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#### ACCUMBENS PROCESSING OF A FOOD ASSOCIATED CUE IN A RAT MODEL OF BINGE EATING

by

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#### ABSTRACT OF THE DISSERTATION

# ACCUMBENS PROCESSING OF A FOOD ASSOCIATED CUE IN A RAT MODEL OF BINGE EATING By JOSHUA PAUL STAMOS Dissertation Director:

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Binge Eating Disorder (BED) is characterized by repeated episodes of loss of control over eating, negative self-image, and feelings of diminished self-worth (APA, 2013). BED is the most common of all eating disorders and effects between 0.6 and 7 percent of the general population (Marzilli, Cerniglia, & Cimino, 2018; Kim et al, 2018; Dahlgren, Wisting, & Ro, 2017). It has been shown that binging eating (BE) behavior, a key component of BED, can lead to acute elevations in synaptic dopamine levels in Nucleus Acumbens (Nac) similar to what may observed as a result of drugs of abuse such as cocaine or amphetamine (Rada, Avena, Hoebel, 2005; Unberg, Shader, Hsu, & Greenblatt, 2012; Wang et al, 2011). It is possible that the act of binging may lead to addition like effects in a similar way to what is observed in drug abusers. It has long been believed that reward cues play an important role in addiction. In this study we examine the effects of a history of BE on cue processing. Female Sprague Dawlay rats were subjected to a six week BE pretreatment. Following which these rats were tested in a 10 day pavlovian experiment where a tone cue signaled availability of a sucrose reward. Single unit recordings were taken in both Nac Core and Shell during the experiment. Additionally, we recorded ultrasonic vocalizations (USV) during the experiment in order to gain a measure of the subjects affect. BE rats exhibited significant decreases in number of cued head entries when compared to a control group. Additionally, analysis of neural firing during the pavlovian task indicated decreased Nac

Core processing of the reward associated cue in BE animals. Analysis of firing during consumption indicated increased processing during consumption when compared to a motor matched control behavior. This result held for both BE and control rats and in both Nac Core and Shell. Finally, Linear trend analysis of USVs indicated a decrease in positive affect in the BE group. Taken together these results indicate that BE treatment results in decreased tone processing in Nac Core, and possible decreases in positive affect. Finally, our results indicate that Nac processing increases during consumption when compared to a control behavior and that this result was unaffected by BE treatment.

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### Introduction

Binge eating (BE) is characterized by repeated episodes of loss of control over eating. Recurrent binge eating is a key component of several eating disorders, including Bulimia Nervosa (BN) and Binge Eating Disorder (BED). BN is characterized by binge eating with inappropriate compensatory measures. These can include voluntary vomiting, excessive dieting behavior, or bouts of excessive exercise (APA, 2013). BED is characterized by periods of binge eating without inappropriate compensatory behaviors. Instead BED is associated with feelings of loss of control over eating, and depression like symptoms which include negative affect and feelings of diminished self-worth (APA 2013; O. Kim, M. Kim, J. Kim, Lee, Jung, 2018). BED is the most common of all eating disorders (Kessler et al, 2013) affecting between 0.6% and 7% of the population (Marzilli, Cerniglia, & Cimino, 2018; Kim et al , 2018; Dahlgren, Wisting, & Ro, 2017). Approximately 25% of obese individuals that are seeking treatment are diagnosed with BED (Yanovski, 2003), indicating that BED is an eating disorder present in some, but not all obese individuals. BED is also associated with negative self-image and embarrassment over eating (APA 2013; Brauhardt, Rudolph, & Hilbert, 2014). Both BN and BED have a complex nature which includes body image perception, social, and physiological components. The complex nature of these disorders has made it difficult to successfully treat BN and BED (Bergh et al, 2013).

Feeding behavior is mediated by complex brain gut interactions. An important brain region involved in the control of eating is the Nucleus Accumbens (Nac), a component of the mesolimbic dopamine system (Rui, 2013; Yamaoto & Ueji, 2011). The Nac medial shell projects GABA to the lateral hypothalamus (LH), a brain region that has long been implicated with regulation of feeding (Stratford & Kelley, 1999; Stratford & Wirtshafter, 2012), as well as to the parabrachial nucleus, an area that has been shown to be taste sensitive (Choi, Campbell, Balsam, & Horvitz, 2012). In turn LH projects glutamate to the ventral tegmental area (VTA) which projects dopamine (DA) to the Nac (Richardson & Aston-Jones, 2012). Decreased Nac activity has been observed in response to an unconditioned reinforcer like food, particularly in the medial shell (Nicola, Yun, Wakabayashi, & Fields, 2003). It has also been shown that deactivation of the Nac medial shell by either the use of a GABA agonist or a Glutamate antagonist results in intense eating behavior in satiated rats (Maldonado-Irizarry, Swanson, & Kelley, 1995; Reynolds & Berridge, 2003). It is possible that deactivation of Nac medial shell causes a disinhibition in LH and other feeding related regions which may account for the increase in feeding behavior.

The mesolimbic DA system is most commonly associated with reward learning (Ambroggi, Ghazizadeh, Nicola, & Fields, 2011; Hoffmann, & Nicola, 2014; Nicola et al, 2003). There is substantial evidence of pathology in the dopaminergic system in patients both with BED and with BN. In a seminal study G.J. Wang and colleagues showed that obese individuals have decreased levels of striatal dopamine D2 receptor availability compared to normal weight controls (Wang, Volkow, & Fowler, 2002). In a later study Wang and colleagues showed enhanced dopamine activity in the striatum in response to food cues in BED patients but not in non-BED controls and that this effect was not related to BMI (Wang et al., 2011). Homovanillic acid (HVA) is the major DA metabolite in humans. HVA concentration is taken to be a measure of DA turnover in the brain. It has been shown that HVA levels in BN patients' cerebrospinal fluid are lower than controls and that this effect is related to binge frequency (Jimerson, Lesem, Kaye, & Brewerton. 1992; Kaplan, Garinkel, Warsh, & Brown, 1989; Kaye et al, 1990). In addition, a particular allele of the DAT 1 gene has been associated with both BN and BED (Davis et al, 2007). In a study of risk taking behavior comparing BED patients to amphetamine users and people with alcohol dependence results indicated that BED patients tended to engage in risky judgments compared to controls in a way similar to what was shown for drug dependent people

(Voon et al, 2014). From this, one might hypothesize that a history of binging on sweet fatty foods may lead to pathologies similar to those seen in various drugs of abuse such as cocaine and amphetamine which are known to act, at least in part, by inducing synaptic DA flooding (Rada, Avena, Hoebel, 2005; Unberg, Shader, Hsu, & Greenblatt, 2012; Wang et al, 2011).

In another study BED patients scored significantly higher on the power food scale than did obese subjects, suggesting that they are more susceptible to the rewarding effects of food. There were also substantial differences in D2 and opioid receptor alleles between BED and obese control groups. The A2 allele which is associated with a 30-40% increase in D2 receptor expression was more common in BED subjects. Eighty percent of subjects that expressed A1-/G+ alleles, which are associated with enhanced hedonic activation, were from the BED group. while 65% of A1+/G- which are associated with decreased hedonic reaction were from the control group (Davis et al, 2009).

#### **Reward Stimuli in the Limbic System**

There has been substantial work done in behavioral experiments examining limbic system reactivity to reward stimuli. Early experiments demonstrated that neurons from the VTA that project DA to the Nac respond to unexpected reward stimuli (Hollerman & Schultz, 1998; Schultz, Dayan, & Montague, 1997). Interestingly when a natural reward (unconditioned stimulus) becomes expected by use of a predictive cue (conditioned stimulus) the VTA neurons cease to respond to the unconditioned stimulus (US) and instead become responsive to the conditioned stimulus (CS) (Hollerman & Schultz, 1998; Shultz et al, 1997). Similarly, Nac neurons have been shown to be responsive to a reward associated tone cue, and the magnitude of the response was greater on trials in which the animal responded to the tone (Hoffmann & Nicola, 2014; Nicola, Yun, Wakabayashi, & Fields, 2003). It has been demonstrated that dopamine plays a key role in reward processing. Both D1 and D2 antagonists, microinjected into Nac, lead to significant decreases in behavioral as well as neural responding to a tone cue in the Nac in an operant task. Interestingly, D2 but not D1 receptor activation was necessary for a neural response to a tone not associated with sucrose reward (Hoffmann & Nicola, 2014).

In another study, it was shown that Nac Core and Shell have different roles in processing a response to a food cue. Glutamate blockade in Nac Core led to decreased responsivity to the tone, longer response latency on trials in which the animal did respond, and an overall decrease in the number of head entries into the sucrose receptacle. Glutamate blockade in in the Nac Shell did not affect cue responsivity, latency to respond or total number of head entries into the dipper port. However, glutamate blockade in Nac Shell led to increases in unrewarded sucrose seeking behavior which included increased reactivity to a tone cue not associated with sucrose availability, an increased number of unrewarded active lever presses, as well as an increase in inactive lever pressing. Nac Core glutamate blockade led to nominal increases in these three measures none of which reached significance (Ambroggi et al, 2011). Taken together it appears that Nac Core is more directly involved in responding to a known reward while Nac Shell may be more involved in general seeking behavior.

In a pavlovian experiment it was shown that a D1 but not D2 antagonist leads to decreases in both the cued head entry as well as responses during the intertrial interval (uncued head entry) (Choi, Morvan, Balsam, & Horvitz, 2009). Similarly, it was shown that D1 but not D2 antagonism led to decreases in the response rate in an operant task (Choi et al, 2009). D1 antagonism in the Nac core and the DMS, as well as systemic injection, but not antagonism in the mPFC, led to decreases in baseline head entry frequency (uncued head entries). Missed trials were not affected by microinjections in any of these brain regions. However systemic D1 antagonism led to increased numbers of missed trials (decrease in CR). High doses of D1 antagonist did lead to decreased locomotor counts in all groups except for the one injected in the DMS (Choi, Campbell, Balsam, & Horvitz, 2011). Taken together it is clear that the Nac, and particularly its dopaminergic innervation, plays a key role in processing of food associated cues and natural rewards.

As noted above there is substantial evidence that BE associated disorders such as BED and BN affect the Nac DA system's processing of food rewards and their associated cues. However, it is unknown whether this change in Nac processing is due to a preexisting pathological state inherent to the disorder or is the result of repeated binging behavior. It has been well established that drugs, such as cocaine, which affect the Nac DA system can chronically interfere with how the Nac processes reward signals (Volkow et al., 1993; Volkow et al., 1997). It is believed that cocaine acts by raising striatal DA levels (Kuhar, Ritz, & Boja, 1991) and has been shown to result in similar DA pathologies to those observed in BED (Jimerson, Lesem, Kaye, & Brewerton, 1992; Kaplan, Garinkel, Warsh, & Brown, 1989; Kaye et al, 1990; Volkow et al, 1993; Volkow et al, 1997; Wang, Volkow, & Fowler, 2002; Wang et al, 2011). It is possible that the repeated hyper-stimulation of the Nac DA system as a result of repeated binge behavior may lead to a similar effect. Unfortunately, it is difficult in human studies to separate the symptoms of BE related disorders from their underlying causes and therefore difficult to determine what if any disease related pathologies are the results of the pre-existing condition or are derived from disease related behavior. With regard to the latter, there has been some promising work in recent years in modeling binge eating in rodents (Corwin, & Babbs, 2012; la Fleur, Luijendijk, van der Zwaal, Brans, & Adan, 2013). Rat models of BE can help to enable mechanistic investigations into disorders which involve BE. Advantages of these rat models include 1) the subjects do not have any preexisting BE associated disorder and so it is possible to

use them to study the effects of binge behavior isolated from these disorders, and 2) wild-type animals are randomly assigned to experimental and control groups, enabling isolation of effects resulting from the experience of binging.

#### Rat Models of BE

An early study carried out by Hagan and Moss (1997) demonstrated that a history of food restriction can lead to permanent changes in food intake and preference for palatable food. It was shown that a behavioral feeding treatment was capable of creating persistent, longterm changes to feeding behavior that were not easily reversed by returning to normal feeding patterns. Additionally, while their methodology may have been somewhat confounded by familiarity with cookies (the palatable diet used in this study) in some of the groups, this study gives evidence that a history of chronic access to palatable food can significantly alter food intake long after the palatable food treatment has ceased (Hagan & Moss, 1997).

Building on this, there have been a large number of animal models which use various feeding manipulations to model eating disorders. In particular, eating disorders that involve BE can vary widely. Due to the large variability, there are a wide variety of rat BE models. Rebecca Corwin describes three general categories of BE related models in her review paper "Feeding and reward: perspectives from three rat models of binge eating" (Corwin, Avena, & Boggianom, 2011). The first of these is the sugar addiction model. There is evidence that some people are specifically addicted to sugar or other highly processed food, and that people use these foods to self-medicate to reduce negative affect (Ifland et al, 2009). A sugar addiction animal model was created in order to model this behavior. In this model rats are food restricted for 12 hours and allowed access to 25% glucose, 10 % sucrose solution as well as rodent chow for 12h. Within a few days of the onset of this paradigm these rats begin to exhibit sugar intake escalation (Avena, Rada, & Hoabel, 2008; Avena, Rada, & Hoeble, 2006a; Avena, Rada, & Hoeble, 2006b). An

opiate antagonist causes signs of withdrawal in sugar binging rats, indicating that sugar binging leads to dysregulation in the opiate system (Colantuoni et al, 2002). Sugar binging animals also show signs of increased anxiety. In addition to this sugar binging rats show cross sensitization with some drugs of abuse. Sugar binging rats have been shown to exhibit locomotor sensitization to amphetamine which is not seen in animals that were allowed to binge on ordinary chow or given ad libitum access to sugar (Avena, & Heobel, 2003). Finally, sugar addiction animals show increased alcohol intake when they no longer have access to their binge food (Avena, Carrillo, Needham, Leibowitz, & Hoebel, 2004; Corwin, Avena, & Boggianom, 2011). This model demonstrates that addiction like neurological phenotypes can be achieved through a diet manipulation.

The history of dieting with a stress model was particularly created as a model for BN. There is evidence that stress can foment BE behavior. While both BN and BED are associated with stress, BN is more strongly associated with stress than BED (Kestler et al, 2013). In this model rats are divided into four experimental groups based on whether they are given access to high fat diet and or are subjected to a stressor. Rats are placed on a cycle of 5 days where they are partially food restricted and then 2 days of ad lib chow. Animals that were allowed access to high fat diet were given cookies during this refeeding period. The stressor used in this model is a foot shock administered immediately prior to testing. (Artiga et al, 2007). After 4 cycles, the high fat diet (HD)+ stress group has been shown to eat significantly more food during the first 4 hours of the test than the other groups (Hagan et al, 2002). It was also demonstrated that when food was restricted and then access was given to both cookies and chow the HD+ stress group had a disproportional increase in cookie intake compared to the other groups (Hagan, Chandler, Wauford, Rybak, & Oswald, 2003; Corwin, Avena, & Boggianom, 2011). This model demonstrates that stress can be used to initiate feeding in a similar fashion to the way in which it can be used to initiate cocaine reinstatement (Boutrel et al, 2005; Sinha, Garcia, Paliwal, Kreek, & Rounsaville, 2006). It also demonstrates that a history of stress can have long lasting effects on feeding behavior.

