow, evaporated milk and sugar. Animals were tested under satiated conditions in which they were given fresh mash diet one hour prior to testing. Animals were injected with either 0.3µL of aCSF or N-methyl-D-aspartate (NMDA) (1.0, 3.3 & 10 nmol/0.3 µl aCSF), the NMDA receptor-selective agonist, or picrotoxin (17, 33, & 133 pmol/0.3 µl artificial CSF), the $GABA_A$ receptor antagonist. Picrotoxin is a known convulsant. With this in mind, there was a mid concern for convulsing behavior in the animals following injection of the highest dose of picrotoxin. The animals were closely monitored for such behavior and did not demonstrate any seizure-like activity. Injections will be conducted every other day through the cannula using a stainless steel micro-injector connected to a glass syringe via polyethylene tubing. Notably, the micro-injector extends 1.0 mm beyond the guide cannula. Measurements of food consumption were taken at, 60, 120 minutes and

240 minutes post-injection and documented onto a table. These values were then transferred onto the Excel table.

Tissue Preparation

After the experiment was complete and food intake was measured following each treatment, animals were fatally anesthetized and transcardially perfused with formalin. Brains were then extracted, post-fixed overnight, frozen with dry ice, and sectioned into 100 μm coronal slices. Slices were then mounted and immediately and underwent cresyl violet staining. At the end of experiments, location of cannula placement will be verified by cresyl violet staining. Cresyl violet staining consists of multiple steps that are concluded with a final rinse and soak in xylenes as the now dyed sections are ready to be covered with a thin glass coverslip adhered to the glass microscope slide using paramount solution. The now mounted slides are left to dry and later the excess paramount can be scraped off using a razor blade, rendering the final slides complete for microscopic analysis.

Analysis

Food measurements were taken for the first part of the experiment in which the animal subject's food bowls were weighed at 1 , 2, and 4 hours post injection. The food weights were entered into a Microsoft Excel spreadsheet programmed to calculate benchmark statistical values. This data was then used to run t-tests comparing aCSF food intake with the food intake at 1, 2, and 4 hours post injection. Targeted coronal section images on slides were compared to the published Rat Brain Atlas. Specific brain slices showing the deepest penetration of the cannula were noted in a notebook and thoses coordinates were cross-checked and identified using the Rat Brain Atlas to determine the accuracy of the cannula placement and approximate location of the drug injection.

Lastly, the slides with the final injection site were imaged using a Zeiss Axiocam camera using Axiovision 4.8 software.

Results

Figure 1 - Food Intake Measurements Post-Injection (NMDA)

