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Characterizing ingestive behavior through licking microstructure: Underlying neurobiology and its use in the study of obesity in animal models

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Abstract

Ingestive behavior is controlled by multiple distinct peripheral and central physiological mechanisms that ultimately determine whether a particular food should be accepted or avoided. As rodents consume a fluid they display stereotyped rhythmic tongue movements, and by analyzing the temporal distribution of pauses of licking, it is possible through analyses of licking microstructure to uncover dissociable evaluative and motivational variables that contribute to ingestive behavior. The mean number of licks occurring within each burst of licking (burst and cluster size) reflects the palatability of the consumed solution, whereas the frequency of initiating novel bouts of licking behavior (burst and cluster number) is dependent upon the degree of gastrointestinal inhibition that accrues through continued fluid ingestion. This review describes the analysis of these measures within a context of the behavioral variables that come to influence the acceptance or avoidance of a fluid, and the neurobiological mechanisms that underlie alterations in the temporal distribution of pauses of licks. The application of these studies to models of obesity in animals is also described.

1. Introduction

The decision to engage in the consumption of food requires the coordination and integration of intricate peripheral and central signaling processes and is dependent upon a variety of variables, which include: the animal's energy needs; the palatability of the food, and the energy acquired from it (Davis and Levine, 1977; Davis and Campbell, 1973; Davis et al., 1994; Morton et al., 2006; Shires et al., 2011; Treesukosol et al., 2011). Together these variables come to affect food consumption based on the innate or learned, liking or disliking of food (Berridge, 2000; Dwyer, 2012; Johnson, 2013; Spector, 2015). Studies that characterize the pattern of intake made by rodents while consuming a solution have provided significant insight into the variables that control ingestive behavior. In this review, I discuss findings from studies of licking microstructure, in which the pattern of rhythmic tongue movements are recorded to enable analysis of distributions of the intervals between consecutive licks (interlick intervals; ILIs) (Davis and Smith, 1992; Spector et al., 1998; Smith, 2001). These studies are described with respect to ingestive behavioral and learned

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factors controlling the acceptance, preference and overeating of food. The remainder of this review will describe the underlying neurobiological systems controlling alterations in the temporal distribution of ILIs, and the use of these techniques in the study of obesity in animal models.

2. Licking behavior and its analysis

The overall intake or size of a meal is commonly used to examine the acceptance or avoidance of food, however this measure reflects the aggregate of numerous behavioral variables that are under the control of distinct physiological mechanisms. In early studies of licking behavior, John Davis provided rats with access to a range of sucrose, glucose and fructose concentrations (Davis, 1973). During these 30 s brief access tests, the rate of licking was found to increase monotonically with the concentration of each tastant. Licking rate was also influenced by the interval between previous test sessions, as it was significantly reduced following a 30 min interval, compared to drinking tests conducted immediately after a meal. Thus, Davis (1973) identified two opposing features controlling meal intake—a stimulatory effect on licking rate that is driven by the palatability of the tastant, and a delayed post-absorptive inhibitory effect.

In addition to characterizing the rate of licking, the temporal distributions of pauses of licking that ultimately produce the change in lick rate have also proved a useful tool for distinguishing stimulatory and inhibitory influences over ingestive behavior (Davis and Smith, 1992; Spector et al., 1998). In a seminal paper, John Davis and Gerard Smith examined the distribution of ILIs and observed three regions that were readily distinguishable. The first accounted for the vast majority ($\approx 90\%$) of licks (ILIs < 250 ms) and displayed a sharply peaked distribution characterized by an equivalent steep decline either side of the peak. This distribution is thought to be under the control of a central pattern generator (CPG) in the hindbrain and reflects rhythmic tongue movements of the rodent (Wiesenfeld et al., 1977). A shift in the distribution of these within-burst ILIs suggests alterations in oromotor function that are independent of palatability and/or post-ingestive effects (Davis et al., 1994; Aja et al., 2001; Swick et al., 2015). The two other regions that were identified reflected ILIs of 251–500 ms and those > 500 ms. Pauses between 251 and 500 ms were thought to reflect brief interruptions of licking, such as lateral tongue movements (Grill and Norgren, 1978), whereas those > 500 ms indicated longer interruptions of licking due to the active engagement of other competing behavior (e.g., grooming behavior) (Davis and Smith, 1992). The ILIs between 251 and 500 ms and > 500 ms were referred to as interburst and intercluster intervals, respectively. The number of licks occurring (or time spent consuming) between interburst intervals reflects the size (or duration) of licking bursts, whereas the number of licks (or time spent consuming) between intercluster intervals reflects the size (or duration) of clusters (Figure 1a). These measures display a monotonic relationship with increases in concentrations of maltose and sucrose solutions (Davis and Smith, 1992; Davis and Perez, 1993; Spector et al., 1998) and have also been shown to decrease monotonically when a palatable solution is made more aversive by adulteration with the bitter tasting solution quinine (Hsiao and Fan, 1993). The size of bursts and clusters are not simply reflective of the overall intake of a solution, which is generally highest at more intermediate concentrations (i.e., an inverted-U-shaped/preference-aversion