One of the DSM criteria for BED is eating large quantities of food in the absence of hunger (APA, 2013). To model this a third category of BE models were developed. These are broadly categorized as limited or intermittent access models (Corwin et al 2011). While these models can vary slightly, all such models allow the subjects unlimited access to ordinary chow and water. In this way they differ from the models described previously. The subjects are given intermittent short duration access to palatable food. In these short access periods animals are given unlimited access to palatable food for a duration usually between 30 minutes and two hours (Bello et al, 2009; Bello, Patinkin, & Moran, 2011; Dimitriou, Rice, & Corwin, 2000; Wojnicki, Johnson, & Corwin, 2008).

In an experiment where a limited access group was compared to a continuous access group, the limited access group had increased food intake during a 2-hour palatable food access period, when compared to continuous access and chow control groups. The continuous access group gained the most weight. The limited access and chow control groups did not differ from each other in body weight. In a reward devaluation task both the control and continuous access groups showed reward devaluation, while the limited access group did not. Limited access rats had higher c-fos in the DLS than the other groups. There was no difference between groups in the DMS. The limited access group had significantly higher c-fos than the control (but not the continuous access group) in infralimbic cortex, cingulate/motor cortex, and somatosensory cortex. Finally, CNQX an AMPA blocker had the effect of restoring sensitivity to the reward that was lost in the devaluation task. This treatment had no effect on the control group. It is worth noting that for the limited access binge group while devaluation did not affect lever pressing, the main measure of devaluation, it did affect consumption. One interpretation of these results would be that the restrict binge group has a greater desire or drive for food but this does not translate into increased consumption (Furlong, Jayaweera, Balleine, & Corbit, 2014).

In a study designed to examine the effects on the reward system of both daily binged, and continuous high fat diets, it was shown that continuous access rats developed a significant increase in reward threshold when compared to baseline, as determined by electrical stimulation in Lateral Hypothalamus. Reward thresholds were shown to be stable over a period of several weeks following the completion of diet treatments. It was shown that a heavy body weight subgroup of limited access rats as well as all extended access animals had significantly decreased D2R levels. A lentivirus D2 knock down did not effect control rats but significantly increased the reward threshold in the extended access group. Finally, it was shown that a D2 lentivirus administration prevented decrease in caloric intake brought on by a punishment associated tone in the extended access rats but not the controls. However, limited access rats were not tested with the D2 lentivirus (Jonson & Kenny 2010). Here we see differential effects of continuous vs short access to palatable food. Only the extended access group had a significant increase in reward threshold. Additionally, extended access as well as heavy limited access rats had decreased D2R in striatum. Taken together it would appear that D2R downregulation and likely related changes in hedonic threshold may be in some way related to weight or at least be more strongly influenced in in extended access models. It is also worth noting that some of the effects observed in diet groups are similar to what has been observed in drugs of abuse (Nader et al, 2006; Volkow et al, 1993).

Limited access models can be subdivided into two subcategories. These are daily and intermittent access models. The difference between these two subcategories is that while in daily access models subjects are given limited access to palatable food on a daily basis,

intermittent access models give access to palatable food normally between two and three times a week (Corwin et al, 2011). In a meta-analysis comparing these two subcategories, ten studies were examined to compare an intermittent access binge model to a model where animals binged daily for a similar period of time. One group of rats was given daily 1-hour access to shortening (D) while the intermittent access group (INT) was given access Monday, Wednesday and Friday. All rats were given unlimited access to ordinary lab chow and water at all times. On week five the INT group universally had higher shortening consumption than the D group. In nine out of ten studies the INT group exhibited greater escalation of food intake then the D group. All INT groups had significant positive escalation slopes while this was the case for only 6/10 D groups. There were two unlimited access to fat groups that both showed negative escalation of fat consumption. Considering the fact that a cardinal feature of binging is a significant increase in palatable food intake, this demonstrates intermittent access enhances the BE model when compared to continuous and daily access groups. Furthermore, it was demonstrated that limited intermittent access to palatable food alone is insufficient to induce the increase in palatable food intake necessary for BE model validity. It was also shown that INT treatment elicits significant changes in in expression of genes associated with the dopaminergic system in the VTA and Prefrontal Cortex (Corwin et al 2016) In another experiment rats were given a maximum of 2g of palatable food per binge session in an intermittent access model. After five weeks on this paradigm subjects did not exhibit an increase in palatable food intake when tested during the sixth week (Babbs et al, 2012). This demonstrates that unlimited access during binge periods is necessary for a viable model.

There is a well-documented connection between food restriction and binging behavior (Hagan & Moss, 1997). One possible explanation for the connection between binge eating and dieting was proposed by Bart Hoebel. He theorized that since food restriction decreases

extracellular Nac DA levels (Pothos, Creese, & Hoebel, 1995), and since feeding in a once deprived rat elevates DA levels in the Nac Shell to amounts outlasting the feeding period it is likely that anhedonia created by food deprivation may prime the subject to binge on food (Pothos et al, 1995; Hoebel, & Teitelbaun, 1962; Hernandez, & Hoebel, 1988; Corwin et al, 2011). In line with this Boggiano and colleagues showed that food restricted rats had neurochemical changes consistent with anhedonia (Chandler et al, 2007). Additionally, it has been shown that binging has stress reducing properties (Dallman, Pecoraro, & la Fleur, 2005) and corticosterone has been shown to augment DA release in Nac (Graf et al, 2013). Finally, HD+stress rats if given a small morsel of palatable food can be primed to binge on chow after foot shock (Hagan, Chandler, Wauford, Rybak, & Oswald, 2003).

Binge models which incorporate periods of food restriction have been designed in order to model the sorts of compensatory measures observed in BN patients. It has been shown that escalation of food intake induced by a binge paradigm persists for several weeks after the treatment has been discontinued. It was shown that a group with a cyclic food restriction followed by a binging treatment had elevated c-Fos in the caudal and medial Nucleus of the Solitary Tract (NTS) . Both the Binge-Restrict and Continuous access groups showed increased c-Fos in the intermediate NTS (Bello et al, 2009; Bello et al, 2012). Following a 6-week pretreatment, restrict-binge animals show higher total caloric intake during a 2-hour binge period. However, a non-restricted binge group had a higher caloric intake from palatable sweetened fat. It was shown that there was decreased u-opioid mRNA in the NTS for continuous access and restrict-binge groups. Additionally, it was shown that a group that had been exposed to a twice weekly food restriction treatment had elevated u-opioid mRNA compared to bingerestrict, ordinary binge (no food restriction), high fat diet, and control animals (Bello et al, 2011). Following a 6-week treatment animals given continuous access to palatable diet expressed increased CB1 mRNA in the NTS when compared to restrict-binge, and Naïve groups, but not when compared to a non-restricted binge group. In the Cingulate Cortex restrict-binge, rats exposed to a food restriction paradigm, and ordinary binge animals had decreased CB1 mRNA when compared to a continuous access group. Finally, in Nac, restrict-binge, non-restricted binge, and continuous access animals all had decreased CB1 mRNA compared to a restrict only group which was not significantly different from a naive control group (Bello et al, 2012). Here we see that dietary manipulations which include binging can effect the opioid and cannabinoid systems as well as immediate early gene activation. It is worth noting that unrestricted access to palatable food, food restriction, and binge treatments all affect these systems in varying fashions, and so it may be correct to view the restrict-binge treatment as a hybrid treatment.

#### Rationale

For our experiment we chose to use a simplified version of the rat model for dietaryinduced binge eating (Bello et al, 2011; Bello et al, 2012). In this version of Bello's model, we opted to use a slightly modified version of the non-restrict binge group described above and the naïve group. Hereafter we will refer to them as the Binge Eating (BE) and Chow Control (CC) groups respectively. We chose to use our Binge group because, being that animals in this group are given unlimited access to ordinary chow at all times, we can be assured that during their binge periods they are eating in the absence of hunger, and as stated earlier, eating in the absence of hunger is a component of certain types of BED (APA, 2013). An additional advantage to this model is that binging and non-binging controls are weight-matched, which allows us to focus on the consequences of eating patterns without the metabolic influences of weight gain (Bello et al 2011). Additionally, we chose an intermittent access model because, firstly, human BED patients often binge less than once a day (APA 2013) making this an accurate model for many cases of the human disorder. Secondly, intermittent access models have been shown to exhibit greater escalation of binge food intake over days than rats with daily access (Babbs et al 2012). Escalation of food intake during binge sessions is a key component of a binge model and so one could argue that intermittent access models have greater model validity. While this model does not include social and body image components, it does model BE as an isolated behavior allowing us to further our understanding of this one component of BE related disorders. BE related disorders are accompanied by a set of distinct eating behaviors that contribute to the sustaining nature of these pathologies, which revolve around the hypothesized sensitization of the mesolimbic DA system (Pothos et al 1995; Hoebel & Teitelbaun, 1962; Hernandez, & Hoebel,I 1988, Corwin et al 2011). Using an animal model of dietary-induced binge eating will allow us to investigate the consequences of these aberrant eating patterns on the chief target of mesolimbic DA projections, the nucleus accumbens.

Additionally, BED has been associated with a plethora of mood associated disorders (APA 2013). BED as well as BN have a significant comorbidity with major depression, bipolar disorder, various anxiety disorders, and attention deficit hyperactivity disorder (Kessler et al 2013). In addition, BED has been associated with increases in subjective reports of liking binge foods (Nasser, Evans, Geliebter, Pi-Sunyer, & Foltin, 2008; Nasser et al,2013). There is some question as to whether these effects are the result of experience, i.e., repeated binge behavior, or an underlying pathology that may contribute to the initial cause of the binge behavior. In our rat model, as noted above, we will examine the effect of binging behavior in the absence of any preexisting condition or comorbidity. Affective state can be effectively studied using modern technology for recording and analyzing ultrasonic vocalizations (USVs). Rats vocalize in two distinct frequency ranges, 22 kHz and 50 kHz, which have been strongly correlated with negative and positive affective state, respectively. These vocalizations can be analyzed for their characteristics which can be used to obtain an indication of the rat's affective state (Knutson, Burgdorf, & Panksepp, 2002). Given the extreme time- and labor-intensive nature of analyzing USVs, we chose to examine 50 kHz calls because heightened levels of 50 kHz calls during the sucrose task could be considered to be indicative of heightened sucrose liking. Additionally, decreased levels of 50 kHz calls have been linked to depressive symptoms as well as depressive phenotypes in rats (Perez-Sepulveda, Flagel, Garcia-Fuster, & Slusky, 2013). In this way using one measure we were able to determine whether depression like symptoms were related to BE as well as if BE animals had increased subjective liking for sucrose.

In addition to this, it is possible to use behavioral measures, particularly in a motivated task, to gain information about a subject's motivation. Here we quantify the binge animal's 50 kHz USVs during the pavlovian task and compare them to those of the control group in order to ascertain if there is a difference brought about by the experience of repeated binge behavior (Fig. 1). Additionally, we examine behavioral measures within the pavlovian task in order to gain an indication of the subject's motivated state.

Finally, while there is substantial interest in the Nac control over eating (Volkow, Wang, Tomasi, & Baler, 2013) there have been very few electrophysiological experiments elucidating the Nac's involvement in feeding regulation. To our knowledge there have been no studies that used the high temporal and spatial resolution of single unit recording to study the Nac in animal models of binge eating, making this study the first of its kind. As stated above, the Nac has been strongly associated with motivated behavior. Considering that eating disorders such as BED and BN are largely disorders of motivated behavior, this warrants examination of how BE may affect Nac processing. Furthermore, it has been shown that people with BED have heightened striatal reactivity to food cues (Wang et al., 2011). In this study we expand on this. Firstly, because our rats have no preexisting disorders, we were able to ascertain what portion of this change is solely due to the act of binge eating. Secondly, our electrophysiology techniques have massively higher spatial and temporal resolution than those used in the aforementioned study (Wang et al 2011). Because of this we are able to distinguish between Nac core and shell, sub-regions that have been shown to process reward behavior differently (Ambroggi et al., 2011). Additionally, due to the excellent temporal resolution of our electrophysiological techniques, it was possible for us to study the complex nature of neural firing in relation to specific behaviors. We divide a simple pavlovian cue response into neurologically differentiable phases which are: cue processing, approach, and consumption. Because of our technique's high temporal resolution we can study these with precise delineation between the three events. Lastly, this study is, to our knowledge, the first in depth electrophysiological study of Nac in female subjects. In recent years there has been heightened interest in the neurological differences between males and females, and this study will be enlightening in this regard. Here we have made a step towards improving understanding of BE and related disorders such as BN and BED. A better understanding of neural mechanisms that underlie these disorders may lead to improved treatments for patients who suffer from these disorders.

#### **Experimental Design**

Here we studied neuronal activity in the Nac in a well-established rat model of binge eating disorder. We pretreated a group of female Sprague Dawley rats with a 6-week binge eating design (Bello et al., 2011, Bello et al., 2012), for comparison to an age-matched control group of females fed only standard lab chow. On completion of pretreatment, all rats had an array of 16 micro wires implanted into the Nac. The rats were tested in a simple Pavlovian task in which a tone cue (CS) was paired with presentation of a sucrose reward (US). It has been shown that responding in this task is disrupted by microinjection of dopamine D1 receptor antagonists into the Nac (Choi et al., 2011), establishing the importance of Nac firing in the task (Nicola, 2010). Here we studied neuronal activity in the Nac during cue processing, approach to the sucrose port, and sucrose consumption. Additionally, USVs during the task were analyzed as an aid in interpreting the neuronal and behavioral data.

### **Materials and Methods**

#### Animals

Adult female Sprague Dawley rats (n=9 per group, 150g-200g at the start of the experiment) were acquired (Harlan Laboratories, Frederick, MD), individually housed, and placed on a 12h/12h light dark cycle (lights off at 1700 hours). All animals had unlimited access to standard lab chow (LabDiet 5012) and water at all times unless otherwise noted. All experiments were done in accordance with the Institutional Care and Use Committee of Rutgers University and in accordance with NIH guidelines.

#### **Binge Eating Paradigm**

A mixture of vegetable shortening (Crisco<sup>®</sup>, 25% saturated fat, 50% polyunsaturated fat, and 25% monosaturated fat) and sucrose (8.6 kcal/g) was used to make a highly palatable "sweetened fat" solution. The sweetened fat mixture was comprised of 10% sucrose by weight. All animals were given unlimited access to the sweetened fat solution for 24 hours, 7 days before the onset of any experimental procedures. Rats were divided into Binge Eating (BE) and Chow Control (CC) groups without any significant difference with respect to body weight and initial preference for the sweetened fat mixture. The BE group was given unlimited access to the sweetened fat solution for a duration of 30 minutes at 1530 hours on days 2 and 5 of each week. This procedure was carried on for 6 weeks before any animal surgery was performed, and resumed after surgery. Vaginal cytology was also taken on days when data were collected to ensure that stages of the estrous cycle were not influencing our measurements.