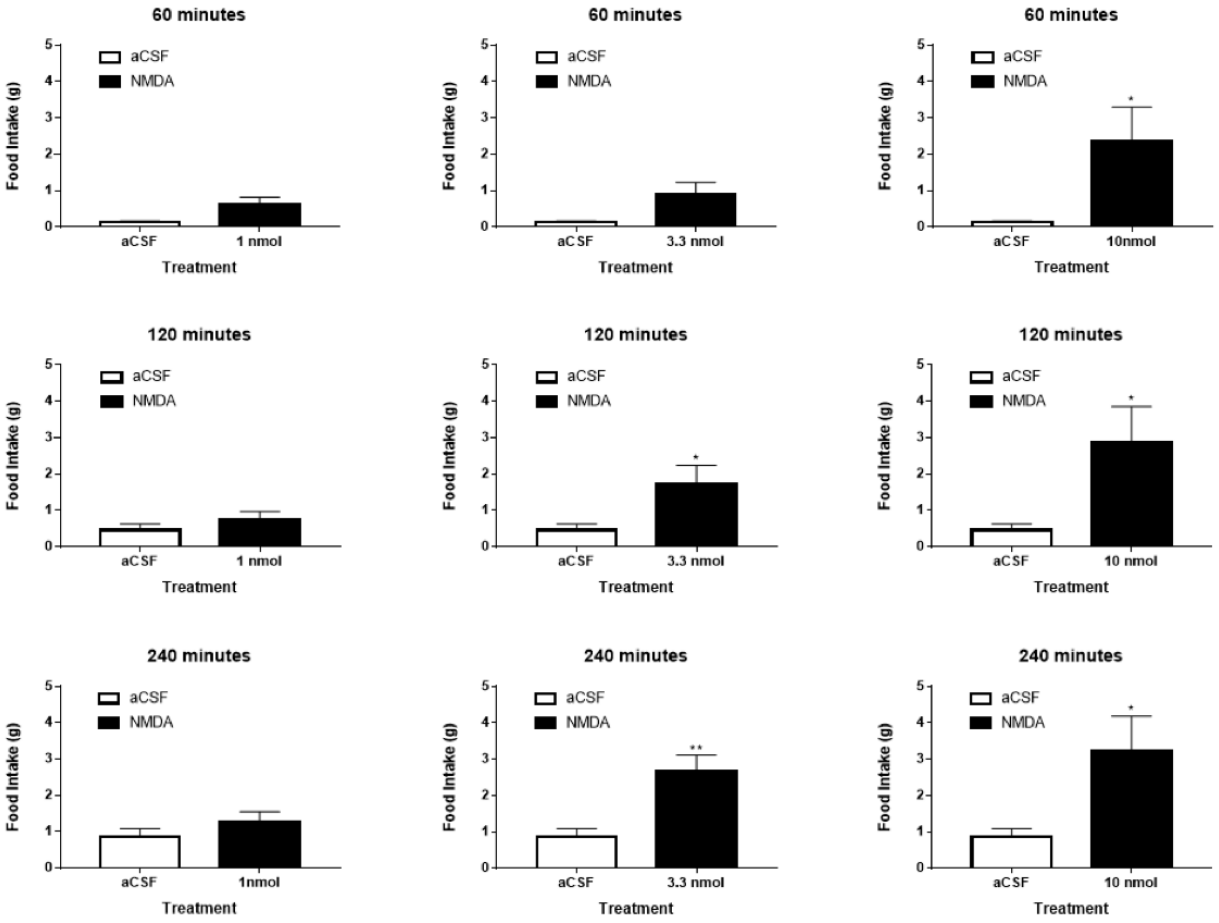

Figure 1: At 60, 120, and 240 minutes post-injection, there was no significant difference in food intake for aCSF animals compared to animals that received 1nmol NMDA. At 60 minutes there was no significant difference between aCSF animals and animals that received 3.3 nmol NMDA. However, there was a significant effect of NMDA on food intake when compared at 120 minutes post-injection, $p=0.0311$ and at 240 minutes post-injection, $p=0.0096$. At 60, 120, and 240 minutes, there was a significant effect on food intake, $p=0.0415$, $p=0.0292$, $p=0.0391$, respectively.

Figure 2: Food Intake Measurements Post-Injection (Picrotoxin)