function; Richter and Campbell, 1940; Stellar, 1960). Moreover, these measures are unaffected by sham feeding preparations (Davis and Smith, 1992) in which fluid is prevented from reaching the gastrointestinal (GI) tract via an esophageal or gastric fistula (Davis and Campbell, 1973; Hull et al., 1951; Schneider, Gibbs & Smith, 1986; Mook et al., 1983). Thus, burst and cluster size reflects pre-ingestive evaluation of the perceived palatability of the consumed tastant (Smith, 2001) (Figure 1b). The initial lick rate (e.g., 1st min lick rate; Spector et al., 1998) also reflects the pre-ingestive evaluation of the tastant as it is typically greater for more palatable solutions and occurs prior to the accumulation of fluid in the GI tract (Davis, 1973; Davis and Levine, 1977; Davis and Perez, 1993; Spector et al., 1998). On the other hand, the number of bursts and clusters can be used to observe behavioral changes resulting from post-ingestive inhibitory feedback (Figure 1b). Unlike the number (or duration) of licks occurring prior to a pause, the frequency with which a rodent initiates licking behavior after a specified pause (i.e., the number of bursts or clusters) displays an inverted-U-shaped function that mirrors the pattern seen with overall intake (Davis and Smith, 1992). Moreover, testing under sham feeding preparations significantly enhances this measure due to the removal of inhibitory signaling from the GI tract (Davis and Smith, 1992). In addition to the use of 251–500 ms and > 500 ms pause criteria, other studies use a > 1000 ms pause criterion for elucidating pre- and post-ingestive licking microstructure variables associated with meal intake (Spector et al., 1998; Johnson et al., 2010; Figure 1a).

3. Tastant evaluation and experience

3.1. Acquired acceptance, preference and overeating

When initially exposed to a food, animals will show hesitation in consuming it. This neophobia can be observed with palatable novel foods and reflects a survival-based mechanism to prevent consumption of potentially toxic substances. Following continued experience, animals show an increase in the acceptance of the food as long as no aversive consequences result from its ingestion. Using analyses of licking microstructure, studies have revealed that the increase in intake after experience with the food reflects enhanced initial lick rate (Monk et al., 2014), and cluster size (Austen et al., 2016) suggesting that the palatability of the tastant appears to increase as it becomes more familiar and/or the expectation of its delivery increases. Furthermore, these studies have revealed that the process of habituation occurs more rapidly than originally presumed from studies that only measured overall intake (Green and Parker, 1975), with swift alterations in stimulus palatability detected within minutes of exposure to the novel fluid (Monk et al., 2014).

As rodents increase their acceptance of a particular food, post-ingestive positive feedback of ingestion can also occur (Sclafani, 2001). An example of this can be observed in flavor preference studies, where animals learn to prefer flavors that are associated with positive outcomes (Sclafani and Nissenbaum, 1988). In these studies, one flavor (conditioned stimulus, CS+) is associated with a nutritive solution (e.g., sugar or glucose; unconditioned stimulus, US) that can be either consumed orally or infused directly into the GI tract (Dwyer et al., 2009; Myers and Sclafani, 2001a, 2001b). A second flavor (CS–) is paired with a less- or non-nutritive solution, such as water. During the test stage, it is typically observed that the

CS+ becomes accepted and preferred (relative to the CS-) (Sclafani and Nissenbaum, 1988; Sclafani, 2001), which is in part due to an increase in initial lick rate and cluster size (Myers and Sclafani, 2001a; Dwyer, 2008; Dwyer et al., 2009). It is thought that the increase in these measures of palatability reflect conditioned enhancements in the hedonic evaluation of the flavor, in a way that is similar to that normally elicited in an unconditioned manner by sugars.

Conditioned increases in palatability also appear important for driving overeating of food. In cue-potentiated feeding (CPF) studies, food-deprived rodents are trained to associate an exogenous cue (e.g., tone; CS+) with sucrose and a second cue (e.g., noise; CS-) with no reward. Following a period of access to food to ensure testing under non-deprived conditions, presentation of the CS+ cue evokes an increase in sucrose intake significantly beyond that seen following CS- presentation or periods when no stimuli are presented (Johnson, 2013; Sherwood et al., 2015; Dailey et al., 2016). Using licking microstructure analyses, we have revealed that the basis for this overeating in part reflects conditioned increases in the palatability of food as revealed by an increase in burst size (using the 1000 ms pause criterion) that is specific to CS+ evoked consumption of the sucrose US (Sherwood et al., 2015; Sherwood et al., 2015; Johnson, 2017).

In addition to classical conditioning, instrumental actions can also influence outcome palatability (Lydall et al., 2010; Johnson and Gallagher, 2011). In a series of effort-based studies Johnson and Gallagher trained mice to respond on two separate levers. Pressing one of the levers (low effort) resulted in the presentation of a particular auditory cue and the delivery of a particular food. The other lever (i.e., high effort) had to be pressed fifteen times to affect the delivery of a different auditory cue and food. When mice were later given free access to each food separately, they exhibited increased acceptance of the food previously associated with high effort. Moreover, in a two-bottle choice test, mice also preferred the reinforcer that was previously associated with the high effort lever. An examination of licking microstructure revealed that the augmented acceptance and preference of the high effort food reflected features of licking thought to reflect orosensory stimulation, including initial lick rate and burst size (Johnson and Gallagher, 2011). Thus, through the refined analysis of meal intake, it was revealed that working hard for food enhances its taste, which implies that the changes in reinforcing value and preference of food may have derived from palatability changes induced by contrasting effort.

3.2. Aversion

Ingestive defense mechanisms have evolved to defend against continued consumption of toxic food. One well-characterized phenomenon is conditioned taste aversion (CTA) (Garcia et al., 1955; Garcia and Koelling, 1966), in which the taste of food (CS +) is paired with a US illness-inducing agent (e.g., lithium chloride; LiCl) (Garcia and Koelling, 1966). Subsequently, when presented alone, prolonged diminished consumption of the CS+ is revealed, relative to a control group that received CS and US in an unpaired manner (Rosas and Bouton, 1996). Analyses of licking microstructure revealed that this conditioning procedure dampens the hedonic evaluation of the CS+ (Baird et al., 2005; Dwyer et al., 2008; Arthurs et al., 2012). Given the intense visceral reaction elicited by CTA, it is perhaps

unsurprising that consumption of the LiCl-paired CS can be relatively permanent, when compared to an unpaired control group (Rosas and Bouton, 1996). Although the aversion to the CS+ can be resistant to extinction, interestingly cluster size recovers to unpaired control levels (Dwyer, 2009), which suggests the hedonic evaluation of the CS+ does not drive the continued aversion to it.