#### **Pavlovian Apparatus**

Pavlovian trials were conducted in a custom acrylic chamber (31.0 cm length, 25.5 cm width, 45.0 cm height). This chamber was housed inside a larger sound attenuating cubicle (6249C, Med Associates; St. Albans, VT). The sound attenuating cubicle was illuminated by a series of LED lights on the ceiling which served as house lights during the experimental

procedure. The Pavlovian chamber was equipped with a rectangular opening (3.0 cm wide × 14.5 cm high × 3.0 cm deep) that served as a head entry port on the right sidewall of the chamber. This "dipper port" was equipped with a sucrose dipper (H1405R, Coulbourn; New York, NY) that could be raised and lowered within it, such that when the dipper was raised the animal could access the dipper's cup by placing its head within the dipper port but when the dipper was lowered the animal could not reach the dipper. The dipper was connected to a remote syringe pump (R-E, Razel; Fairfax, VT) which delivered a 32% sucrose solution into the dipper's cup. The syringe pump was located outside of the sound attenuating cubicle underneath a heavy counter, the intent of this being to make the syringe pump's operation inaudible. A cue light was located inside the dipper port. An infrared photocell (H2093SP01, Coulbourn; New York, NY) was located 1 cm within and 1 cm up from the floor within the dipper port, the purpose of which was to record head entries to the dipper port. The chamber was equipped with a digital camera that was used to record the entire session of 56 Pavlovian trials. Sessions in this chamber were controlled by a computer running Datawave (Datawave Technologies, Loveland CO) software which acquired all neural, behavioral and video data.

#### Surgery

Briefly, animals were anesthetized with an i.p. injection of sodium pentobarbital (50 mg/kg i.p.). Anesthesia was maintained by ketamine hydrochloride (60 mg/kg, i.p.) as necessary. Atropine methyl nitrate (50 mg/kg, i.p.) and penicillin G (75,000 U/0.25 ml, i.m.) were administered before surgery. A 16 micro-wire array (2X8; Shapiro; West Windsor, NJ) was implanted into the animal's Nac in the right hemisphere (for details on surgical procedures see Barker, Root, Coffey, Ma, and West (2014)). Arrays were implanted by rectangular craniotomy. The coordinates for the corners of the craniotomy window were [(0.6 A-P, 0.5 M-L), (1.8 A-P, 0.5 M-L), (0.6 A-P, 3.0 M-L), (1.8 A-P, 3.0 M-L)] measured in millimeters lateral of the centerline, and

anterior relative to bregma. Arrays were constructed from 50 um stainless steel and were coated in Teflon insulation. All arrays were made up of 2 columns of 8 wires. Within each column all wires were 250 um apart and the distance between the two columns was 750 um. Arrays were lowered using a motorized stereotaxic device (Coffey, Barker, Ma, & West, 2013) to a depth of 6.7 mm below the skull surface. In addition to this a stainless-steel ground wire was implanted, with 5 mm of exposed wire, to a depth of 5 mm below the skull surface.

#### **Pavlovian Task**

After completion of surgery animals were allowed 1 week to recover before the onset of Pavlovian training, during which time BE animals were maintained on their binge eating schedule. After recovery, animals were placed in the Pavlovian chamber for 10 consecutive daily, Pavlovian sessions which averaged one hour in duration. Each session comprised 56 trials in which a tone was activated for .5 seconds. At the midpoint of the tone (.25 sec after tone onset), the cue light was activated and the dipper was raised delivering a small volume of 32% sucrose solution. The light remained on and the dipper remained in the raised position for 4 seconds after a head entry or 10 seconds in the absence of a head entry. Trials were spaced by a pseudorandom time interval which averaged 1 minute. Each session was recorded by a digital camera for further analysis. We did not interdigitate a second, unpaired tone as "CS-" because a sequence of + and – trials generates local expectancies and affects neural activity (West, Christian, Robinson, & Deadwyler, 1982). It is already well established that rats learn to respond to a CS+ but not to a CS-; the present interest is to explore whether a tone paired with sucrose evokes accumbens firing in binged rats differently from controls. We used a 32% sucrose solution instead of the sweetened fat used during binge to prevent any effect of flavor familiarity between BE and CC groups.

#### **Ultrasonic Vocalizations**

A condenser microphone (CM16/CMPA, Avisoft Bioacoustics, Berlin Germany) was inserted into the sound attenuating cubicle so that it was suspended approximately 2.5 cm above a set of small holes in the top of the Pavlovian chamber. Baseline vocalizations were recorded for each animal in a separate chamber from the Pavlovian chamber prior to any Pavlovian sessions. Recordings were also taken throughout each Pavlovian session. Recordings were taken at a 92-kHz sampling frequency (16-bits) using Avisoft Recorder software and hardware (Ultrasound Gate 116H, Avisoft Bioacoustics). Following each session recordings were saved as WAV files and stored for offline analysis. Analysis was performed using Raven Pro 1.5 software (Bioacoustics Research Program, 2011; Cornell Lab of Ornithology, Ithaca, NY).

#### Analysis of Ultrasonic Vocalizations

Sound files recorded during the Pavlovian sessions were represented graphically as spectrograms in which the frequency, duration, and intensity of spectrograms can be inspected visually. WAV files were first run through custom software that identified possible 50 kHz calls. Spectrograms exhibiting characteristics of USVs were transposed to 5% of their normal frequency so that they could be played back for a secondary auditory verification. Only calls that exhibited both visual and auditory characteristics indicative of a USV were included in the analysis. For each animal its baseline recording was compared to recordings taken during the pavlovian trials (Fig. 1); in this way each animal served as its own control, in order to control for the large individual differences in rates of calling. Comparisons were also made between BE and CC groups.

#### Vaginal Cytology

Phase of Estrous cycle was determined by the use of vaginal cytology. This was performed following data acquisition on all days on which experimental data was taken. Briefly, vaginal cytology was performed via vaginal lavage with .9% sterile saline solution. Cells were characterized by vaginal epithelial cell morphology. Proestrus and estrus were determined by the presence of nucleated epithelial cells and of cornified cells. The presence of leukocytes was used to classify Diestrus. For the purpose of analysis proestrus and estrus were considered to be similar (Bello, Walters, Verpeut, & Caverly, 2014).

#### Histology

On completion of the experiment all animals received a lethal injection of sodium pentobarbital (150-200 mg/kg i.p.) after which time a current (50 μA for 4 sec) was passed through each of the 16 microwires in order to mark their locations. Animals were perfused with paraformaldehyde and their brains were removed and sunk in paraformaldehyde for 48 hours. Following this they were placed in a 30% sucrose solution for 48 hours. The Accumbens (plus an extra ~1.5 mm rostral and caudal) was sectioned coronally into 50µm slices and mounted on slides. Following sectioning all slices were stained with calbindin d-28k in order to differentiate between Accumbens Core and Shell. Following this, sections were soaked in 5% potassium ferrocyanide and 10% HCl in order to stain the iron deposits left at the marked microwire recording tips. Our criterion was set such that, each of the 16 iron deposits must be able to be positively identified with its corresponding microwire. It was necessary as part of our criteria that the microwire's entire track from its entry into cortex to its tip must be accounted for. If we were unable to do so with any wire then the data recorded from that wire were discarded. The precise locations of the microwire tips were estimated as the centers of the deposit markers. Centers located within 100µm of any Core/Shell border area were considered to be in a border area. Microwire tips that bordered extra-accumbal structures were eliminated (Fig 2.).

#### Video Analysis

Video analysis was performed in order to study Accumbens firing during approach to the sucrose dipper port. The animal's approach (cued or uncued) was defined as the time between when the animal initiated her approach to the dipper port and when her head entered the port. The approach was measured retrogradely from the time when the animal's head broke the photobeam in the dipper port. From the photobeam break the video recording was played backward frame by frame (30 frames/sec) until the frame in which the movement started in the direction of the dipper port was identified. We required that during approach the animal's movement was continuous in the direction of the dipper port. In lieu of a photobeam break in the dipper port, three consecutive video frames in which the rat's head remained motionless in the dipper port were counted as a head entry, in which case the precise time of approach termination was measured from the first frame in which the animal's head was motionless in the port. The animal's approaches were divided into two categories: cued and uncued. Cued approaches were defined as approaches that were initiated after the onset of the tone cue and terminated while the dipper was still available to the rat. Uncued approaches were defined as approaches that took place during the inter-trial interval (Fig 1).

#### **Behavioral Measures**

The primary behavioral parameters examined were the proportion of hit trials, and number of uncued head entries. We defined the hit trial proportion as the total number of trials in a session in which the animal succeeded in making a head entry while the sucrose dipper was present in the up position (10 sec), referred to as hit trials, divided by the total number of trials per session (56). The number of uncued head entries was defined as the total number of head entries that the animal made during inter-trial intervals in a session. These behavioral measures were analyzed by the use of generalized linear mixed modeling (see below).

#### Analysis of Electrophysiology

Signals recorded by microwires in the Nac were analyzed offline using commercially available software (Datawave). Spikes were sorted in order to eliminate electrical noise and so

that possible multiple neurons recorded on a single electrode could be distinguished from one another (~95% of wires record only one neuron's waveform). Spikes were differentiated from electrical noise as well as signals from other neurons by the shape of their wave form. The parameters used to classify a neuron's wave properties were peak time, peak amplitude, spike time, spike height, principle components, and two custom voltage cursors. Electrical signals recorded from a given microwire were considered to be neuronal and resulting from a single neuron if there was a signal to noise ratio of at least 2:1, all spikes had a similar shape and consequently similar values for the above-mentioned parameters, and there was an inter-spike interval (ISI) of no less than 2ms corresponding to a natural refractory period.

In circumstances similar to those outlined in this experiment, it is possible to longitudinally record from the same neuron on a given wire over days (Tang, Pawlak, Propenko, & West, 2007; Tang et al., 2009; Tolias et al., 2007; Dickey, Suminski, Amit, Hatsovoulos, 2009; Fraser and Schwartz, 2012; McMahon, Bonder, Afuwape, Ide, Liopold, 2014; Coffey et al 2015). In cases where a neural waveform was consistently recorded over days on a single microwire, it was concluded that the recordings came from the same neuron if the following criteria were satisfied. First, there must be a correlation of .9 or greater between the average waveforms (average waveform voltage during the spike) recorded from the microwire on any two consecutive days of the given period (i.e., the waveforms have a similar shape). Second, the difference between spike height from one session to the next was less than 20%, and the difference in peak time from one session to the next was less than .04 ms. Finally, alternative explanations may be ruled out using a combination of logical inferences based on properties of MSNs including average spacing between somas and average spike amplitudes. The above criteria have been shown to be a statistically valid method for determining if a neuron was held over days in the following way. In a previous study the intra-wire waveform stability was assessed by calculating the area displaced under the curve for the average waveform for the recordings on each day. This was compared to the expected difference between waveforms assuming that the microwire recorded from a different neuron each day. This was done by randomly selecting neurons from different wires on different days and comparing them to the recordings from a single microwire. It was shown that, using the above criteria for neuronal stability, there was a large statistically significant difference between neurons selected with our criteria from ones randomly selected from the same brain region (Coffey et al 2015).

To characterize Nac neuron sensitivity to reward seeking behavior, firing rate was analyzed during five reward related behaviors. These were cue processing, cue-evoked approach, non-cued approach, sucrose consumption (i.e., cue-evoked head entry and consumption), and pseudoconsumption (i.e., the time period while the animal's head was in the dipper port after a non-cued head entry). These five behaviors were divided into two distinct categories, namely cued and uncued behaviors. A cued behavior began with a tone cue and the associated cue processing. It was followed by a cued approach and concluded with a consummatory behavior. We defined cue processing as the time beginning with the onset of the tone cue and ending either at the onset of approach or the end of the tone cue, whichever occurred first. It is important to note that tone evoked firing in response to tones that did not result in an approach were not considered to be related to cued behavior. We defined a cued approach as the time period beginning with a cued approach onset and ending when the animal's head entered the dipper port. For an approach to be considered a cued approach the approach onset must take place after the onset of a tone cue and must terminate before the dipper was withdrawn. Consumption was defined as the time period between the onset of a cued head entry and either the following head exit or the retraction of the dipper, whichever occurred first. However, firing during these "non-responded to" tones (misses) were used

during analysis for comparison to tones that did elicit an approach (hits). Uncued approach and pseudoconsumption were both uncued behaviors that were observed using video analysis to be motorically similar to cued approach and consumption respectively. Uncued approaches were defined as the time period beginning with the onset of an uncued approach (as determined by video scoring) and ending with an uncued head entry. For an approach to be considered uncued it must both begin and end during the inter-trial interval. Pseudoconsumption consisted of the time interval between an uncued head entry and the corresponding head exit, provided that there was no tone during this interval. Firing rates for each cued behavior were compared to those exhibited during their analogue uncued behavior. In this way we were able to eliminate possible motor confounds from our analysis of firing. Furthermore, firing rates for all five reward related events were compared to temporally matched periods of baseline firing taken from a time period immediately before the given event in which no other reward related event took place. This was done on an event-by-event basis such that each individual event had its own baseline, of the same duration, for comparison of firing. Firing rates (FR) during all reward related behaviors were compared to baseline FR sampled from the inter trial interval before the onset of the behavior (when the animal was not engaged in any of these five behaviors, see next paragraph). FR during each reward associated behavior was measured in spikes per second (i.e., total number of spikes during a given behavior divided by the duration of the behavior on that trial). Event related FR was visualized using raster plots and peri-event time histograms (PETH) (Fig 3), and was analyzed quantitatively via generalized linear mixed modeling (see below).

#### **Data Analysis and Statistics**

Having divided the cued behavior into three events: cue (tone), approach, and consumption, separate statistical models were used to analyze neuronal FR for each event. Analysis of event related FR in all phases, with the exception of cue processing, utilized "motor matching" in order to eliminate or minimize any confounds related to motor differences. Because cue processing takes place before the animal initiates the approach, there was no direct motor match for cue processing. Nonetheless, motor confounds were minimized or eliminated because the unpredictability of tone onset assured that motor behavior was asynchronous up until the time of the first tone-evoked movement, at which time our cue processing window was terminated. Instead, cue processing events were divided into hit and miss trials. Hit trials were defined as trials which, on hearing the tone cue the animal initiated an approach followed by consummatory behavior. A miss trial was defined as a tone trial in which the animal did not respond to the tone cue with an approach. Due to their likely difference in processing, we compared firing during hit and miss trials in our cue processing statistical analysis. FR during cued approach was compared to FR during non-cued approach. FR during consumption was compared to FR during pseudoconsumption. For the purposes of comparing baselines to event firing we in some cases calculated change scores. Change scores were calculated as follows (Event firing rate-Baseline firing rate)/(Event firing rate + Baseline firing rate + c) where c is a small constant, which in this case was .1. c was added to the denominator of all change scores in order to prevent division by zero in cases where the baseline and event firing were both zero.

**General Approach Towards Statistical Analysis.** All data were initially examined using exploratory data analysis (EDA) techniques. EDA was carried out using the ggplot2 package (Wickham, 2016) for R (R Core Team, 2018) (cite). The goal of the EDA was to do an initial examination of the data for relevant and interesting patterns, and to determine linear and/or curvilinear trends were present that would make the data suitable for parametric statistical modeling. The EDA consisted of histograms, density plots, bloxplots, and scatterplots.