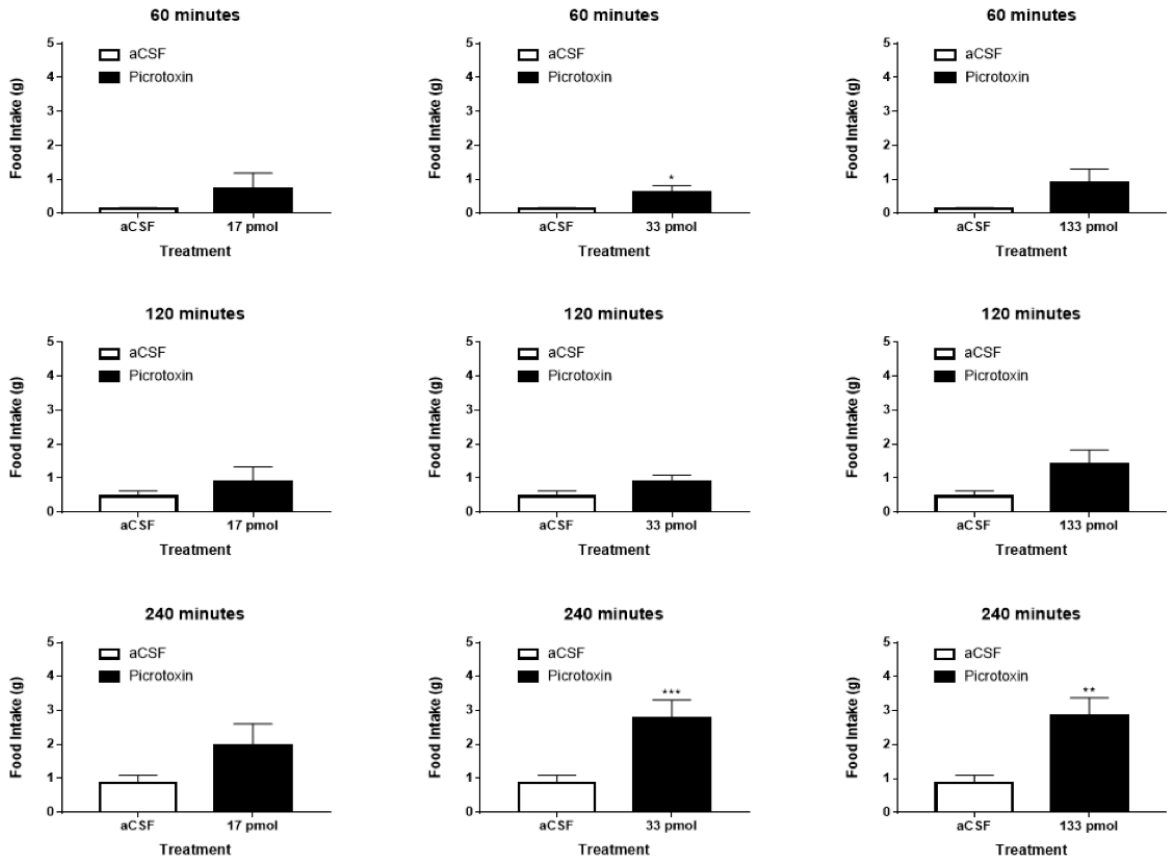

Figure 2: At 60, 120, and 240 minutes post-injection, there was no significant difference in food intake for aCSF animals compared to animals that received 17pmol picrotoxin. At 120 minutes there was no significant difference between aCSF animals and animals that received 33 pmol. However, there was a significant effect of picrotoxin on food intake when compared at 60 min, p=0.0398, and 240 minutes post-injection, p=0.0008. At 60 and 120 minutes, there was no significant effect on food intake, but at 240 minutes post-injection, there was a significant effect of picrotoxin on food intake, p=0.0074.