CTA's can also be revealed through other chemical techniques (Lett and Grant, 1996; Parker, 1982; Zalaquett and Parker, 1989). For example, pairing a taste with drugs of abuse such as amphetamine, cocaine or nicotine leads to a subsequent reduction in consumption of the CS+. However, previous studies employing taste reactivity procedures revealed that this reduced consumption was not accompanied by changes in appetitive taste reactivity responses suggesting that the hedonic evaluation or palatability of the taste CS+ was unaffected by the drug pairings (Parker, 1995). This was taken to reflect the idea that drug-induced reduction of CS+ intake reflected conditioned taste *avoidance*, rather than CTA (Parker, 1995). However, findings from licking microstructure question this account, given that exposure to a taste CS followed by amphetamine injections resulted in less consumption of the CS+ due to reduced cluster size (Lin et al., 2012). This has also been demonstrated with a range of CS's and drug US's (Arthurs et al., 2012; Lin et al., 2012), and comparable findings have also been revealed when wheel running was used to drive vestibular disorientation and promote aversion to the CS+ (Dwyer et al., 2008). The discrepancy between the appetitive taste reactivity findings (Parker, 1995; Parker, 1995, 2003; Parker et al., 2009, 1995; Parker, 1995, 2003; Parker et al., 2009) and licking microstructure (Arthurs et al., 2012; Dwyer et al., 2008; Lin et al., 2012) may reflect in the former approach, the involuntary nature of intake that occurs when fluid is injected directly into the mouth via the indwelling oral cannula, which (unlike studies that use voluntary intake and subsequent licking microstructure analyses) leads to intake behavior that is independent of the desire to consume the fluid.

4. Post-ingestive inhibitory and motivational state influences

4.1. Unconditioned and conditioned negative feedback

Under real feeding conditions, fluid intake is controlled both by the orosensory stimulation of the food and post-ingestive inhibition that develops as fluid accumulates in the GI tract (Davis and Levine, 1977). Removing this inhibitory influence via sham feeding preparations should reflect the control of ingestive behavior via direct orosensory stimulatory effects. Based on this premise, intake rates should be maximal the first time that a rat is tested with the sham-feeding procedure (Davis and Smith, 1990). However, Davis and Campbell first reported that although sham-feeding led to an immediate increase in intake compared to the previous real feeding session, maximum intake was not seen until at least four 30 min sham feeding sessions were provided (Davis and Campbell, 1973). This transient inhibition did not reflect adaptation as it was observed in rats that had previous experience with the sham-feeding procedure using a different fluid (Davis and Campbell, 1973). Instead the critical variable determining whether rats displayed this form of negative feedback was found to be their previous real feeding experience with a given food (Weingarten and Kulikovsky, 1989). Thus, during consumption conditioned negative feedback develops due to an association

between the sensory features and the post-ingestive consequences of food—exposure to sham feeding leads to a gradual extinction of this conditioned inhibitory control, which accounts for the progressive increase in intake that is observed after repeated testing under sham-feeding conditions.

An analysis of the rate of licking revealed that the conditioned negative feedback signal operates within the first 6 min of intake (Davis and Smith, 1990). Following repeated testing and subsequent extinction, the rebound in intake that results reflects an increase in intake during this period of time due to an elevation in the number but not size of clusters, indicating the release from transient learned inhibitory control (Davis et al., 2001). This extinction has been demonstrated with different nutritive solutions (Young et al., 1974; Mook et al., 1983; Weingarten and Kulikovsky, 1989; Davis and Smith, 1990) though it is dependent upon the concentration of the fluid as it is revealed when rats experience a higher (e.g., 0.4 M sucrose) but not lower solution concentration (Davis and Breslin, 2000; Davis et al., 1999; Davis et al., 2001). Thus, with lower sucrose concentrations intake reaches its maximum threshold in the first sham-feeding test, which implies the immediate loss of the unconditioned negative feedback signal. By comparison, with higher solution concentrations the build up of gastrointestinal inhibition is likely greater (e.g., due to the increased energetic and colligative properties of the solution) and sets the endogenous conditions that persist past direct post-ingestive inhibition.

4.2. Motivational influences: effects of hunger on licking microstructure

Increasing the hunger of an animal by restricting access to its regular maintenance diet leads to increases in overall intake when tested with a palatable tastant (Richter and Campbell, 1940; Smith and Duffy, 1957; Campbell, 1958). Two studies have systematically examined the influence of deprivation status and solution concentration on licking microstructure to reveal that the two variables interact to affect the volume consumed (Davis and Perez, 1993; Spector et al., 1998). Although increases in overall intake were revealed in both studies, rats in the Davis and Perez study showed deprivation-induced elevations only at lower concentrations of sucrose (Davis and Perez, 1993). Furthermore, in this study although no effects of overall intake were revealed at higher concentrations (> 0.4 M sucrose), increases in the size of clusters were observed and were taken to reflect deprivation-induced increases in the palatability, which was compensated by a reduction in cluster number, such that overall intake was unaffected by hunger (Davis and Perez, 1993). On the other hand, Spector and colleagues reported no effects of deprivation on palatability measures (initial lick rate, burst size with 1000 ms pause criterion), though hunger did increase meal intake by enhancing the number of bursts (Spector et al., 1998). The basis for these discrepancies may reflect procedural differences such as the duration of testing (30-min, Davis and Perez, 1993; 60-min, Spector et al., 1998), deprivation (17-h, Davis and Perez, 1993; 23-h, Spector et al., 1998), and/or the statistical techniques used to analyze the distribution of the ILIs.