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Much of the neural and behavioral data exhibited nesting in its organization. Nesting, or clustering, is the sampling phenomena in which multiple individual observations are grouped by instances of certain higher order variables, such as individual firing rates associated with a specific neuron or repeated behaviors observed for a specific animal (Raudenbush & Bryk, 2002; Stevens, 2009; Stroup, 2012). Standard ANOVA/regression statistical models are not suitable for this type of data because the nested structure of the data causes a violation of the assumption of independence and thus leads to a massively inflated Type I error rate (Raudenbush & Bryk, 2002; Stevens, 2009; Stroup, 2012). Generalized linear mixed models (GLMM), also known as hierarchical linear models (HLM), were designed to handle the presence of nesting by modeling them as random effects (Raudenbush & Bryk, 2002; Stroup, 2012; West, Welch, & Gałecki, 2006). In the present study, whenever nesting was present in the structure of the data, GLMM was used. All GLMM analyses were carried out using PROC GLIMMIX in SAS 9.4 (SAS Institute, Cary NC). A key advantage of GLIMMIX is that it allows for normal and nonnormal distributions to be specified for the outcome variable (Stroup, 2012). In certain instances, the outcome variable showed a skewed distribution, in which case, a gamma distribution with a log link was specified for the GLMM. In all other cases a normal distribution was specified for the GLMM.

For each GLMM, all possible combinations of independent (fixed effects) variables were specified. All GLMM analyses utilized robust standard errors because robust standard errors are less sensitive to departures from normality (Raudenbush & Bryk, 2002). Planned post-hoc comparisons where specified a priori and were carried out only if the relevant associated omnibus interactions and main effects were statistically significant, i.e., a "protected t-test" approach was utilized for post-hoc testing (Howell, 2001). Holm-Sidak corrections for multiple comparisons were specified for each family of post-hoc tests to control for Type 1 error. If a GLMM included a covariate, i.e., a continuous independent variable, then essentially a multiple regression model was formed in which each possible combination of different levels of the model's fixed effect categorical variables was associated with a specific regression of the dependent variable and a covariate. In such cases, the regression line for each combination of fixed effects levels was computed and confidence intervals were computed for the intercept and slope. The confidence intervals were used to test whether the slopes and intercepts were different from certain relevant values. For instance, if a slope showed no statistical difference from 0, i.e., the confidence intervals did not encompass 0, then no relationship between the covariate and the independent variable was observed for that particular subgroup among the levels of the independent variables. In addition, post-hoc tests comparing slopes and intercepts among different subgroups of independent variables were tested. All confidence intervals were adjusted using the Holm-Sidak post-hoc correction for multiple tests in order to control for Type I error rates.

Analysis of Behavior. EDA graphical analysis was performed as described above. Two separate GLMMs were performed as described above for proportion of hit trials. The first had independent variables being Diet, Phase of Estrous, and Session. The second GLMM had dependent variables being Diet and Session. Session was treated as a continuous variable. Because proportion of hit trials was skewed, a gamma distribution with a log link was specified. Both linear and curvilinear regression analysis were performed with respect to the variable of Session on the BE and CC groups. Additionally, multiple post hoc comparisons were performed where appropriate.

EDA graphical analysis was also performed for rate of uncued head entries. Two separate GLMMs were performed as described above. The first GLMM had Session, Phase of Estrous, and Diet as independent variables. The second GLMM had Session and Diet as
independent variables. Session was treated as a continuous variable. Additionally, both linear and curvilinear analysis was performed with respect to Session on both BE and CC groups. Finally, multiple post hoc comparisons were performed when appropriate.

Statistics for ultrasonic vocalizations. EDA graphical analysis was performed using the above described techneques. Two separate GLMMs were performed for 50 kHz call rate. The first had independent variables being Diet, Phase of Estrous, and Session. The second GLMM had dependent variables being Diet and Session. Session was treated as a continuous variable. Additionally, linear and curvilinear regression analysis with respect to Session was performed. Post hoc analyses as described above was performed as appropriate.

Statistical analysis of Electrophysiology. Identical separate analysis were performed for all neural data for Nac Core and Shell. For each of the three events, a GLMM was used to analyze firing rate (FR). FR exhibited a nested structure in which multiple FRs, each representing a single neuron, were observed across each trial. For this reason, for the purpose of analysis, FR was nested within neuron. Because of this, the GLMM approach enabled individual differences in the pattern of FRs observed across neurons to be captured and statistically assessed.

The GLMM for each event had FR as the outcome variable, and the independent variables were: 1) for the tone event, eating condition (BE vs. CC), reward-related events (baseline vs. cue(tone)), and hit vs miss, 2) for the approach event, eating condition (BE vs. CC), event (baseline vs. approach event), and approach (cued vs. uncued), 3) for the consumption event, eating condition (BE vs. CC), event (baseline vs. consumption event) consumption (consumption vs. pseudoconsumption). All possible interactions were specified for each model. There was a two level nested structure for the GLMM based on individual trials (1<sup>st</sup> level), and neuron (2<sup>nd</sup> level). All GLMM analyses were carried out in SAS PROC GLIMMIX. Furthermore,

comparisons were made in behavioral data across sessions to ascertain if there was an effect of treatment on reward learning.

Analysis of Cue Processing. EDA graphical analysis was performed using the above described techneques. A GLMM was performed with Diet, Session, and Trial Type (hit vs miss) as independent variables. A GLMM was performed with independent variables being Baseline Firing Rate, Diet, Trial Type (hit vs miss), and Session. Session was treated as a continuous variable. Post hoc analysis as described above were performed as appropriate. Additionally, we performed two regression analysis' the first comparing baseline firing rates between hit and miss trials and the second comparing event firing during hit and miss trials.

Analysis of Approach Firing. As before EDA graphical analysis was performed as described above. A GLMM was performed with Baseline Firing Rate, Diet, Session, and Trial Type (cued vs uncued) as independent variables. As before post hoc comparisons were performed where appropriate. Similarly, to what was done for cue processing, two regression analysis' were preformed, the first comparing baseline firing rates for cued and uncued approaches, the second comparing approach firing between cued and uncued approaches.

Analysis of consumption firing. As before EDA graphical analysis was performed as described above. A GLMM was performed similar to the ones described for cue processing and approach with Baseline Firing Rate, Diet, Session, and Trial Type (consumption vs pseudoconsumption) as independent variables. As before post hoc comparisons were performed where appropriate. Similarly, to what was done for cue processing and approach, a regression analysis was conducted comparing baseline firing rates for cued and uncued approaches, as well as a second regression analysis comparing event approach firing between cued and uncued approaches. Additionally, a simple a two-way ANOVA was run with factors

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being Diet and Trial Type (consumption vs pseudoconsumption) comparing the change scores during Consumption/Pseudoconsumption.

# Results

## **Behavior**

**Proportion of Hit Trials.** Based on a graphical analysis comparing the proportion of Hit trials over days between BE and CC animals we observed that BE animals appeared to be deficient in learning the sucrose task, as measured by lower numbers of Hit trials over days (Fig 4). In order to quantify this result we ran a generalized linear mixed model (GLMM) with factors Diet, and Session. It was found that there was a significant main effect of session (p<.0001, F(9,98)=34.16), as well as a significant interaction between Diet and Session (p<.0001, F(9,98)=6.76), indicating that there was a change in the number of hit trials as the sessions progressed and that there was a difference between diet groups in the progression of this learning. To further elucidate this difference we fit both linear and curvilinear models to the data. It was shown that there was a significant linear trend for both Control (p<.0001, t(98)=-10.78) and Binge (p<.0001, t(98) =-9.31). Additionally, there was a significant curvilinear relationship between proportion of hits and session for the Control animals (p<.0001, t(98)=8.70). However, the curvilinear relationship for the Binge rats was not significant (p=.0938, t(98)=1.69). Furthermore, comparisons between Binge and Control animals were significant for the curvilinear regression (p<.0001, t(98)=-5.53) and nearly significant for the linear regression (p=.058, t(98)=1.92). These observed differences in regression trend indicate that there is a significant difference in how the BE and CC animals learn the task.

In addition to this we did a multiple comparison analysis where the interaction between diet and session was compared between session 1 and every subsequent session. We used session 1 as a baseline comparison for each animal in order to limit effects of variance between subjects. It was shown that there was a significant difference between BE and CC rats in comparisons between sessions 1 and sessions 3 (p=.0284, t(98)=-2.82), 5 (p<.0001, t(98)=-5.42), 6 (p=.0085, t(98)=-3.28), 8 (p=.0028, t(98)=-3.71), and 9 (p=.0065, t((98)=-3.42). Additionally,

there was a marginally significant comparison on session 4 (p=.0627, t(98)=-2.45) (Fig 4). Finally, we chose to do a similar analysis to the above, but in this case, we collapsed sessions 1 through 3 into an "early" period. This period was chosen because the median hit to miss trial ratio had exceeded 90 percent by this point. The interaction between Diet and Session was compared between the early period and every subsequent session. It was shown that there was a significant difference between Binge and Control in the difference between early sessions and sessions 5 (p<.0001, t(98)=-5.58), 6 (p=.0192, t(98)=-2.88), 8 (p=.0063, t(98)=-3.37), and 9 (p=.0165, t(98)=-3.01) (Fig 4).

Finally, we did a graphical analysis of the Hit data parsed by estrous phase. We did not observe any differences between estrus and diestrus within either diet group. To statistically verify our observation, we ran a three-way GLMM. In this case the three factors were, Session Epoch (Early vs Late), Diet, and Phase of Estrous. As we had done previously we chose to describe the first three sessions as 'Early' sessions. Sessions 4-10 were taken to be Late sessions. We did this in this case in order to maintain statistical power. As one might expect, omnibus statistics indicated that there was a significant main effect of Session Epoch (early vs Late) (p<.0001, F(1,10)=101.76). In addition to this there were significant interactions between Diet and Session Epoch (p=.0014, F(1,10)=6.56), and Diet and Estrus Phase (p=.0283, F(1,10)=6.56). Multiple pairwise comparisons were calculated in order to further investigate these interactions. Analyses were done comparing Phases of Estrous sliced within Diet and Session Epoch. It was shown that Phase of Estrous had a significant effect only during the Early Epoch in the Control animals (p=.0167, F(1,6)=10.79). In addition to this a pairwise comparison where the interaction between Diet and Estrous phase was examined. It was shown that there was a significant effect of Estrous Phase only for the Control animals (p=.0452, F(1,10)=5.23) (Fig 5).

Taken together we see that the effects of estrous only effects CC animals during early sessions. Considering the fact that our neural analysis only considers sessions 4-10, estrous phase was therefore irrelevant for our neural analysis. Furthermore, there were significant differences in the overall regression equations between BE and CC animals indicating differences in learning between diet groups in overall learning. With respect to proportion of hit trials, the BE animals overall exhibit an overall retarded learning pattern when compared to the CC group.

**Uncued Head Entries.** Initial observations indicated that there may be a difference between diet groups during later sessions, in the tendency to enter the sucrose port between trials (Fig 6). To investigate this, we ran a GLM with factors being Diet and Session. Omnibus statistics revealed that there was a significant main effect of Session (p<.0001, F(9,98)=19.85), and a significant interaction between Diet and Session (p<.0001, F(9,98)=5.46). To further investigate this, we used linear and curvilinear trend analysis. There was no significant linear trend for either CC (p=.067, t(98)=-2.33), or BE (p=.067, t(98)=-2.42). Nor were there significant curvilinear trends for CC (p=.1971, t(98)=1.64) or BE (p=.87, t(98)=.16) animals. We compared BE and CC animals in both linear (p=.90, t(98)=.12) and curvilinear (p=.56,t(98)=-.97) trends, neither of which were significant. We performed post hock comparisons in which BE and CC groups were compared on each consecutive day. However, none of these comparisons were significant. In addition to this we made comparisons between the rate of uncued head entries on session 1 and each consecutive session. These differences were compared between BE and CC animals. However, none of these comparisons were significant. In addition to this we collapsed sessions 1-3. These sessions were considered to be early sessions where learning was still taking place. These were compared to every consecutive session following session 3 (4-10). The differences were calculated and comparisons were made between BE and CC groups. However, none of these comparisons were significant (Fig 6). Taken together, while omnibus

statistics indicated a significant interaction between Diet and Session, no post hoc analyses were able to reveal any significant differences between BE and CC animals. Thus, uncued head entries, while expected during early training sessions, continued to occur throughout all sessions, even after animals in both groups had acquired the conditioned response to the tone (Choi et al, 2011).

As before we performed graphical analysis with both diet groups parsed by estrous phase. Based on our graphical analysis we did not find any effect of estrous on either diet group (Fig 7). To test this observation, we ran an additional GLM which included estrous phase. Here the ten experimental sessions were collapsed into early (sessions 1-3) and late (sessions 4-10) epochs. The factors for the GLM were Diet, Session Epoch, and Phase of Estrus. Omnibus results yielded no significant effects, Diet (p=.5311, F(1,72)=.40), Estrous Phase (p=.5598, F(1,72)=.34), Session Epoch (p=.128, F(1,72)=2.37). Based on this we concluded that estrous had no effect on uncued head entries.

Ultrasonic Vocalizations. Our observations based on graphical analysis indicated a downward trend in calls in BE animals that was not shared by the CC animals. In order to test this observation, we ran a GLM with factors Diet and Session. Omnibus statistics revealed that there was a significant main effect of Session (p<.0001, F(10,124)=5.52), and a significant interaction between Diet and Session (p<.0001, F(10,124)=6.84). Linear and curvilinear trends were calculated for BE and CC animals. However, the only significant trend was that of the curvilinear trend for the BE animals (p=.0016, t(124)=-3.65). Comparisons were made between BE and CC animals in both linear trends. It was found that there were significant differences between BE and CC animals in both linear (p=.047, t(124)=-2.16) and curvilinear (p=.047, t(124)=-2.29). Comparisons were made between session zero (baseline) and all ten trial sessions for both BE and CC animals. It was found that for the CC animals there was a significant

difference between baseline and sessions 1 (p=.0073, t(124)=3.4) and 2 (p=.0039, t(124)=3.62) as well as sessions 7 (p=.024, t(124)=2.86), 8 (p=.0095, t(124)=3.28), 9 (p=.0036, t(124)=3.02). For BE animals there were significant differences between baseline on session 3 (p=.0004, t(124)=4.23) only. Additionally, we made comparisons between BE and CC animals on each session. However, BE and CC animals were only significantly different on session 10 (p=.0005, t(124)=-4.23). The comparisons did reach near significance on session 8 (p=.052, t(124)=-2.84) (Fig 8). All of this taken together indicates that there was a slight but significant downward trend observed within the BE animals that did not exist for the CC animals.

We next chose to analyze USVs parsed by estrous phase. Based on initial graphical analysis we did not observe any difference between estrus and diestrus within either diet group (Fig 9). In order to verify this observation we ran an additional GLM with factors being Diet, Session Epoch, and Estrus Phase. As before, sessions were divided into Session Epochs where early sessions (sessions 1-3) as well as late sessions (sessions 4-10) were combined. Omnibus statistics revealed a significant main effect of Session Epoch (p=.036, F(1,172)=4.48) as well as a significant interaction between Diet and Session Epoch (p=.0026, F(1,172)=9.35) (Fig 9). Based on this lack of significant results involving estrous phase we concluded that our graphical observations were correct and that phase of estrous did not play an important role in vocalizations.

## **Neural Activity.**

Over the course of ten sessions we recorded from a total of 492 neurons. These neurons were divided between Nac Core and Shell as well as between BE and CC groups, as follows: 166 neurons in BE Core, 95 in CC Core, 128 in BE Shell, and 103 in CC Shell.

