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Parsing the hedonic and motivational influences of nociceptin on feeding using licking microstructure analysis in mice

Ian A. Mendez, Nigel T. Maidment, and Niall P. Murphy

Hatos Center, Department of Psychiatry and Biobehavioral Sciences, Semel Institute for Neuroscience and Human Behavior, University of California at Los Angeles, Los Angeles, CA, USA

Abstract

Opioid peptides are implicated in processes related to reward and aversion; however, how specific opioid peptides are involved remains unclear. We investigated the role of nociceptin (NOC) in voluntary licking for palatable and aversive tastants by studying the effect of intracerebroventricularly (i.c.v.) administered NOC on licking microstructure in wild-type and NOC receptor knockout (NOP KO) mice. Compared to wildtypes, NOP KO mice emitted fewer bouts of licking when training to lick for a 20% sucrose solution. Correspondingly, i.c.v. administration of NOC increased the number of licking bouts for sucrose and sucralose in wildtype, but not NOP KO mice. The ability of NOC to initiate new bouts of licking for sweet solutions suggests that NOC may drive motivational aspects of feeding behavior. Conversely, adulterating a sucrose solution with the aversive tastant quinine reduced licking bout lengths in wildtype and NOP KOs, suggesting that NOC signaling is not involved in driving voluntary consumption of semi-aversive tastants. Interestingly, when consuming sucrose following 20 hours of food deprivation, NOP KO mice emitted longer bouts of licking than wildtypes, suggesting that under hungry conditions NOC may also contribute to hedonic aspects of feeding. Together, these results suggest differential roles for NOC in the motivational and hedonic aspects of feeding.

Keywords

Nociceptin; intracerebroventricular; sucrose; quinine; licking; knockout mouse

Introduction

Several decades of research have implicated endogenous opioids in controlling feeding and related processes such as incentive learning, motivation, and reward (Olszewski et al., 2011). Interest in such a role has recently intensified as disorders of eating, especially overeating, are heavily implicated in driving the obesity epidemic sweeping many developed countries (Hill and Peters, 1998). In general, activation of mu opioid receptors by opiates such as morphine, and endogenous opioids such as enkephalins and beta-endorphin, promote

Corresponding author: Ian A. Mendez, Ph.D., Department of Psychiatry and Biobehavioral Sciences, University of California, Los Angeles, 760 Westwood Plaza, Los Angeles, CA 90024-1759, Phone: (310) 206-7890, FAX: (310) 825-7067, imendez@ucla.edu.

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feeding, generally considered to result from increases in the incentive and hedonic properties of orally ingested stimuli (Mendez et al., 2015; Olszewski et al., 2011; Ostlund et al., 2013).

Another endogenously occurring opioid peptide is nociceptin (NOC), known also as orphanin FQ. This peptide binds and activates its cognate G protein-coupled receptor, known variably as NOP, ORL1, or OP4. Unlike mu and delta opioid receptors, which bind various endogenous opioid peptides, NOC appears to be the only endogenously occurring ligand capable of activating NOPs. In doing so, NOC modulates numerous processes, including learning and memory, anxiety, water homeostasis, and of particular relevance to the current study, feeding and reward (see Witkin et al., 2014 for review). Many studies show that administration of NOC agonists promote eating (Polidori et al., 2000a; Pomonis et al., 1996; Stratford et al., 1997). There is evidence that this action may involve inhibition of anorexigenic signaling (Witkin et al., 2014), but given that a major motivator for feeding behavior is the pleasure experienced during food consumption, the ability of NOC to stimulate feeding may also involve effects on the motivational and hedonic aspects of food stimuli. Indeed, there has been considerable recent discussion over the relative roles of incentive motivational (often termed “wanting”) versus hedonic (often termed “liking”) processes, in driving feeding (Berridge, 2009).

When voluntarily licking solutions, rodents usually cluster their licks into discrete bouts of varying lengths. Analysis of this licking microstructures is frequently used to tease apart elements driving feeding behavior (Mendez et al., 2015; Ostlund et al., 2013). Particularly, while the total number of licks within a given time can provide a general measure of feeding activity, the total number of bouts may reflect the incentive motivational properties of the stimulus (D'Aquila, 2010; Higgs and Cooper, 1998). In contrast, the average length of bouts may reflect the hedonic properties (i.e. orosensory pleasure) of a food stimulus (see Dwyer, 2012 for review and Discussion for further elaboration).