5. Neurobiology underlying licking microstructure responses

Licking in rodents is a highly stereotyped behavior and the within-burst rhythmic tongue movements produced as rodents ingest a fluid are under CPG control (Grill and Norgren,

1978; Wiesenfeld et al., 1977). While the CPG is thought to be located in brain stem nuclei and may include hypoglossal motoneurons (Wiesenfeld et al., 1977), alterations in the temporal distribution of licks that correspond to oral stimulatory and post-oral inhibitory ingestive influences reflect control from numerous neurotransmitter and hormonal signals in the periphery and the central nervous system (CNS).

5.1. Dopamine

Dopamine (DA) signaling has long been implicated in learning and motivation, yet its precise role remains a contentious subject. Early studies suggested that DA functioned as a pleasure neurotransmitter (Wise et al., 1978; Wise, 1980), however following the discovery that DAergic manipulations have no influence on appetitive taste reactivity responses to sweet tasting solutions (Berridge et al., 1989; Wyvell and Berridge, 2000), the idea that DA functions in the hedonic evaluation of food has been questioned and much research has focused on its role in reward learning. In particular, DA seems important for the attribution of incentive motivational value to reward stimuli (Berridge and Robinson, 1998), and consistent with this finding systemic injections of amphetamine lowers sucrose intake due to reductions in the number of licking bursts initiated (Asin et al., 1992). These findings are compatible with the view that DA is associated with the motivation to engage in consummatory behavior (e.g., ‘wanting’), but not measures associated with the evaluation (i.e., burst size) of sucrose (e.g., ‘liking’) (Berridge and Robinson, 2003). On the other hand, a number of licking microstructure studies suggest that the nature of the influence of DA is dependent on the targeted receptor. Dopamine binds to both excitatory D1-like receptors (D1 and D5) and inhibitory D2-like receptors (D2, D3 and D4) (Missale et al., 1998). In general, disruptions in D1 receptor signaling lead to reductions in sucrose or sodium intake due to reductions in the number, but not size of licking bursts (D’Aquila et al., 2012; Galistu and D’Aquila, 2013), whereas D2 receptor manipulations affect intake by altering the evaluation of the test diet (Galistu et al., 2011; Genn et al., 2003; Hartfield et al., 2003; Liao and Ko, 1995; Schneider et al., 1990), which may correspondingly influence the number of bursts initiated (Robles and Johnson, 2016). These effects have been suggested to reflect a D2 receptor-mediated reward evaluation (re-boosting) process that occurs in response to consummatory contact and serves to facilitate further behavioral activation or inhibition via D1 receptor signaling (Paolo, 2010).

5.2. Opioids

Numerous studies implicate the brain opioid system in hedonic evaluation (Berridge and Kringelbach, 2015). The μ -opioid receptors (MOR) are a class of opioid receptors that are expressed throughout the CNS and display a high affinity for endogenous opioids such as beta-endorphin (Mansour et al., 1988). In mice, gene-targeted deletion of beta-endorphin reduced burst duration, however this was only observed when mice were food deprived or tested with high sucrose concentrations (Mendez et al., 2015). Similarly, deletion of MOR mainly resulted in disruptions in bursts of licking, though these effects were also dependent on the motivational status of the animal (Ostlund et al., 2013). When tested under non-deprived conditions, MOR deletion had little effect over intake of sucrose or the temporal disruption of ILI’s, however in knock out mice upshifts in motivational state accomplished through food deprivation were less effective in augmenting overall intake and had little

effect on sucrose evaluation compared to wild-type mice (Ostlund et al., 2013). Thus, MOR's in part underlie the influence of food deprivation on sucrose palatability (Davis and Perez, 1993). Alternatively, infusions of the MOR-specific agonist DAMGO into the shell region of the nucleus accumbens (ACB) (a 'hedonic hotspot') (Berridge and Kringelbach, 2015) revealed a correlation between increased sucrose consumption and licking burst duration, indicating that DAMGO-evoked hyperphagia reflected increased palatability of the test diet (Taha et al., 2009). Similar stimulatory palatability effects on consumption have been revealed with intralipid ingestion, however these effects are driven by the ACB core, but not shell (Katsuura et al., 2011).

Not all manipulations of opioid transmission influence tastant evaluation. Knockout mice for the endogenous occurring opioid peptide, nociceptin (NOC) showed reduced number of bursts for sucrose under non-deprived conditions, whereas intracerebroventricular (ICV) infusion of NOC in wild-type mice increased this measure (Mendez et al., 2016). Thus, NOC signaling appears important for supporting the motivation to engage in solution intake under non-deprived conditions. Similar effects have been reported with another endogenous opioid peptide, enkephalin. In proenkephalin knockout mice reduced bouts of licking behavior were revealed, which were suggested to reflect motivational deficits (Mendez et al., 2015). This latter observation is also consistent with the broad opioid receptor antagonist (naltrexone), which suppresses sucrose and saccharin licking due to reductions in the number, but not size of clusters (Frisina and Sclafani, 2002).

5.3. Endocannabinoids

There is extensive evidence to support a role for the endocannabinoid system in ingestive and reward behavior (Williams et al., 2001; Crombag et al., 2009). The endocannabinoid system is dependent on the actions of its two receptors: CB1 and CB2. Several pharmacological studies have been conducted to evaluate the influence of this system on feeding behavior using licking microstructure (Grey et al., 2012; Higgs et al., 2003; Thornton-Jones et al., 2007). Overall these studies point to a complex relationship between cannabinoid manipulations and licking responses. With respect to alterations in stimulus evaluation, in rats systemic treatment with the exogenous cannabinoid ⁹-tetrahydrocannabinol (THC) or the endogenous cannabinoid anandamide resulted in an increase in burst size and duration but not number (Higgs et al., 2003).