### **Cue Processing.**

Nac Core. We began our analysis of cue processing in Nac Core by graphing the log transformed tone evoked firing rates regressed against their respective baselines. Bayesian weighted regression lines were added to the graphs in order to aid in the analysis. Based on initial observations, it appeared that only the regression for CC Miss trials was different from the line of no change (Fig 10). It is worth noting here that a regression line being not significantly different from the line of no change (slope of one) indicates that there was no difference in firing from baseline. In the opposite sense, a regression line having a slope of zero indicates that baseline and event firing are not related, i.e., are different from each other. For our GLM analysis, a significant effect of baseline indicates that baseline and event firing are significantly correlated. Baseline and event firing being significantly correlated implies that the two are related but does not imply that here is no change from baseline to event. For our analysis we will utilize an analysis of confidence intervals associated with the regression line for baseline and event firing. This will allow us to ascertain if the regression line is actually significantly different from the line of no change or from the zero line. We performed a GLMM with effects being Average Baseline Firing Rate (Bayesian weighted mean), Response (i.e. whether the cue elicited an approach or not), and Diet. For our analysis significant effects of diet or response indicate that these factors significantly effected the event firing. We chose to do analysis from sessions 4 onward. This was done because we were interested in how a history of BE affected processing of a known cue. By session 4, animals had learned the task (Fig. 4) and therefore all trials were considered to be similar. Omnibus statistics revealed that there was a significant main effect of Baseline Firing Rate (p<.0001, F(1,19000)=169.72) indicating that there was a significant connection between the neurons' baseline firing rates and their activity during the tone. There were also significant interactions between Diet and Response (p=.0376, F(1, 19000)=4.32) as well as Baseline Firing Rate and Response (p=.0013, F(1, 19000)=10.41). A post hoc analysis of regression lines was performed in order to elucidate these statistics. It was shown that there was a significant difference in the head to head comparison between Hit vs Miss trials within the

CC group (p=.0009, t(19000)=-3.69)). An analysis of confidence intervals for these regression lines was performed. It was found that the slopes of the regression lines did not differ from the line of no change for all four groups, CC Miss (slope=.71, lower bound=.40, upper bound=1.01), CC Hit (slope=.97, lower bound=.65, upper bound= 1.29), BE Miss (slope=.93, lower bound= .76, upper bound=1.10), or BE Hit (slope=1.05, lower bound=.80, upper bound=1.30) (Fig 10). It is worth noting that while the slope of the regression line for CC Miss trials was not significantly different from the line of no change, this is likely due to high variance. And while this was the case, there was still a significant difference in the slope of the regression line between Hit and Miss trials within CC animals (Fig 10).

Because it is well established that Nac is involved in pavlovian tasks with trained subjects (Ambroggi et al 2011), we chose to investigate the lack of cue processing observed during Hit trials (Fig 10). We hypothesized that firing could reflect pre-processing, in which processing begins before the tone onset, rendering animals "ready for" the next trial, or anticipating the tone. In order to test this, we performed an analysis comparing baseline firing rates for Hit and Miss trials, the idea being that if there was preprocessing for Hit trials but not Miss trials there should be some sort of observable difference between baselines during Hit and Miss trials. We performed a regression analysis in which firing rate during Hit trials was regressed against that of Miss trials. A regression line was calculated and the slope was analyzed. It was shown that the grand regression line (collapsing BE and CC animals) was significantly different from the line of no change (slope=.54, lower bound = .42, upper bound = .67). We further calculated the regression lines for BE and CC animals separately. Further, the regression line slopes for both BE (slope=.58, lower bound=.38, upper bound=.77) and CC (slope=.51, lower bound=.39, upper bound=.68) were significantly different from the line of no change (Fig 12). This indicates that there was a significant difference in baseline firing depending

on whether the animals were about to initiate a response to the imminent tone or not. Thus it is likely that Nac core neurons are involved in pre-processing before the onset of a tone cue on trials in which the animal initiates an approach. This may partially explain the aforementioned absence of any further change in firing at tone onset.

Nac Shell. We began our analysis of Nac Shell firing rates in response to the cue in a similar fashion to that for Nac Core. We first graphed log transformed firing rate data comparing baseline and cue evoked firing (Fig 11). Finally, we fit a Bayesian weighted regression line to the graph. We observed that there was a substantial clockwise rotation in regression lines during Miss trials which was not observed during Hit trials. In order to quantify this result we ran a three factor GLMM on cue processing data recorded from Nac shell. The effects were Average Baseline Firing Rate (Bayesian weighted mean), Response (Hit vs Miss), and Diet. As before analysis was done for sessions 4 onward. Initial omnibus results indicated that there were significant main effects of Baseline FR (p<.0001, F(1, 20000)= 129.8) and Response (p=.0017, F(1,20000)=9.86). There was also a significant interaction between Baseline FR and Response (p<.0001, F(1, 20000)=23.25). In addition, post hoc analysis was performed. It was shown that there were significant head to head comparisons between Hit and Miss trials within BE animals (p=.0006, t(20000)=-3.81) and within CC animals (p=.003, t(20000)=-3.29). Additionally, an analysis of confidence intervals for the slopes of the individual regression lines (baseline vs event) revealed that the slope of the regression line during Hit trials was not significantly different from the line of no change for both CC (slope=.94, lower bound=.69, upper bound= 1.18) and BE (slope=.82, lower bound=.60, upper bound=1.03) animals (Fig 11). Taken together this indicates that there was a significant difference in processing between Hit and Miss trials and that, similar to what we had seen in Core, Hit processing was not different from

baseline firing while Miss firing showed a robust difference from baseline. Unlike what was observed in Nac Core, there was no effect of diet.

As before because of the observed lack of cue processing during Hit trials we performed an analysis comparing baseline firing rates for Hit and Miss trials. We performed a regression analysis in which the firing rate of animals during Hit and Miss trials were regressed against each other. The slope of the grand regression line was significantly different from the line of no change (slope=.27, lower bound=.17, upper bound=.37). Further, the regression line for Hit vs Miss trials was significantly different from the line of no change for both BE (slope=.26, lower bound=.09, upper bound=.43) and CC (slope=.28, lower bound=.12, upper bound=.44) neurons (Fig 12). This indicates that, similar to Nac Core, baselines were different for Hit and Miss trials, which supports our hypothesis that Nac firing in Shell as well as Core reflects preprocessing on Hit trials.

#### Approach.

Nac Core. We began our analysis of Nac Core response to Approach by regressing the log transformed firing rate data for baseline against approach, and plotted the Bayesian weighted regression line. Based on initial graphical analysis we observed that here was a substantial clockwise rotation in CC animals when compared to BE. In order to quantify this observation, we ran a three factor GLMM on Nac Core approach data (Fig 13). The effects were Average Baseline Firing Rate (Bayesian weighted mean), Diet, and Trial Type (Cued verses Uncued). As before, analysis was done for all sessions from session 4 onward. Omnibus statistics revealed that there was a significant main effect of Baseline firing rate (p<.0001, F(1,31000)=33.44), and Trial Type (p=.0003, F(1,31000)=13.39). In addition to this there was a significant interaction between Diet and Baseline FR (p=.0387, F(1,31000)=4.28). These results

indicate that event firing rate was significantly correlated with baseline, demonstrating that

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baseline and event firing are related, and that there was a significant effect of diet. Multiple pairwise comparisons were performed comparing the slopes of the regression lines as event firing was regressed against baseline firing. It was found that there was a significant difference between BE and CC animals during Uncued Approach (p=.023, t(31000)=-2.76). Additionally, an analysis of confidence intervals for the regression lines revealed that the confidence interval for CC neurons on cued trials was not significantly different from zero (slope=.32, lower bound= -.20, upper bound=.84) (Fig 13). Thus, firing on cued approach in CC animals was different from baseline firing. Taken together this indicates a significant difference in approach processing between BE and CC animals.

We also observed that firing rates appeared to increase more from baseline during cued approach than during uncued approach (Fig 13). In order to test whether this was true, we first tested whether baseline firing rates were different between cued and uncued approach. When baseline firing rates for both cued and uncued approaches were regressed against each other (Fig 15), the grand slope was not significantly different than the line of no change (slope= .858, Lower Bound=.682, Upper Bound= 1.034). This relationship also held between binge and control groups: BE (slope=.922, lower bound=.625, upper bound=1.218) and CC (slope=.7942, lower bound =.523, upper bound=1.056). Having ascertained that there was no difference in baselines between cued and uncued approaches we next used regression analysis to determine if the event firing was different between cued and uncued approaches. The results indicated that the grand slope of the regression line was different from the line of no change (slope=.8834, lower bound=.7963, upper bound=.9705). The regression line was different from the line of no change for CC (slope=.8762, lower bound= .7978, upper bound= .9545), but not for BE (slope= .8906, lower bound=.7076, upper bound=1.0735). BE and CC groups were not significantly different from each other (p=.871, t=-.16). Taken together it seems safe to say that if there is an effect here it is a very small one (Fig 16).

Nac Shell. We began our analysis of approach firing in Nac Shell with the same graphical approach that we used for Core. After our initial visual inspection it appeared that all regression slopes did not deviate from the line of no change (Fig 14). In order to verify our observations, we ran a three factor GLMM on Nac Shell approach data. As before the effects were Average Baseline Firing Rate (Bayesian weighted mean)), Diet (BE vs CC), and Trial Type (Cued verses Uncued). Analysis was done for all sessions from session 4 onward. Omnibus statistics revealed significant main effect of baseline firing rate (p<.0001, F(1,30000)=171.35), indicating that firing during approach was significantly correlated with baseline. In addition to this multiple pairwise comparisons were performed comparing the slopes of the regression lines as event firing was regressed against baseline. However, none of these comparisons were significant. Additionally, analysis was done comparing the confidence intervals for the regression slopes to the line of no change and it was found that the regressions for CC neurons were not different from the line of no change during cued (slope=.84, lower bound=.50, upper bound=1.18) or uncued (slope=.91, lower bound= .77, upper bound=1.05) behavior (Fig 14). Taken together this indicates that there was very little processing in Shell during approach.

We in order to do this we first regressed the also observed that there were possibly larger increases from baseline during cued approaches than uncued approaches (Fig 14). In order to test this first we determined if there was a difference in baselines between cued and uncued approaches. To do this we ran a linear regression analysis comparing the two baselines, similar to the one performed for Nac Core. The results demonstrated that the grand slope of the regression was significantly different from the line of no change (slope= .7512, lower bound= .6697, upper bound=.8328). This indicates that when both diet groups are taken together there was a significant difference in baseline firing rates between cued and uncued approaches. To further investigate this, we regressed cued and uncued approach baselines for the two diet groups separately. We found that the slope of the regression line was significantly different from the line of no change for both BE (slope= .6643, lower bound=.5597, upper bound= .7690) and CC (slope=.8381, lower bound=.6840, upper bound=.9923) animals. We next compared the baseline regressions for the two diet groups with each other to see if there was an effect of diet. We found there was a significant difference between the baselines for BE and CC animals (p=.0369, t(227)=2.10) (Fig 15). This indicates that there was a difference in baseline firing rate in Nac Shell depending on whether the animal initiated its approach in response to the to a tone cue or not and this effect was greater in BE animals.

While the above described differences in baselines could potentially explain eventrelated changes *from* baseline between cued and uncued approaches, we needed to ascertain if such a difference existed. We performed a regression analysis comparing event firing during cued and uncued approaches. Results showed that the grand slope was significantly different from the line of no change (slope=.7671, lower bound= .7040, upper bound= .8302) indicating that when both diet groups are taken together there was a significant difference between cued and uncued approach firing. Further, both BE (slope= .7197, lower bound=.6256, upper bound= .8137) and CC (slope=.8145, lower bound=.7052, upper bound=.9238) regression slopes were significantly different from the line of no change, indicating that firing rates were different between cued and uncued approaches for both diet groups (Fig 16). Finally, we compared the two diet groups directly and found that they were not significantly different from each other (p=.1401, t(220)=1.48). These results indicate that there was a significant difference in processing between cued and uncued approaches in Nac Shell, but this effect was not related to diet.

## Consumption

Nac Core. We began our analysis of consumption firing in Nac Core with a similar graphical approach to that which we used for approach firing. Based on graphical observations it appeared that there was a difference in firing rate between Consumption vs Pseudoconsumption (Fig. 17). In order to test this a GLMM was performed with factors Average Baseline Firing Rate (Bayesian weighted mean), Diet (BE vs CC), and Trial Type (Consumption vs Pseudo-consumption) on Nac Core neural data. As before analysis was done for all sessions from session 4 onward. Omnibus statistics revealed that there was a significant main effect of Baseline FR (p<.0001, F(1,26000)=25.19), and Trial Type (p<.0001, F(1, 26000)=40.04). In addition to this there was a significant interaction between Baseline FR and Trial Type (p=.0001, F(1, 26000)=14.99). Multiple pairwise comparisons were performed to investigate these main effects, comparing the slopes of the regression lines for baseline firing rate and event firing rate. There was a significant difference between consumption and pseudoconsumption within BE (p<.0001, t(26000)=6.72) subjects. The slope of the regression line for CC neurons was not significantly different from the line of no change during Pseudoconsumption (slope=.76, lower bound=.18, upper bound= 1.33). Additionally, the regression line for CC neurons on consumption trials was not significantly different from zero or the line of no change (slope=.45, lower bound=-.24, upper bound=1.13) (Fig 17). These statistics confirm our observation that there was a significant difference in firing between Consumption and Pseudo-consumption. It is worth noting that while there was no significant difference between Consumption and Pseudoconsumption within CC animals the difference was marginally significant (p=.0695, t=2.26) and this failure to reach significance is likely due to high variance (standard error= .136). We can also see this high variance in the CC group reflected in the fact that the confidence

interval for the consumption regression line encompasses both the zero line and the line of no change (slope=.45, lower bound=-.24, upper bound=1.13).

Based on overall graphical observations it appeared that there may have been an overall higher firing rate during Consumption than Pseudoconsumption (Fig 17). In order to test this, additional analysis was performed comparing firing during Consumption to firing during Pseudoconsumption. Consumption and Pseudoconsumption firing rates were regressed against each other and the slope of the regression line was analyzed. When both diet groups were taken together it was shown that the slope of the grand mean was significantly different from the line of no change (slope=.84, lower bound=.73, upper bound=.96). When we analyzed the two diet groups separately we found that the slope of the regression lines was significantly different from the line of no change for BE (slope=.80, lower bound=.65, upper bound=.92) but not CC (slope=.89, lower bound=.65, upper bound =1.12) animals (Fig 21). We compared the event firing rates between the two diet groups directly. It was found that there was no significant difference between BE and CC animals in their comparisons between event firing (p=.4849, t(215)=.70) (Fig 21). In order to determine if this effect might be accounted for by differences in baseline, we also ran a similar regression analysis comparing baselines for Consumption and Pseudoconsumption. There was no difference between Consumption and Pseudoconsumption for the grand slope (slope=.9916, lower bound=.9232, upper bound=1.0599). This result also held for both BE (slope=.9775, lower bound=.897, upper bound=1.058) and CC (slope=1.0056, lower bound=.8951, upper bound=1.1161) animals. This indicated that there above mentioned observed effect was not due to a difference in baselines and so that there was no significant difference between raw firing rates. Finally, a two-way ANOVA with factors being Diet and Trial Type (Consumption vs Pseudo-consumption) compared the change scores for neuronal firing during Consumption/Pseudo-consumption (see methods

section for a description of how change scores were calculated). It was found there was a significant main effect of Trial Type (p<.0001, F(1, 731)=38.93) (Fig 19). This indicates that, relative to baseline, there was significantly higher firing during Consumption than during Pseudoconsumption in Nac Core.