Figure 3 – Cresyl Violet Stained Coronal Section

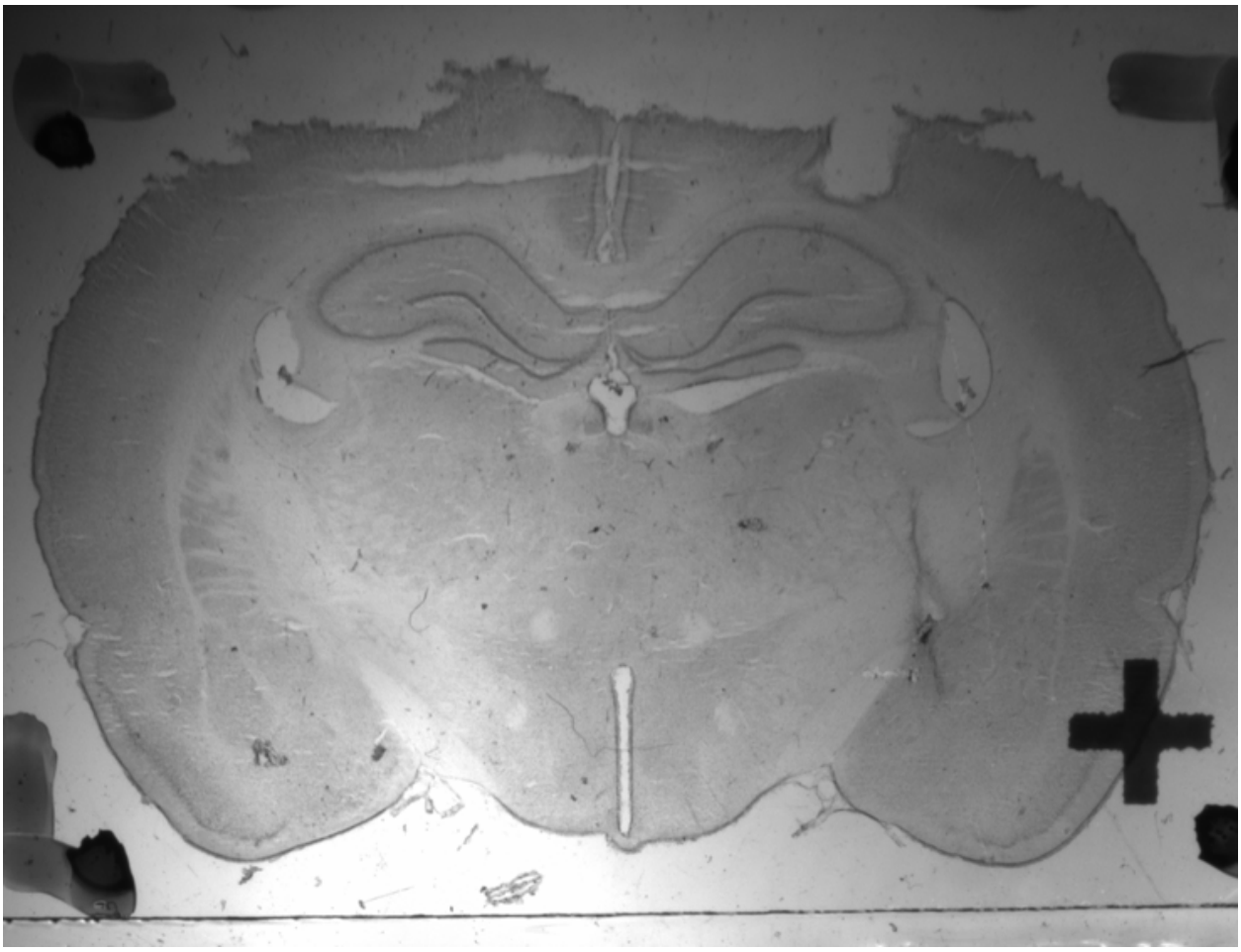

Figure 3: After staining the 100 µm coronal slices, the slides were analyzed using microscopic analysis to verify cannula placement. The sections with the deepest cannula penetration demonstrated the injection site. This slide depicts the injection site in the target zone, the central nucleus of the amygdala.