Conversely, treatment with the CB1 antagonist SR141716 has been shown to decrease palatability for sucrose (Higgs et al., 2003), whereas this antagonist reduced the motivation to initiate bursts of consumption to an intralipid solution (Thornton-Jones et al., 2007). By comparison, in mice systemic injections of THC or CB1/CB2 agonists CP55 and Win 55, each increased the intake of 15% condensed milk by increasing bout number with minimal effects in the size of licking bursts, whereas the inhibition of condensed milk intake that followed SR141716 treatment resulted in decreased intake due to decreased burst number (Grey et al., 2012). Thus, findings suggest that the effects of pharmacological manipulations over the endocannabinoid system on licking microstructure point to an interaction between the gustatory and nutritive properties of the food and the specific binding characteristics of the pharmacological agents that are employed (Grey et al., 2012).

5.4. Serotonin

A substantial body of literature suggests that 5-hydroxytryptamine (5-HT) plays a significant role in the regulation of feeding behavior. Typically, the administration of compounds designed to stimulate 5-HT neurotransmission lead to anorexigenic effects (Clifton et al., 1989; Simansky, 1996). Serotonergic agonists d-fenfluramine and fluoxetine have their actions on 5-HT 2C receptors (Vickers et al., 2005) and lead to reductions in sucrose intake due to a reduction in burst and cluster size (Asin et al., 1992). Conversely, d-fenfluramine reduced intake of a glucose saccharin solution by reducing the number but not size of licking bursts (Clifton et al., 2000). Similar effects were reported following the use of the 5-HT1B receptor agonist CP-92,253 (Lee and Simansky, 1997). Thus, as with endocannabinoids, the effects of pharmacological manipulations to this system on the evaluation or motivation to engage in consumption appear to be partly dependent on the interaction between test diet and drug targets.

5.5. Neuropeptides: orexigenic

In the periphery the gastric feeding signal ghrelin is the only orexigen that has been identified (Wren, 2000). We observed a reduction in sucrose intake that reflected reduced burst size but not number in food-deprived mice that received systemic treatment with the ghrelin receptor antagonist GHRP-6 [D-Lys³] (Johnson et al., 2009). Conversely, in non-deprived rats, ICV infusion of ghrelin enhanced intake of a test diet (composed of intralipid, maltodextrin, sucrose and protein), however it did so without influencing initial lick rate or the size of clusters (Overduin et al., 2012). Further studies are required to establish whether peripheral and central ghrelin-signaling influences the motivational or evaluative aspects of feeding behavior, and how these effects interact with the energetic status of the animal and the nutrient content of the test diet (Perello and Zigman, 2012; Johnson, 2013).

The hypothalamus plays a pivotal role in central appetite regulation, with the arcuate nucleus (ARC) functioning as a critical system in the integration of hormonal signals for energy balance. Located at the ventral tip of the hypothalamus and adjacent to the median eminence (Banks, 2006), the ARC responds to circulating hormones such as ghrelin. One population of neurons in the ARC expresses the orexigenic peptides agouti-related peptide (AGRP) and neuropeptide Y (NPY). Forebrain treatment of NPY accomplished by 3 V ICV infusion enhanced sucrose but not saccharin consumption, and it did so primarily by increasing the number of bursts in a meal, with mixed effects over measures of tastant evaluation (Baird et al., 2006a). Thus, although there was a tendency for NPY to enhance initial lick rate for higher (1 M) sucrose concentrations, NPY had minimal effect over burst size for water, saccharin or weaker concentrations of sucrose (Baird et al., 2006a).

Neurons in the ARC project to the lateral hypothalamus (LH), which has traditionally been viewed as the brain's feeding center (Delgado and Anand, 1953) and more recent approaches have identified populations of neurons responsible for triggering appetitive behaviors in this region (Stuber and Wise, 2016). Two distinct LH feeding signals are orexin and melanin concentrating hormone (MCH) (Qu et al., 1996; Peyron et al., 1998). Orexin ligands are synthesized into two forms, orexin A and orexin B. Orexin A shows a ten fold higher affinity for the orexin receptor OxR1, whereas both ligands show similar affinity to

the orexin receptor OxR2 (Peyron et al., 1998). ICV infusions of orexin A resulted in an increase in sucrose intake via burst number without affecting either the initial lick rate or mean burst size (Baird et al., 2009). These effects of orexin A infusion were dependent on area postrema and the nucleus of the solitary tract (NTS), as excitotoxic lesions to these brain stem structures abolished the stimulatory effects of 3V orexin A infusion (Baird et al., 2009), which is consistent with orexin A input to these sites (Zheng et al., 2005). This dependence of hindbrain circuitry on the stimulatory effects of feeding was further supported by 4 V hindbrain orexin A infusions that also enhanced sucrose intake primarily by an increase in the number of licking bursts (Baird et al., 2009). Thus, orexin A stimulates feeding by reducing postingestive feedback inhibition, resulting in enhanced motivation to initiate food intake, which is likely dependent on ORX1 receptor projections to brain stem structures. In addition, midbrain structures are also important for orexin A feeding, as ventral tegmental area (VTA) infusions of this ligand have been shown to increase sucrose intake (Terrill et al., 2016). In this study, the authors divided the 30-min sucrose intake test into thirds and established that although effects on increased licking and burst size were revealed they were only evident in the final portion of the meal. Accordingly, this may suggest that the effects of orexin A stimulation in VTA are dependent on fluid accumulation in the GI tract and under these conditions, treatment with orexin A serves to suppress post-ingestive inhibitory feedback, leading to control of feeding through orosensory mechanisms (Terrill et al., 2016).