Taken together our initial GLMM demonstrates that that there was a significant difference in consumption and pseudoconsumption firing. Our head to head consumption vs pseudoconsumption analysis along with our change score analysis demonstrated that there is increased consumption firing compared to pseudoconsumption.

Nac Shell. As before, we began our analysis of Consumption firing in Nac Shell with a graphical analysis regressing firing rate during Consumption vs Pseudoconsumption. Based on observations of the regression lines it appeared that in all cases with the possible exception of CC Pseudoconsumption, the slopes of the regression lines were not different from zero (Fig 18). This pattern would indicate that the event firing rate was not related to baseline firing rate. To test these observations, a GLMM with factors Average Baseline Firing Rate (Bayesian weighted mean), Diet (BE vs CC), and Trial Type (Consumption vs Pseudo-consumption) was performed on Nac Shell neural data. As before, analysis was done for all sessions from session 4 onward. Omnibus statistics revealed there was a significant main effect of Trial Type (p<.0001, F(1,25000)=33.21). In addition to this there were significant interactions between Diet and Trial Type (p=.0288, F(1,25000)=4.78), and Baseline FR and Trial Type (p=.0054, F(1,25000)=7.74). It is worth noting here that there was no significant main effect of baseline indicating that overall, baseline firing rate was not related to, or not predictive of, event firing rate. Multiple pairwise comparisons were performed comparing the slopes of the regression lines when baseline was regressed against event. There was a significant difference between Consumption vs

Pseudoconsumption within CC neurons (p=.04, t(25000)=2.55). Additionally, analysis of

confidence intervals for the regression lines revealed that the slope of the regression lines was not different from zero for CC Consumption (slope=.03, lower bound=-.50, upper bound=.55), BE Pseudo-consumption (slope=.21, lower bound=-.12, upper bound=.53), or BE Consumption (slope=.08, lower bound=-.30, upper bound=.47) (Fig 18). Taken together these results confirm our original observation that there was no significant correlation between baseline and event firing within all groups with the exception of CC Pseudoconsumption.

As before we observed a possible difference in event firing between Consumption vs Pseudoconsumption. Firing rates during Consumption and Pseudoconsumption were regressed against each other and the slope of the regression line was analyzed. When both diet groups were considered together it was shown that the slope of the regression line was significantly different from the line of no change (slope=.80, lower bound=.75, Upper Bound=.85). When BE and CC animals were considered separately it was found that the slope of the regression line was significantly different from the line of no change for both BE (slope=.91, lower bound=.88, upper bound=.94) and CC (slope=.69, lower bound=.58, upper bound=.79) animals (Fig 21), indicating that there was a significant difference between Consumption and Pseudoconsumption.

To test if that difference was related to a difference in baselines, we ran a similar analysis comparing baselines head-to-head during Consumption vs Pseudoconsumption. The regression line for CC animals was significantly different from the line of no change (slope=.7672, lower bound=.6748, upper bound= .8596). However, the BE and CC regressions in Nac Shell were not significantly different from each other (p=.4636, t(229)=-.73). This indicates that while there was a difference between Consumption and Pseudoconsumption baselines for CC, it was small. However, it may partly account for the above mentioned difference between BE and CC animals in Consumption and Pseudoconsumption (Fig 21). It is worth noting that in the standardized change plot nearly all firing rate changes lie above the line of no change (Fig 19). Importantly, this indicates that neurons fire faster during Consumption than Pseudo-consumption. It is also worth noting that not a single neuron both increased during Pseudo-consumption and decreased during Consumption (Fig 19). To further analyses this a two-way ANOVA was run with factors being Diet and Trial Type (Consumption vs Pseudo-consumption) comparing the change scores during Consumption/Pseudo-consumption (see methods for details on how change scores were calculated). There was a significant main effect of Trial Type (p<.0001, F(1,659)=47.87) (Fig 19). This indicates that, when baselines were taken into account, there was a significant difference in firing rates between Consumption and Pseudoconsumption.

Our initial GLMM demonstrated that there was no correlation between baseline and event firing indicating a massive effect of consumption behavior on Nac Shell firing. It is also worth noting that while there was a significant difference between consumption and pseudoconsumption firing in the CC group there was no difference between consumption and pseudoconsumption within the BE group. This indicates that BE animals process nonconsummatory reward seeking behavior in a fashion more similar to consumption in Nac Shell than to CC animals. Additionally we showed that there ware significant differences in firing rates during consumption and pseudoconsumption. This was true for both f BE and CC animals, although the effect was smaller for the BE group. Finally, our analysis of change scores demonstrated that firing during consumption was higher than that observed during pseudoconsumption.

# Discussion

# **Behavior**

Ultrasonic Vocalizations. BED has been strongly correlated with depression in humans (Kessler et al 2013; Linde et al 2004). However, it is unknown whether this comorbidity is directly related to the bingeing behavior as such, or is a symptom of a preexisting condition, or, in a third possibility, is a social correlate (ie derived from negative social consequences of being a binge eater). Fifty kHz USVs are a reliable index of positive affect in rats (Knutson et al 2002). Rats selectively bred for low 50 kHz USV call rates exhibit decreased motivation for maternal interaction as measured by preference for their mother's scent (Harmon et al 2008). Early life maternal separation has been linked to depression symptoms in rats (Lee et al 2007). Taken together it is reasonable to think that a low 50 kHz calling rate may be an indication of depression like symptoms in rats.

Microinjection of amphetamine, a DA agonist, into Nac leads to an increase in the rate of 50 kHz USVs (Burgdorf, Knutson, Panksepp, & Ikemoto, 2001). Furthermore, it has been shown that playing a recording of 50 kHz calls elicits phasic DA release in Nac (Willuhn et al, 2014). From this it is possible to infer that a decrease in 50 kHz calls may be reflective of hypoactive Nac DA activity. In the present study, USVs were recorded during a baseline session as well as all 10 pavlovian sessions. Analysis of vocalizations indicated that the BE group exhibited a modest downward trend in 50 kHz calls compared to the CC group. The regression equations were different for BE and CC animals, although the two groups became significantly different from each other only on session 10. It is possible that this may be representative of mild depressive symptoms exhibited by BE rats. If this is the case we can say that, at least in part, the depressive symptoms associated with BED may be related to the bingeing behavior.

**Cued Head Entries.** It has been shown that in an operant task, microinjection into Nac of either a dopamine D1 or D2 antagonist led to decreases in both neural and behavioral responses to a tone CS in well trained rats (Hoffmann and Nicola 2014). Additionally, it has been shown that microinjection of a glutamate antagonist into Nac Core but not Shell leads to decreases in cued head entries in animals trained in an operant task (Ambroggi et al., 2011). The BE animals in our experiment exhibited decreases in cued head entry rate when compared to the controls on days 3, 5, 6, 8, and 9. In addition, there was an overall difference in the curved linear trend between the two groups, indicating that BE animals emitted fewer cued head entries than CC animals. Considering that this task has been shown to be Nac Core dependent, this could be indicative of an overall decrease in Nac Core activity in the BE animals. Furthermore, it is possible to decrease cued head entries in well trained rats by antagonizing dopamine D1 or D2 receptors. Taking this together with our above discussion of downregulated 50 kHz calls, dopamine circuity and depression like symptoms, it is likely that BE animals have some sort of deficiency in dopamine circuity. When we compare this result with above mentioned studies showing that BED patients have altered DA circuity (Davis et al 2007; Majuri et al 2017; Wang et al 2002) we can infer that at least part of this alteration may be due to the binging behavior. It is also worth noting that BED has been linked to a particular allele of the Dat1 gene (Davis et al., 2007), indicating a genetic component to BED. Taken together, it seems reasonable to infer that BE behavior may be partly responsible for DA pathology in BED patients.

Uncued Head Entries. When we compared BE and CC animals' rates of uncued head entries, none of the pairwise comparisons revealed significant differences. There were also no differences between the BE and CC groups' linear or curved linear regression lines, possibly influenced by a high level of variance within the BE animals. These results indicate that group differences between BE and CC animals in uncued head entries are subtle or nonexistent. It has been shown that if animals are trained in an operant task, microinjection of a glutamate antagonist into Nac Shell leads to increases in uncued head entries (Ambroggi et al., 2011). Judging from the minimal differences in uncued head entry behavior between the BE and CC groups it is likely that the BE treatment had minimal effect on Nac Shell. Combining this with our result for cued head entries it is likely that effects from binging behavior are largely limited to Nac Core.

# **Electrophysiological Results**

We chose to examine the effects of a history of binge eating behavior on reward cue processing in the Nac Core and Shell. We used a simple pavlovian task in which a tone CS was pared with a sucrose US. We chose this task because of its simplicity for studying Nac processing of a reward. In all cases, we chose to use temporally matched baseline taken from immediately before the tone CS for firing rate comparisons. We chose to do this rather than taking a longer baseline similar to the one seen in Abroggi 2011 because the Nac can be responsive to various behaviors which may include general locomotor activity or exploration of various places in the chamber. Because of this a longer baseline may represent Nac responsivity to several events. We chose instead to take a relatively short period immediately preceding the CS as the most relevant baseline. It is worth noting at this point that one disadvantage of our short baseline method was that in some cases it may be difficult to get an accurate measurement of the firing rate for slow firing neurons within a single baseline. However, there are two relevant points to be made here. Firstly, since baselines are temporally matched to their respective events, this same difficulty is unavoidably encountered for the corresponding event firing. Thus, baseline firing rates were measured with the same level of accuracy as event firing rates. Secondly, we were able to overcome this problem by averaging firing rates of the same neuron over many trials across sessions, in this way getting accurate firing rates.

Given the potential of Nac firing to vary with behavior from moment to moment, it was necessary to divide each Cued trial period into three phases. These were tone processing, approach, and consumption. This was done because, while the three phases combined represent a single action, they are neurologically heterogeneous (Ambrogii et al 2011). Additionally, we used uncued approach and Pseudo-Consumption behavior ("Pseudoconsumption") as motor controls for cued approach and consumption behavior respectively. We chose these as a control behaviors because with respect to overt behavior, they are similar to the cued behavior without the same cue response component.

We chose to limit our neural analysis to late sessions (sessions 4-10). This was done because our primary interest was in how a history of BE affected processing of a learned appetitive cue, not how it affected initial learning. To our knowledge there is little to no evidence that BE or BE related disorders have any effect on learning. On the other hand, as we have outlined above, there is substantial evidence that BE related disorders as well as a history of BE itself may lead to the sort of dopaminergic dysregulations that can lead to changes in food cue processing. For instance, it has been shown that BED patients exhibit increased DA response to learned food cues (Wang et al 2011). Behavioral analyses included all ten sessions in order to demonstrate that learning had taken place, and had asymptoted by session 4. Having demonstrated that, we chose to limit our neural analysis to the latter seven sessions. We divided cue processing behavior into two categories. These were Hit and Miss trials. We defined a Hit trial as a cue trial that was followed by a cued approach and subsequent consumption. A Miss trial was defined as a trial that was not followed by an approach. We chose this division because of the likely difference in cue processing in trials where the animal responded to the cue vs ones where it did not.

We used linear regression analysis in which the baseline firing rates were regressed against the cued firing rates and a regression line was plotted for these data points. In this way we were able to make more nuanced connections between baseline and event firing of individual neurons than would be possible with a traditional ANOVA. In particular, a regression line with a slope of zero indicates that there is no connection between the baseline and event firing. On the other hand, a slope of one indicates that there is no change from baseline to event firing. This was useful for the population approach to neural firing analysis that we took. We found that in many cases neural responses to a given behavior were comprised of slow firing neurons increasing their firing rates while fast firing neurons tended to decrease their firing rates. While both of these reactions represent neural processing of a given behavior, if taken together in a more traditional mean-based analysis these effects could cancel each other. There are, of course, multiple methods of dealing with this problem. One method would be to compare changes in firing rate variance between baselines and events. However, this method is unsatisfactory considering that a change in variance is difficult to interpret. A more commonly used method would be to separate the neurons by their firing patterns into like categories (Ambroggi et al 2011). While this approach has the advantage of being able to use straight forward mean-based analysis to examine neuronal behavior it fails to take into account the overall gross processing in Nac during a given behavior. It is worth noting that Nac is a complex brain region that receives inputs from many different regions, has efferents to several different brain regions, as well as being populated by histologically distinct neuronal subclasses. It is unknown whether these specific Nac subclasses result in a particular firing phenotype. Analysis of this question is beyond the scope of this study. It would be speculative to assume that a neuron having a particular response to a given behavior (i.e., increasing or decreasing) is

indicative of its having a particular histological or anatomical phenotype. In light of all of this, we took a population approach to our analysis and examined the overall activity of Nac neurons.

Cue Processing. We found that during Hit trials there was minimal deviation from the line of no change (slope of one). An analysis of confidence intervals demonstrated that there was no significant difference between the slope of the regression lines for Hit trials and the line of no change. This relationship held for both BE and CC animals in Nac Shell as well as Core. This indicates that there was little to no change from baseline FR during cue presentation on Hit trials. Based on this initial result, two possibilities were considered. Firstly, it was possible that Nac was not involved in processing of the reward CS. However, this was unlikely considering that it is possible to interfere with the behavioral response to a reward cue by inactivating Nac (Ambroggi et al 2011). The other possibility was that pre-processing prior to the tone cue onset facilitated the behavioral response on Hits. We performed an analysis comparing baseline firing rates for Hit and Miss trials and demonstrated that the baseline firing rates were significantly different. It is worth noting here that this baseline comparison was done using regression analysis similar to that which we used to compare Hit and Miss trials to their relative baselines. As stated before, this method of analysis has the advantage of being able to accept both increasing and decreasing data into the model without their canceling each other out. Based on graphical analysis it can be seen that difference in baseline firing rates for Hit and Miss trials can manifest as either increased or decreased firing depending upon the neuron (Fig 12). Therefore a more standard mean-based analysis would not have not been appropriate for this analysis.

This lack of difference between baseline and tone evoked firing was not observed by the Fields group (Ambroggi 2011). Instead they report tone evoked increase in Nac activity. There are several points to be made here. Firstly, Fields group used a prolonged tone cue. They reported that the tone evoked firing did not begin for several hundred milliseconds following the onset of the tone cue. A key difference is their lack of using video analysis of each trial, as used in the present study. The time period that we defined as our cue processing phase was at most 500 milliseconds (the tone duration) and as low as 150 msec, terminating at the onset of the first tone-evoked movement. The increased firing observed by the Fields group would, in most cases, take place after the tone processing had already ended, as defined by the present design. Secondly, Fields' group did not distinguish between tone processing and approach. It is likely that much of what Fields' group counted as cue processing would be considered to be approach using our analysis. Finally, Ambroggi et al (2011) describe an operant experiment, whereas we chose to use a pavlovian task. While these tasks are similar they are not the same. It is possible that the Nac response to a CS is different from that to a DS. To our knowledge this question has not been adequately studied and is beyond the present scope.