Figure 4 – Coronal Section Brain Atlas Reference Map

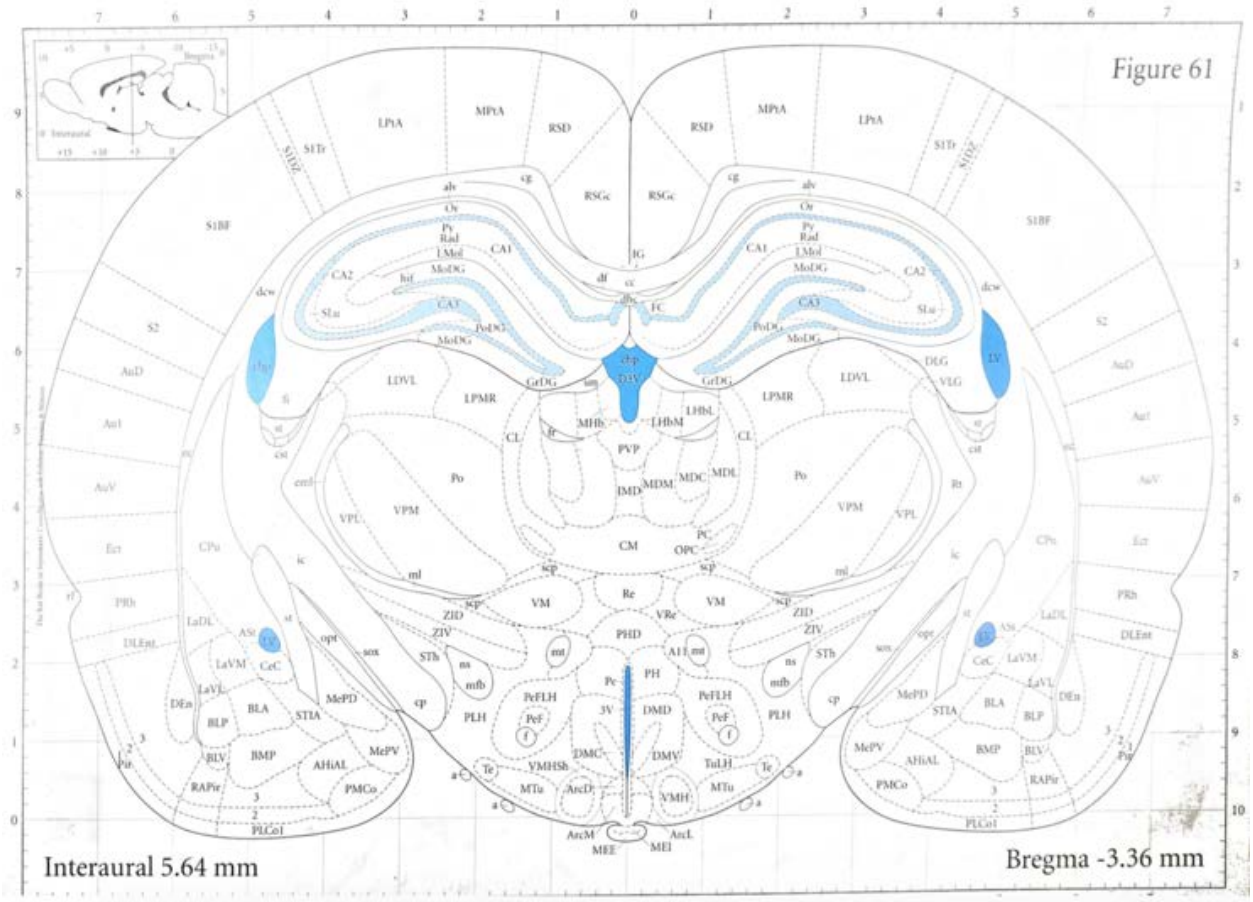

Figure 4: After staining each of the sections, microscopic analysis of the slides was conducted in comparison to the interaural 5.64mm reference map depicting the central nucleus of the amygdala, the injection target site.

Discussion

After performing statistical analysis of the food intake values at 1, 2, and 4 hour post-injection of NMDA and picrotoxin, statistical significance was shown at various time points and drug concentrations. Histological analysis suggests that this subset of subjects had injections just medial and dorsal to the central nucleus of the amygdala. Due to the fact that this study was in fact a pilot study to prompt research of the amygdala in relation to modulation of feeding behavior, it is important to have direction for effective and valuable follow-up studies. Thus, a follow-up study is planned to identify the specific brain site of action for this feeding stimulatory effect. Upon finishing this follow up study with verification of the new stereotaxic coordinates and cannula placement, microscopic analysis, in conjunction with food intake statistical analysis, will determine if another set of adjusted coordinates should be tested or if different experimental methods can be conducted in determining the effects of glutamate and GABA on feeding behavior.

In this follow up study, more animal subjects will be used. The larger sample size allows to have a higher confidence level when determining statistical significance of food intake. With this added data, the overall food intake measurements can be analyzed using an analysis of variance test or ANOVA. Again, if the data does not appear to show clear results and animals with a different set of coordinates not at the target site elicit more feeding behavior than those in which the coordinates are nearly exact, the same procedure should follow.

As each of these studies is performed, the role of the amygdala or any neighboring nuclei becomes more clear in its place in neural mechanisms of feeding behavior modulated through the overall neural circuitry attempting to be mapped by our laboratory. The more brain nuclei that could possibly have a role in feeding behavior, the closer the community can become to truly understanding neural modulation of eating behavior. Such an understanding paves way for

pharmaceutical interventions for obesity. A decline in obesity ideally reduces the risk of diabetes, hypertension, and coronary artery disease, which are some of the leading causes of death in the United States.

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