Although orexin and MCH are both synthesized in the LH and produce stimulatory effects over feeding behaviors, studies of licking microstructure have revealed that these two orexigens stimulate feeding in different ways. Unlike orexin, 3V MCH infusions leads to an elevation in sucrose intake that is modulated primarily by increases in the hedonic evaluation of sucrose (Baird et al., 2006b). This is consistent with CPF studies that show the overeating evoked by an auditory CS+ is disrupted in MCH receptor (MCH-1R) knock-out mice, due in part to a decrease in the capacity for this cue to enhance the palatability of sucrose (Sherwood et al., 2015). More generally, these findings are also consistent with the observation that MCH-immunoreactive fibers project to numerous areas important for gustatory function including gustatory cortex, the ventroposteromedial thalamus and the parabrachial nucleus (Saito et al., 2001). In addition, unlike the effects of 4V orexin A stimulation, hindbrain MCH infusions have no effect on sucrose intake or licking microstructure (Baird et al., 2008). Collectively these results suggest that although the hypothalamic feeding signals, orexin, MCH and NPY stimulate food intake, they do so via separate behavioral influences over meal ingestion which likely points to the distinct extra-hypothalamic sites of actions of these feeding signals.

5.6. Neuropeptides: anorexigenic

A variety of peripheral and central anorexigenic signals function to reduce and inhibit food intake. The incretin and anorexigenic signal glucagon-like-peptide 1 (GLP-1) is expressed in gut endocrine cells and is also found in the caudal brain stem (Turton et al., 1996). Expression of its receptor, GLP-1R, is distributed throughout central circuitry underlying regulation of appetite control (McMahon and Wellman, 1998; Hayes et al., 2009; Vrang and Larsen, 2010). CNS infusions of GLP-1 or Exendin-4 (a potent agonist of the GLP-1R)

leads to pronounced hypophagia (Lambert et al., 1993; Turton et al., 1996; Szayna et al., 2000). This anorexigenic effect is modulated by reductions in the palatability of the ingested tastant (Asarian et al., 1998; Dossat et al., 2013) as under sham-feeding conditions, central infusion of GLP-1 decreased intake via a reduction in initial lick rate, burst and cluster size (Asarian et al., 1998). In the CNS, manipulations of GLP-1R also appear to affect tastant evaluation as infusions of the GLP-1R antagonist exendin-9 in the ACB increased sucrose intake by increasing the size of bursts and clusters without effect on the number initiated (Dossat et al., 2013). These effects of GLP-1R antagonism within ACB were dependent on nutritive solutions as ventral striatal exendin-9 infusion had no effect on intake of saccharin, thus nutrients in the GI tract appear necessary for the palatability influences following manipulations to GLP-1R signaling (Dossat et al., 2013).

An additional anorexigenic signal that is secreted in the GI tract is the duodenal peptide CCK, which functions as a satiety signal and is secreted in response to nutrients in the intestinal lumen (Gibbs et al., 1973a; Smith and Gibbs, 1992). Peripheral injections of CCK inhibited intake of a 12.5% glucose solution and it did so by reducing the size but not number of licking bursts and clusters (Moran et al., 1996), consistent with the inhibitory effect of this satiety signal on 1st minute lick rates for sucrose (Waldbillig and O'Callaghan, 1980). CCK has its effects over two receptors: CCK-1 and CCK-2. While treatment with CCK reduces orosensory feedback (Moran et al., 1996), a disruption in CCK-1 signaling leads to reductions in post-ingestive inhibitory feedback (Moran et al., 1998). Otsuka Long-Evans Tokushima Fatty (OLETF) rats lack synthesis and expression of intact CCK-1 receptors in either the periphery or CNS owing to a spontaneous null mutation in the gene encoding the CCK-1 receptor (Funakoshi et al., 1995). When the rate of licking of OLETF rats during intake of a liquid diet (Ensure) was examined to fit a Weibull function, this revealed a higher slope and lower shape (Moran et al., 1998). In other words, while initial lick rates were similar between OLETF and controls, OLETF rats continued to maintain a higher rate of licking for a more protracted period of time and that rate of decline occurred more slowly than controls, which is commonly attributed to reduced negative feedback (Smith, 2001). This finding is also consistent with the observation of increased mean burst number in OLETF rats (Moran et al., 1998).

Within the CNS, a number of anorexigenic neuropeptides also exist to reduce intake. In the ARC, a population of anorexigenic neurons produces a peptide encoded by the cocaine and amphetamine related transcript (CART) (Elias et al., 2001); and melanocortins, which are a group of peptide hormones that result from cleavage products of a common precursor peptide, proopiomelanocortin (POMC) (Williams et al., 2001). The melanocortin system works through actions of its receptors (MC1R-MC5R) and has been described as important for influencing meal cessation. Forebrain infusion of the MC3/4-R agonist MTII produces a dose-dependent inhibition of food intake (Fan et al., 1997; Thiele et al., 1998; Baird et al., 2011), which may reflect a more rapid rate of decline during intake and reductions in the number of bursts (Baird et al., 2011; Williams et al., 2002), thus suggesting an increased sensitivity to post-ingestive inhibitory control. These effects are independent of motoric impairments, as rats under the influence of MTII appropriately adjusted their licking rates when lick volume was altered from 8 to 4 μ l (Williams et al., 2002). MTII administration also influences tastant evaluation, though these effects appear more varied. Thus, MTII

decreased burst duration when examined over a 30 min consumption test, however when the mean duration of the first three bursts in the meal were examined (when presumably minimal post-ingestive influences occurred), MTII had no effect on this measure (Williams et al., 2002). This finding is consistent with the effects of gene-targeted deletion of MC4R in mice that show normal taste responses to sucrose in brief access taste response tests (Eylam et al., 2005) in which fluid is available for a short time (e.g., 5s) and thus limits post-ingestive influences due to minimal accumulation of fluid into the GI tract. In contrast, using the brief access test in rats, 3 V MTII infusion reduced intake of sucrose and saccharin (but not water), indicating a possible influence over tastant evaluation (Baird et al., 2011). These results could reflect discrepancies between the approaches and species used, thus further characterization of MTII on tastant evaluation is required. However, one possibility is that deletion of MC4R (and potential associated compensatory influences) leads to effects that are more dependent on hindbrain, as 4 V MTII infusion in rats has no effect on brief access licking, though similar influences on post-ingestive inhibition to that seen with 3 V infusions were observed (Baird et al., 2011).