In contrast to Hit trials, there was a significant difference in cue processing on Miss trials in Nac Shell. Nac Shell cue processing was, for the most part, not affected by binge behavior. For both BE and CC animals the regressions lines during Miss trials had slopes that were significantly different from the line of no change. In addition to this there were significant differences in regression line slope between Hit and Miss trials within both BE and CC groups. This robust processing indicates that during Miss trials the animals were not simply ignoring the tone.

In Nac Core, during Hit trials, we observed similar neural processing as that seen in Shell. However, during Miss trials there was a notable difference between BE and CC animals. In CC animals, Core neurons behaved in a fashion similar to what was observed in Shell, in that there was a significant difference between Hit and Miss trials. BE Core neurons, on the other hand, exhibited downregulated processing during miss trials, as well as Hit. There was no significant difference in the regression line slopes for BE animals on Hit and Miss trials, and in

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both cases the slope of the regression line was not significantly different from the line of no change.

It is likely that BE Core neurons do not process the tone during Miss trials or at least their processing is significantly decreased. This observation is in keeping with our behavioral results. We observed significant decreases in numbers of cued head entries in the BE group when compared to the CC group. Abroggi 2011 demonstrated that the number of cued head entries performed by well-trained rats in a similar task can be downregulated by microinjecting a glutamate antagonist into Nac Core. This indicates that a deficiency in Nac Core processing leads to a decrease in cued head entries. This is in keeping with our hypothesis that Nac Core processing is inhibited in BE animals. However, this decreased processing is limited to Miss trials. The significance of this may be worth investigating in future studies.

Approach. Due to their nature Miss trials are not associated with an approach, and so there can be no analysis of Miss trials in reference to approach and consumption behaviors. Instead, we divided approaches between Cued and Uncued (see methods section). The advantage of this is that Cued and Uncued approaches are motorically indistinguishable (verified by video analysis), differing only in that Cued approaches are initiated in response to a tone cue while Uncued approaches are generated internally. Because of this we used Uncued approaches as a comparison for Cued approaches in a way similar to our comparison of Hit and Miss trials when we analyzed cue processing.

Our results indicated minimal approach processing in Nac Shell. Our omnibus results revealed no main effects aside from that of baseline firing rate as well as no interactions. This lack of Shell involvement in approach was especially evident in CC animals where an analysis of confidence intervals indicated that the slopes of the regression lines in CC animals for both Cued and Uncued trials were not significantly different from the line of no change, indicating that shell FR during approach was no different than baseline. And while an analysis of confidence intervals for BE animals demonstrated that they were significantly different from the line of no change they were not significantly different from their CC counterparts (Fig 14).

Our experimental design which precisely delineates approach as a separate behavioral phase is somewhat novel (Abroggi et al 2011; Root et al, 2013) and so there is little to compare our result to in the literature. Our analysis of baseline firing rates indicated that there was a significant difference in baseline firing between cued and uncued approaches, indicating that Nac Shell preprocessing differed for cued and uncued approaches (Fig 15). This result is reminiscent of our difference in pre-processing between Hit and Miss trials. As we have observed that different classes of approach (cued vs uncued) exhibit significantly different preprocessing, it is likely that the preprocessing in Nac Shell encodes more than simple preparedness to approach. Analysis of this difference may be an interesting topic for a future study.

We observed a significant difference between BE and CC animals in Shell preprocessing (Fig 15). BE animals exhibited a significantly greater difference in preprocessing between Cued and Uncued trials than did the CC animals. When comparing approach to baseline, BE Shell neurons exhibited a regression slope significantly different from the line of no change while CC neurons did not (Fig 14). Direct comparison between BE and CC regression slopes did not yield a significant result, suggesting a modest effect of diet on approach firing in Nac Shell. While there was a significant difference in baselines between the cued and uncued approaches (Fig 14), and this relationship held for both BE and CC animals, the differences in baselines did not translate into differences in event processing. The meaning of putative pre-processing signals indicated by these differences in baseline may be an interesting topic for future study. Despite the above described diet-related differences in approach, diet had no effect on cue processing in Nac Shell. This reinforces the idea that cue processing and approach are distinct phases that need to be analyzed separately. This also helps to illustrate the complex nature of Nac processing in that a diet treatment can alternately affect or not affect different phases of a given behavior.

In Nac Core, significant processing occurred during approach. All four regression line slopes were significantly different from the line of no change. We also observed a robust effect of binge treatment during approach in Core. There was a significant interaction between Diet and Baseline Firing Rate. There was also a significant main effect of trial type, indicating that there was a significant difference between cued and uncued approach processing in Nac Core. In addition to the significant interaction between diet and baseline firing rate there was a significant difference in the head to head comparison of slope between BE and CC neurons in Uncued trials. However, none of the comparisons between Cued vs Uncued approach, either for BE or CC, were significant. Additionally, the comparison between BE and CC FR of Core neurons during Cued approaches was not significant (Fig 13).

Taken together, these results indicate that, while there was a significant main effect of trial type, the actual effect is difficult to interpret due to the lack of any difference in the head to head comparisons. There was no interaction between trial type and baseline firing rate, which is likely responsible for the lack of difference in regression line slope. We chose to use regression analysis for head to head comparisons because it was necessary to take into account the neuron's baseline firing rate while making these comparisons. This allowed us to normalize the high level of variation that is observed when taking a population approach to neuronal activity.

Based on graphical observations, as well as the above-mentioned lack of significant head to head comparisons between cued and uncued trials, it is clear that the most important effect was diet rather than trial type (Fig 13). There was a significant difference between BE and CC animals during Uncued approaches, and while this comparison did not reach significance during cued trials this likely due to the high level of variance seen particularly in CC animals. This finding is in line with our behavioral results which predicted a large effect of diet in NAc Core.

The BE treatment had the effect of decreasing approach processing. The slope of the regression line was significantly closer to the line of no change for BE animals than CC during Uncued trials. While there was no significant difference between BE and CC animals during Cued trials, the BE regression line was significantly different from zero while the CC regression line was not. This Decrease in Core approach processing in the BE group is similar to what was seen during tone cue processing. This indicates a possible similarity in Nac Core processing of cue processing and approach. As was stated above, diet affected cue processing and approach in a different manner in Nac Shell. This is indicative of possible differences in how these two phases are processed in these two regions of the Nac.

The lack of difference between cued and uncued trials indicates that Nac Core does not process motivation during approach in the same way that Shell does. It is possible that Core firing is exclusively related to the locomotor aspect of the approach. This could explain the lack of difference between Cued and Uncued trials. However, it would of course be incorrect to assert that uncued approach is an unmotivated behavior. The animal is likely Pseudo-Consumption sucrose, although precisely what is being processed is unknown. Our behavioral data clearly demonstrate that the animals had made the CS-US association. Additionally, the dipper was retracted during the inter-trial interval, and thus the numerous uncued head entries provided ample opportunity to learn that there was no sucrose available. It is possible that Nac Core is still involved in the motivational aspect of approach, but does not parse between cued and uncued approach behavior. **Consumption/Pseudoconsumption.** When examining FR in Nac Shell during

Consumption/Pseudo-Consumption, the first observation worth noting is the lack of a significant main effect of Baseline Firing Rate. This indicates that FR during Consumption is not predicted by the neurons' baseline firing rates. This is indicative of massive changes in processing during Consumption. Neurons with different baseline characteristics became coopted in order to fire in ways unrelated to their baseline firing. While this was the overall effect in Shell it is important to note that the regression slope for CC Pseudo-Consumption was significantly different from zero as well as being significantly different from CC Consumption (Fig 16). This indicates decreased processing during uncued head entries when compared to Consumption behavior within the CC group. It is hardly surprising that there would be increased processing in response to Consumption when compared to Pseudoconsumption, considering that it has been long been known that the Nac is responsive to food rewards, although the direction of change in firing rate may need updating (see below).

What is also notable here is that there was no difference in Nac Shell processing between sucrose Consumption and Pseudoconsumption within the BE group (Fig 18). Contrary to what we have seen in Nac Shell in cue processing and approach, here the BE treatment served to intensify processing during uncued head entries. This result indicates that it is likely that Nac processing of a US likely is very different from that of a CS. It is also worth noting that cue and approach processing in Nac Shell, while distinct, are more like each other than they are like consumption/Pseudo-Consumption.

When considering NAc Core activity during Consumption/Pseudo-Consumption, there was a significant main effect of Baseline Firing Rate. This indicates that the change in processing was less intense in Nac Core than what was observed in Nac Shell. There was significantly greater processing during Consumption relative to Baseline within the BE group. However, the

CC group did not follow this trend, possibly because the variance observed during Consumption was so great for CC animals that their regression slope was not significantly different from the line of no change or zero. We also observed minimal or no detectable processing during Pseudo-Consumption within either the BE or CC group (Fig 17). While the BE Pseudo-Consumption regression slope was significantly different from the line of no change it was not significantly different from the Pseudo-Consumption regression slope for the CC animals. Taken together, we conclude that there was minimal Pseudo-Consumption related processing taking place in Nac Core in either treatment group.

Taken together, while we did not compare Core and Shell directly, consumption and Pseudo-Consumption behavior were processed substantially more strongly in Nac Shell than Core as demonstrated by the clockwise shift in regression slopes. We also observed a significant effect of diet in Shell but not in Core. It is possible that this difference is due to the overall greater processing observed in Shell allowing the effect of diet to be more measurable. However, it is worth mentioning that the majority of diet effects during cue processing and approach were seen in Nac Core and this was not the case during consumption. This further illustrates the difference in processing between CS and approach processing vs processing the US.

Based on graphical analysis (Fig 17 and 18) we observed a possible increase in firing during consumption when compared to Pseudo-Consumption. In order to test this we examined change scores for Consumption and Pseudo-Consumption. We found that Consumption firing was significantly greater than Pseudo-Consumption and that this result was true for both Shell and Core neurons and both BE and CC groups. Regardless of whether neurons increased or decreased FR during Pseudo-Consumption relative to Baseline, nearly all neurons fired faster during Consumption of the reward than during its absence, i.e., Pseudo-Consumption (Fig 18). To our knowledge this is the first time that this effect has been demonstrated in this way, i.e., by employing a comparison between two conditions (consuming sucrose vs not consuming sucrose) where motor behavior was matched.

On the face of it this result appears to contradict that which has been reported by Fields' group (Ambroggi et al, 2011). On initial examination of their findings, it appears that there is a significant decrease in Nac firing during consumption. We reported a small increase in mean change score during consumption as well as a nominal decrease in change scores during Pseudo-Consumption behavior. However, a closer examination of the results of their study reveals that there were substantially more neurons that responded to consumption by decreasing firing than ones that responded by increasing firing. Their criteria for a neuron to be considered an increaser or decreaser was a significant increase or decrease in firing rate from baseline with a 99% confidence criterion (Ambroggi et al, 2011). Because of this rather strict criterion they were forced to remove more than half of their data. In keeping with the population approach that we have adopted thus far, we decided to include all of our neural data for analysis. We have shown that, while Fields' analysis is legitimate, failing to take into account the entire data set can lead to misleading results. Furthermore, our approach has the additional benefit of not simply comparing behavior related firing to its baseline. In our analysis we compare firing rates during reward related behaviors to analogous unrewarded behaviors. In this way we were able to deconstruct these reward related events, and study them in a more nuanced fashion than what is seen in other experiments.

### **Estrus**

While phase of estrous was not one of the primary focuses of this study, we recorded estrous phase after all experimental sessions. This was done primarily in order to ensure that estrous was not an experimental confound. However, we chose to include phase of estrous into a second set of analyses to gain additional insight.

**Estrous and Behavior.** Firstly, we chose to collapse session to early (sessions 1-3) and late (sessions 4-10) epochs in order to conserve power for statistical analysis. Similarly, we chose to collapse the four phases of the estrous cycle into estrus (estrus and proestrus) and diestrus (diestrus and metestrus) firstly to increase statistical power, and secondly in order to make the complex results more tractable.

The only effect of estrous on cued head entries that our omnibus statistics revealed was a significant interaction between Diet and Estrous Phase. We ran sliced comparisons and it was shown that in a comparison of Diet sliced by Estrous Phase, there was a significant effect of Estrous in CC animals. A series of three-factor slice comparisons revealed a significant effect of Estrous in CC animals during the early sessions only. For uncued head entries our omnibus statistics failed to find any significant effects of estrous cycle. Taken together, the data imply that any effect of estrous phase on behavior was small and primarily relegated to early sessions. When we included Estrous Phase into our GLM for USVs we did not find any significant main effects or interactions involving Estrous cycle.

Altogether it appears that Estrous phase had little to no effect on the behaviors under study, and whatever effect there may have been seemed to be strictly relegated to early sessions. Considering the fact that for this study we chose to limit our investigation of neural activity to late sessions we felt justified in not examining the effects of estrous phase on neural activity. Considering Estrous' weak effects on relevant behavior and the compelling neural findings that we have already outlined, it seemed reasonable to assume that it was unlikely that Estrous would confound our neural results.

## **Neuronal Phenotypes Recorded**

The primary neuronal type in the Nac is the medium spiny projecting neurons. These neurons project GABA to brain regions which include the VTA and Globus Palidus (Smith & Bolam, 1990). These neurons are further divided ones which express dopamine D1 or dopamine D2 receptors. These these two subcategories are thought to have substantially different connectivity and be responsible for different aspects of reword learning (Francis & Lobo 2017). A natural question for any study involving Nac recording in animals during a learning task would be what are the phenotypes of the individual neurons recorded from. Unfortunately, due to the nature of the study it was imposable to say more about the neurons recorded than their location. This was partly due to the necessity of lesioning the wire tips before brain extraction. Without this procedure it would have been nearly imposable to trace the wire tips. However, even if it were not necessary to lesion the wire tips it still would not have been possible to determine the phenotype of the neurons recorded from because proximity to the wire tip at the time of brain extraction is only a week indicator of which neurons were recorded. This statement becomes apparent when one considers the fact that not all neurons were able to be recorded for the full ten days. A thorough analysis of Nac neuronal activity parsed by phenotype during a pavlovian experiment would be an interesting topic for future investigation but is beyond the scope of this study.

### **Final Comments**

When we designed this study, we sought a method to determine if the addiction like quality seen in BED patients was entirely due to a pre-existing disorder or was due, at least in part, to the binging behavior its self. We did this in order to test the food addiction hypothesis described above. Much of the literature surrounding addiction is concerned with reinstatement of drug taking after a period of abstinence. As such it is widely believed that changes in reward cue processing for drugs of abuse in addicted subjects may hold a key to solving the addiction
problem. To parallel this reasoning, we chose to focus our study on how a history of binge eating may change processing of a food reward cue. We chose to study this in Nac because Nac has been strongly implicated in reward processing and addiction.

We found that our BE subjects exhibited behavior that is indicative of decreased activity in Nac Core. In keeping with this we also found Core processing of both tone CS and approach were decreased in BE subjects (Fig 10,13). Interestingly, we did not see this effect during Consumption or Pseudoconsumption. Instead we saw an increase in processing during Consumption when compared to Pseudoconsumption behavior in BE animals (Fig 17). This was not observed for the CC group. This observation gives an interesting insight into the individual components of our consumption behavior paradigm (cue processing, approach, and consumption) and how a history of BE effects them. The actual act of consumption is a different behavior from cue processing and approach, and the effects of BE on NAc processing appear to be behavior specific. to further alucidate this question could be an interesting topic for a future study.