CART expression in the ARC is a potent inhibitor of food intake (Kristensen et al., 1998), and 3V infusions lead to pronounced reduction in Ensure intake due to reduced initial lick rate with no effect on either the slope or shape parameters as defined by the Weibull function parameter (Aja et al., 2001). These effects over reduced orosensory stimulation may occur in combination with alterations in motoric function as particularly at higher drug doses, CART shifted the distribution of ILI's < 250 ms to the right (Aja et al., 2001), which as described above is thought to rely on the CPG (Wisnfeld et al., 1977).

Finally, Corticotropin-releasing factor (CRF) has been examined in studies of licking microstructure. CRF interacts with two receptor subtypes, CRF-1 and CRF-2 (Behan et al., 1996), however CRF-2 is thought to primarily regulate its anorexigenic influences (Martinez et al., 1998; Smagin et al., 1998). In rats, forebrain infusion of CRF leads to a reduction in intake of a sucrose solution due to a reduction in the number of clusters in a meal, without influencing cluster size (Kochavi et al., 2001), suggesting that CRF may be important for mediating central salience of post-ingestive negative feedback.

6. Relevance for study of obesity in animal models

The precise quantification of the temporal distribution of ILI's may be broadly informative to numerous areas of research including addiction (Barkley-Levenson and Crabbe, 2012, 2015; Eastwood et al., 2014; Robinson and McCool, 2015), mental illness (Hartfield et al., 2003; Galistu et al., 2011; Schepisi et al., 2014) and aging (Zhang et al., 2008). However, the use of licking microstructure is perhaps most readily translatable for animals models of obesity.

6.1. Diet induced obesity

The rapid rise in the prevalence of obesity has been in part attributed to the availability of palatable energy dense food, which results in caloric intake that exceeds energy needs. The diet induced obese (DIO) model has been used to examine the consequences of prolonged access to high-energy diet in animals, which typically result in increased caloric

consumption and adiposity. A number of studies have examined whether these alterations in ingestive behavior reflect changes in the evaluation of taste stimuli or post-ingestive inhibitory control (Johnson, 2012; Duca et al., 2014; Treesukosol et al., 2015). I previously examined licking microstructure in mice that received 12 week exposure to either a 45% high-fat or 10% control diet (Johnson, 2012). The fat content in the former diet is similar to that contained in Western diets in the human population (Drewnowski and Popkin, 2009), and as anticipated, exposure to the high-fat diet in mice led to increased epididymal, perirenal and inguinal adiposity (Johnson, 2012). However, when mice received short-term 20 min drinking tests with a range of sucrose concentrations, DIO mice surprisingly showed reductions in intake, which reflected decreases in both the size and number of bursts (Johnson, 2012). These findings are consistent with subsequent studies in rats in which exposure to a high-energy maintenance diet reduces sucrose intake (Duca et al., 2014; Treesukosol et al., 2015). Consistent with diminished orosensory detection that follows the onset of dietary obesity, high-fat DIO rats show reduced consumption of the non-caloric sweetener saccharin and reduced gene expression for the sweet taste receptor T1R3 (Chen et al., 2010). Moreover, using sham feeding preparations it was revealed that DIO rats showed reduced intake of sucrose (Duca et al., 2014; Treesukosol et al., 2015). Thus, exposure to high-energy diets does not appear to generalize to increases in sensitivity to other palatable foods, but conversely leads to reductions in their acceptance. To account for these findings, Johnson (2012) suggested that maintaining rodents on a high-fat diet may have lead to contrast-mediated suppression of sucrose intake. That is, the palatability of the sucrose is evaluated in relation to that of the more palatable and preferred high-fat homecage diet. In support of this account, when the high-fat diet was replaced with lab chow for DIO mice, intake rates were normalized to control levels, as were the evaluative and motivational measures of licking microstructure (Johnson, 2012). Although additional studies are required to determine whether higher sucrose concentrations (> 20%) may elicit contrast-independent DIO effects, this account is also consistent with other simultaneous (Dwyer et al., 2011) and successive contrast studies (Austen et al., 2016), in which contrast-mediated upshifts or downshifts in intake are accompanied respectively by corresponding increases or decreases in cluster size (Dwyer et al., 2011; Austen et al., 2016).

6.2. Binge eating

A number of animal models have been developed to examine the voracious intake to high-energy diets that occurs within a context of binge eating (Avena et al., 2008; Corwin et al., 2011), which in humans is a core symptom associated with all major eating disorders (APA, 2013) and under certain circumstances leads to obesity (de Zwaan, 2001). Common to all these animal models is the intermittent presentation of an energy dense palatable diet. To examine the variables responsible for the escalation of meal intake, Lardeux et al. (2013) provided rats with 5 weeks access to a liquid emulsion comprised of heavy cream, corn oil and sugar for three times each week for 90 min. The pattern of intake in these rats was compared to a continuous access group that had access to the diet during the same 90 min exposure sessions and at all times in their homecage. An additional group received the same intermittent or continuous access schedules, however they were provided a less caloric diet. As reported previously (Avena et al., 2008; Avena et al., 2008; Bello et al., 2008; Corwin et al., 1998, Avena et al., 2008, Avena et al., 2008; Bello et al., 2008; Corwin et al., 1998)

intermittent access to the high energy diet led to greater intake compared to the continuous access group or rats exposed to the low caloric diet (Lardeux et al., 2013). An analysis of licking microstructure revealed the hyperphagic response in the intermittent high calorie group reflected an increase in the number of bursts compared to the continuous high calorie group. Thus, the effects of intermittent exposure to a high energy diet appear to be driven by increases in the motivation to initiate bursts of licking behavior that may reflect diminished post-ingestive negative feedback. Interestingly, increases in the perceived palatability of the test diet were noted in both high energy diet groups compared to the low energy groups, thus suggesting that access to the diet alone may promote alterations in taste evaluation (Lardeux et al., 2013).

7. Limitations of licking microstructure analyses

The licking microstructure analysis method provides the opportunity to identify ingestive and reward measures associated with tastant evaluation and post-ingestive negative feedback; nevertheless, despite these advantages there are a number of limitations. First, although the initial lick rate is a useful tool for measuring palatability as it reflects pre-ingestive orosensory stimulation prior to fluid accumulation in the GI tract (Smith, 2001), the capacity for this measure to reflect increases in tastant palatability is limited by rats' asymptotic rates of licking, which is between 6 and 7 Hz (Spector et al., 1998) and is under the control of the CPG. Thus, increases in initial lick rate following increasing concentration of sucrose diminish at higher sucrose concentrations (Davis and Perez, 1993), whereas deprivation has been found to increase initial lick rate only at lower concentrations (Davis and Perez, 1993) or not at all (Spector et al., 1998). It is also possible that the limitations in exceeding a certain response rate threshold may account for some of the variability with this measure relative to other indicators of palatability (Baird et al., 2006; Torregrossa et al., 2006; Wirtshafter et al., 2011).

Second, while the mean size of bursts and clusters are less limited by asymptotic motor output, it has been suggested that these measures are only strictly reflective of palatability when they are examined during initial stages of consumption (i.e., prior to post-ingestive inhibition) (Williams et al., 2002). Indeed, adopting this approach has revealed that although MTII hypophagia reduced the duration of licking bursts across 120 min glucose intake tests, this was not observed when the first three bursts of the meal were examined (Williams et al., 2002). In other words, the suppressive effects of melanocortin activation on this measure of palatability were only evident following the accumulation of fluid in the GI tract.

Third, the use of burst or cluster number in studies of learning requires further validation. This measure has been suggested to reflect the incentive motivational properties of the stimulus (Paolo, 2010). Within this account, the attribution of incentive properties to food stimuli and food-related cues can facilitate food-seeking behavior, such that the frequency with which an animal initiates a new burst of licking behavior reflects motivational features of feeding behavior (Paolo, 2010). Obviously, as fluid accumulates in the GI tract, the colligative and energetic properties of the solution enhance satiation and eventually diminish the motivational state of the animal (Spector et al., 1998). However, is this peripheral influence of motivation analogous to the attribution of incentive motivation that occurs to

food stimuli and invigorates food-seeking behavior? We have found burst initiation can be dissociated from other more typical measures of food-seeking, such as conditioned approach to the food source (Sherwood et al., 2015). In this study, deletion of the Melanin Concentrating Hormone receptor, MCH-1R in knock-out mice disrupted CPF by reducing both the mean size and number of licking bursts. Nevertheless, both knock-out and wild-type mice showed comparable elevated food cup entries to the CS+ relative to the CS- (Sherwood et al., 2015). That is, the capacity for a CS+ to evoke conditioned approach behavior can be dissociated from its capacity to initiate bursts of licking, which is problematic for the account proposed by Paolo (2010).

8. Conclusion

Although there are limitations as noted above, the microstructure of licking behavior provides the opportunity to measure and analyze the specific behaviors involved in the ingestion of fluid, and when combined with manipulations to cerebral and peripheral circuitry, identify the physiological mechanism(s) underlying these distinct forms of ingestive behavior. To date, a number of commercially available lickometers exist (Davis and Smith, 1990; Davis et al., 1993; Schoenbaum et al., 2001; Austen et al., 2016); thus, characterizing licking behavior in this precise and quantitative manner can be relatively inexpensive and adapted to numerous experimental settings. Given that licking microstructure provides the opportunity to readily analyze vast amounts of data associated with evaluative and motivational components of meal intake, it is perhaps surprising that relatively few studies have used this technique in assessing ingestive changes that result from or accompany weight gain and obesity. In today's society, the current obesogenic epidemic results from numerous heterogeneous variables, making identification of a particular genetic, physiological or behavioral marker extremely difficult. However, adopting a reductionist approach in animals through refined analyses of meal intake provides the opportunity to identify specific behavioral and physiological variables that underlie the dysregulation of appetite control.

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Fig. 1.

Example of pause criteria used in licking microstructure and interpretation of measures of licking microstructure (a) Hypothetical example of temporal pattern of licking as a rodent ingests a solution. The black lines represent individual licks, with the majority of interlick intervals occurring ≤ 250 ms. (i) Pauses of ≤ 250 ms and > 250 ms are referred to as interburst intervals, and the number of licks occurring prior to this pause criterion reflect burst size (Blue lines; Davis & Smith, 1992). (ii) Pauses of > 500 ms represent intercluster intervals, and cluster size is derived from the number of licks occurring prior to this pause criterion (Green lines; Davis & Smith, 1992). (iii) Other studies have used a > 1000 ms pause criterion, and the number of licks occurring prior to this pause criterion reflect burst size (Red lines; Spector et al., 1998). (b) Measures of licking microstructure and their interpretations.