Studies involving cocaine self-administration demonstrate changes in Accumbens processing of reward cues. This phenomenon is referred to a as cocaine incubation. Cocaine incubation has been associated with modifications in AMPA receptor transmission in Nac (for review see Lowth et al 2014), as well as strengthening of the connection between the Prefrontal Cortex and Nac (Luis et al 2017). Electrophysiological studies has shown that the Nac is responsive to cocaine cues and the this behavior changes over the course of cocaine taking (Coffee et al 2015). However, cocaine and food cues are processed substantially differently. Unlike animals self-administering sucrose, animals taking cocaine tend to be receptive to internal cues over external ones (Coffey et al 2015). This difference makes our results difficult

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compare to those seen involving cocaine. A further examination of this subject would be an interesting topic for future study.

Another interesting result was the apparent lack of processing in Nac during the tone CS during Hit trials. Although this result was counterintuitive to say the least, analysis of pre-tone firing rates showed that there was already a difference in baseline firing between Hit and Miss trials. Based on this we theorized that Nac preprocessing selective for Hit trials may represent a state of readiness to respond. While this pre-processing appeared to obscure or obviate tone-evoked changes in FR on Hit trials, in its absence on Miss trials we identified robust tone processing in Nac shell. This indicates that when animals do not respond to the tone they are not simply ignoring it. Future studies should further elucidate this newly identified pre-processing.

## **Figures**



**Figure 1.** Approach Behavior: (A) Video analysis was used to determine the time of onset of each approach that ended with a head entry into the dipper port. Beginning from each head entry (as recorded by the photosensor in the dipper port) the video was played backward frame by frame to determine the instant of the approach onset. (B) Diagram of the three phase of cued approach behavior: Tone processing was defined as beginning at the tone onset and ending

when either the tone ended (500ms) or when approach began. Approach was defined as beginning at the onset of approach and ending when the animal made a head entry into the dipper port. Consumption was defined as beginning at the start of the head entry and ending when either the animal made a head exit or when the dipper was retracted after 4 sec.



**Figure 2.** Immunohistochemistry: A side by side comparison of a stained brain slice with the corresponding image from The Rat Brain in Stereotaxic Coordinates (Paxinose and Watson 6<sup>th</sup> eddition). Nac Core and Shell are outlined by blue dashed lines and can be distinguished by the difference in the darkness of the calbindin stain. The red arrow denotes the end of a wire trace. The iron deposit left behind from the lesion has been stained green and can be seen in this photograph.



**Figure 3.** Raster PETHs of neural activity during the five phases of behavior from a single neuron: (A) Raster PETH of cue elicited firing from a single neuron. In the raster plot (above) each row represents spikes from a given neuron around the reward event. The vertical red line represents the onset of the tone cue. The blue dots represent the time of approach onset. The PETH plot (below) shows a quantification of the above raster plot. (B) Raster PETH for cued approach related firing. In the raster plot the red line represents the onset of approach and the blue dots represent the head entry. (C) Raster plot describing consumption related firing. In the raster plot the vertical red line represents head entry and the blue dots represent head exit. Taken together these three graphs indicate that this neuron is ordinarily slow firing but is highly responsive to consumption. (D) Uncued Approach: The red line in the raster plot represents the onset of uncued approach and the blue dots represent uncued head entry. (E) Pseudo-Consumption: In the raster plot the red line represents the onset of uncued head entry and the blue dots represent the uncued head exit. We can see that the neuron is nominally responsive to Pseudo-Consumption behavior. However, the response is far less robust when compared to that elicited by the cued head entry.



**Figure 4.** Proportion of Hit Trials: Linear and curved linear trend analysis was performed. It was demonstrated that there was a significant difference in curved linear trends for binge and control animals (p<.0001, t=-5.53).



**Figure 5.** Proportion of Hit Trials with Estrous: Proportion of hit trials (number of hit trials/total number of trials for a given session) for Binge and Control animals separated by estrous and diestrous. Results of statistical analysis indicate there was a significant main effect session epoch (sessions 1-3 vs sessions 4-10) (p<.0001, F=101.76) as wells as significant interactions between Diet and Epoch (p=.0014, F=18.91) and Diet and Estrus phase (P=.0283, f=6.56). Multiple pairwise comparisons demonstrated that the effects of estrus were significant only for the control rats on early sessions (p=.0167, F=10.79). Additionally, the comparison between estrus and diestrus was significant only for the Control animals (p=.0452, F=5.23).



**Figure 6.** Rate of Uncued Head Entries: Initial statistical analysis demonstrated an initial effect of session (p<.0001, F=19.85) as well as a session by diet interaction (p<.0001, F=5.46). However, multiple pairwise comparisons did not yield any significant results.



**Figure 7.** Rate of Uncued Head Entries with Estrus: Omnibus statistics revieled that there was no significant interaction between Diet, Estrous Phase and Session Epoch.



**Figure 8.** Ultrasonic Vocalizations: Initial statistics demonstrated that there was a significant effect of Session (p<.0001, F=5.52) as well as a significant interaction between Diet and Session (p<.0001, F=6.84). Pairwise comparisons were made and it was found that there was a significant effect of diet on session 10 (p=.0005, t=-4.32). It was shown that there was a significant difference between BE and CC animals in both linear (p=.0471, t=-2.16) and curved linear (p=.0417, t=-2.29) trends.



**Figure 9.** Ultrasonic Vocalizations with Estrous phase: Initial statistics revealed there was a significant main effect of Session Epoch (sessions 1-3 vs sessions 4-10) (p=.0357, F=4.48) as well as a significant interaction between Diet and Session Epoch (p=.0026, F=9.35). However, there was no effect of Estrus Phase.



**Figure 10.** Graphs regressing baseline vs event Cue processing FR in Nac Core comparing Hit vs Miss trials as well as BE vs CC data: The four quadrants of the graph represent a regression of the log transformed baseline vs Cue firing separated by Hit vs Miss and BE vs CC. Each black dot represents averaged firing for a single neuron over the course of all late sessions (sessions 4-10). The blue line in each graph represents a simple least squares regression line for the graphed data. The rug plots on the bottom and left-hand sides of each graph represent the density of the graphed data superimposed onto their respective axes. The diagonal dashed line is the line of no change. A dot appearing above the line of no change indicates that there was an increase in

average firing during event when compared to baseline. Likewise, a dot appearing below the line of no change indicates a decrease in FR from baseline to event. Omnibus statistics revealed that there was a significant main effect of Baseline FR (p<.0001, F(1, 19000)=169.72) indicating that there was a significant positive regression between Baseline and Event FR. In addition to this there were significant interactions between Diet and Response (p=.0376, F(1, 18869)=4.32) as well as Baseline and Response (p=.0013, F(1,18869)=10.41). There was a significant difference in the head to head comparison between Hit vs Miss trials within the CC group (p=.0003, t(18869)=-3.69)) indicating that the regression line for Control Hit is different from the regression line for Control Miss. Finally, the slopes of the regression lines were not significantly different from 1 for all four groups, Binge Hit (slope =1.0492, lower bound=.4018, upper bound =1.0143), Binge Miss (slope=.9290, lower bound =.7550, upper bound =1.1029), Control Hit (slope=.9683, lower bound=.6508, upper bound=1.2857), Control Miss (slope=.7081, lower bound=.4018, upper bound=1.0143).



**Figure 11.** Graphs regressing baseline vs event Cue Processing FR in Nac Shell comparing Hit vs Miss trials as well as BE vs CC data: Graph description is outlined in Fig 10. Omnibus statistics revealed that there were significant main effects of Baseline FR (p<.0001, F(1, 20000)= 129.8) and Response (p=.0017, F(1,20000)=9.86), as well as a significant interaction between Baseline and Response (p<.0001, F(1, 19985)=23.25). Indicating that there was an overall positive correlation between Baseline and Event FR and that tone evoked FR was different depending on whether the animal initiated an approach or not. In addition to this post hoc comparisons demonstrated that there was a significant difference in the regression lines between BE Hit and

BE Miss graphs (p<.0006, t(19985)=-3.81 ) as well as a significant difference in the regression lines between CC Hit and CC Miss graphs (p=.003, t(19985)=-3.29). Additionally, the slopes of the regression lines were not significantly different from the line of no change for both BE (slope=.8163, lower bound=.6008, upper bound=1.0318) and CC (slope=.9360, lower bound=.6892, upper bound=1.1827) Hit trials.



**Figure 12.** Comparisons of Hit and Miss trial (cue processing) baseline firing rates in both Nac Core and Shell: For graph description see Fig 10. Regression analysis was performed comparing baseline firing rates for Hit and Miss trials. It was demonstrated that the slope of the regression line in all cases was significantly different from the line of no change, indicating that baseline firing rates for Miss trials were significantly different from those on Hit trials.



**Figure 13.** Graphs regressing baseline vs event Approach FR in Nac Core comparing Hit vs Miss trials as well as BE vs CC data: For detailed graph description see Fig 10. Omnibus statistics revealed that there was a significant main effect of Baseline FR (p<.0001, F(1,30803)=33.44), and Trial Type (p=.0003, F(1,30803=13.39) as well as a significant interaction between Baseline and Diet (p=.0387, F(1, 30803)=4.28). This indicates that there as a significant positive correlation between Approach baseline and event FR as well as a significant difference in FR between Cued and Uncued approach. Additionally there was a significant difference between BE and CC animals. Multiple pairwise comparisons were performed indicating that there was a significant

difference between BE and CC regressions during Uncued Trials (p=.0232, t(30803)=-2.76). The slope of the regression line was not significantly different from zero for CC Cued trials (slope=.3159, lower bounds=-.2063, upper bounds=.8381).



**Figure 14.** Graphs regressing baseline vs event Approach FR in Nac Shell comparing Hit vs Miss trials as well as BE vs CC data: For a detailed graph description see Fig 10. Omnibus statistics revealed significant main effect of Baseline FR (p<.0001, F=171.35) indicating that there was a significant overall positive correlation between baseline and approach FR. In addition to this multiple pairwise comparisons were performed. However, no comparisons were significant. The slopes of the regression lines for CC Cued (slope=.8415, lower bound=.5007, upper bound= 1.1822) and Uncued (slope=.9088, lower bound=.7707, upper bound= 1.0469) were not significantly different from the slope of no change.



**Figure 15.** Cued and Uncued approach baseline firing rates regressed against each other for both Nac Core and Shell: For a detailed graph description see Fig 10. In Nac Core the slope of the regression line for both BE (slope=.922, lower bound= .6250, upper bound= 1.2189) and CC (slope=.7942, lower bound= .5230, upper bound= 1.0645) animals was not different from the line of no change. In Nac Shell the regression line slopes for both BE (slope=.6643, lower bound=.5597, upper bound=.7690) and CC (slope=.8381, lower bound=.6840, upper bound=.9923) neurons were significantly different from the line of no change. Additionally the

slopes of the regression lines for BE and CC neurons were significantly different from each other

(p=.0369, t(227)=2.10).



**Figure 16.** Cued and Uncued approach firing rates regressed against each other for both Nac Core and Shell: For a detailed graph description see Fig 10. The regression line for the BE neurons in Nac Core was not significantly different from the line of no change (slope=.8906, lower bound= .7076, upper bound= .9545).



**Figure 17.** Graphs regressing baseline vs event Consumption/Pseudo-Consumption FR in Nac Core comparing Hit vs Miss trials as well as BE vs CC data: For a detailed graph description see Fig 10. Omnibus statistics revealed that there was a significant main effect of Baseline FR (p<.0001, F(1,25765)=25.19), and Trial Type (p<.0001, F(1, 25765)=40.04) as well as a significant interaction between Baseline and Trial Type (p=.0001, F(1, 25765)=14.99), indicating that there was a significant positive overall correlation between Baseline and Event FR. Multiple pairwise comparisons indicated that here were significant differences in regression lines between Consumption and Psudoconsumption for BE (p<.0001, t(25765)=6.72) animals. The slope of the

regression line for CC Psudoconsumption was not significantly different from the line of no change (slope=.7559, lower bound=.1843, upper bound=1.3275) and for CC Consumption was neither different from zero or the line of no change (slope=.4477, lower bound=-.2368, upper bound=1.1322).



**Figure 18.** Graphs regressing baseline vs event Phase 3 FR in Nac Shell comparing Hit vs Miss trials as well as BE vs CC data: For a detailed graph description see Fig 10. Omnibus statistics revealed there was a significant main effect or Trial Type (p<.0001, F(1, 25335)=33.21) as well as significant interactions between Diet and Trial Type (p=.0288, F(1, 25335)= 4.78) and Baseline and Trial Type (p=.00554, F(1, 25335)=7.74) indicating that there was a significant overall in FR difference between Consumption and Pseudo-Consumption behavior. Pairwise comparisons

revealed that there was a significant difference between Consumption and Psudoconsumption within CC animals (p=.0419, t(25335)=2.55). There no difference between the regression line and zero for CC Consumption (slope=.02633, lower bound= -.387, upper bound= .5522), BE Consumption (slope= .08476, lower bound= -.2192, upper bound= .3887), and BE Psudoconsumption (slope=.08476, lower bound= -.2192, upper bound= .3887).



Figure 19. Change score comparison of Consumption to Pseudoconsumption: The above scatterplot is a comparison between the change scores for BE and CC animals in both Nac Core and Shell. Change scores were calculated by subtracting the baseline firing rate from event firing rate and then dividing this by the sum of the baseline and the event firing rate (Event - Baseline)/(Event + Baseline). In this way a positive change score indicates an increase in firing rate from baseline and a negative change score indicates a decrease in firing from baseline. A dot above the line of no change (slope of 1) indicates that the neuron fired faster during Consumption than Psudoconsumption. We can see from this graph a strong trend towards increased firing during Consumption when compared to Psudoconsumption. Two separate two-way ANOVAs were run for Nac Core and Shell respectively, comparing change scores for Consumption and Psudoconsumption. The factors for these ANOVAS were Diet and Trial Type (Consumption vs Psudoconsumption). It was found that there was a significant main effect of Trial Type in Nac Shell (p<.0001, F(1,659)=47.87) as well as Core (p<.0001, F(1,731)=38.93) (bar plot).



**Figure 20.** Comparison of baseline firing rates during Consumption and Psudoconsumption for both Nac Core and Shell: For a detailed graph description see Fig 10. Only the regression line for Nac Shell in CC animals was significantly different from the line of no change (slope=.7672, lower bound=.6748, upper bound= .8596). However, the BE and CC regressions in Nac Shell were not significantly different from each other (p=.4636, t(229)=-.73).



**Figure 21.** Direct comparison of firing rates during Consumption vs Psudoconsumption (Pseudo-Consumption). For detailed graph description see Fig 10. An analysis of confidence intervals was performed. It was shown that the slope of the regression line for CC Core neurons is not different from the line of no change (slope= .8858, lower bound= .6472, upper bound= 1.1245). There was a significant difference between BE and CC animals in shell (p<.0001, t(191)=-4.52). Note that nearly all neurons fire faster during Consumption than during Pseudoconsumption.

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