Previous studies from our laboratory, using involuntary administration of tastants, found little evidence of altered hedonic taste reactions to sucrose in NOP KO mice, though these mice displayed altered aversive taste reactions to quinine solutions, under certain satiation conditions (Koisumi et al., 2004). Importantly, such studies are poorly suited to assessing the incentive motivational properties of tastants due the tastants being administered involuntarily. Therefore, the purpose of the current study was to expand on the above studies by addressing the ability of exogenously administered NOC to modulate incentive motivational and hedonic responses to tastants during voluntary licking for sweet solutions, and to determine the role of endogenous NOC in these processes. To this end, we studied licking microstructure in wildtype and NOP receptor knockout (NOP KO) licking for sucrose, the aversive tastant quinine (as an adulterant to sucrose), and the non-caloric sweetener sucralose. Specifically, we compared the effects of i.c.v. infusions of NOC on the licking microstructures of these mice, under both sated and hungry states. On the basis of an extensive literature, we used licking bout frequency and licking bout length as measures of the incentive motivational and hedonic properties of the tastants, respectively.

Methods

Subjects

Male wildtype (n=6) and NOPKO (n=6) mice, fully back-crossed over 10 generations onto a C57BL/6J background, were derived from an in-house breeding colony produced by heterozygous-heterozygous matings (Matthes et al., 1996). Mice are back-crossed annually, and experimental wildtype and NOP KO mice are housed as littermates. One NOP KO mouse was removed from the study after cannula surgeries and one wildtype mouse was removed from the study between Experiment 2 and 3, both as a result of complications with the implanted cannula. Mice were group housed in cages of mixed genotypes. Additionally, animals were age-matched across genotype at the start of testing (days from birth; wildtype, M=217.17, SEM=14.20; NOP KO, M=190.60, SEM=20.60). Food and water were provided freely (except as noted below) in a climate-controlled colony room (22°C). All testing took place during the light phase of a 12-h light/dark schedule (lights on 06:00–18:00 h). Experimental protocols were approved by the UCLA Institutional Animal Care and Use Committee and were performed in accord with the “Principles of Laboratory Animal Care” (National Academy of Sciences, New York, NY, USA) and the National Research Council’s Guide for the Care and Use of Laboratory Animals.

Apparatus

Licking microstructure was recorded in 30-min sessions using custom-made lickometers within a designated recording chamber outside of the home cage, during which mice could freely consume solutions through a standard rodent drinking bottle spout. For each session, mice were placed individually in a darkened square chamber (10 × 10 cm) with a stainless steel floor and 20 cm high walls. The chamber had a single hole (1.25 × 2 cm) located on one wall 2 cm above the floor, through which a stainless-steel drinking spout was inserted at an approximately 30° angle to provide access to solutions. The spout had a 5.5 mm internal diameter hole at the tip, and contained an approximately 7.5 mm diameter ball bearing. The spout was connected to a 13.8 V, 3 A DC power supply and, in series, to an analogue-digital converter (Model DI-148U, DataQ Instruments, Akron, OH, USA) attached to a PC sampling at 30 Hz. When licking from the spout, an electrical circuit was completed that registered as electrical deflections on the computer. The current passing through the circuit at the time of tongue contact was estimated to be 0.9 μA. Previous studies with rodents suggest this to be below the mouse sensory threshold (see Ostlund et al., 2013).

Digitized recordings of total number of spout licks, the total number of licking bouts, and the length of individual bouts of licking, over each 30-min session, were analyzed. Licking bouts were defined as a series of two or more licks uninterrupted by a period of more than 1 s, as recent studies show this threshold to be optimal for determining palatability-related changes in mice (see Ostlund et al., 2013). We computed both the total number of licking bouts performed in each 30-min session and the average duration of licking bouts (i.e., time between the first and last lick in a bout, in seconds, averaged across all bouts in a session), in addition to tracking the total number of individual licks. Although all of these measures may reflect stimulus palatability, it has been argued that they do so with varying degrees of

fidelity and may be modulated by distinct processes (D'Aquila, 2010; Davis and Smith, 1988; Frisina and Sclafani, 2002; Higgs and Cooper, 1998; see Discussion).

Acquisition of sucrose licking behavior

To compare the effects of NOC i.c.v. infusions on licking microstructure in wildtype and NOP KO mice, under both sated and hungry conditions, mice were initially trained in the lickometer for 7 consecutive days under 4-h of food deprivation. On all training days, mice were placed in the lickometer for 30 min and allowed continuous and free access to 20% sucrose solution.

Cannula implantation

Surgeries for cannula implantation occurred over 2 days, 6 to 7 days after the last day of initial training. Animals were anesthetized with 5% isoflurane and maintained at 1–2% isoflurane (Summit, Foster City, CA, USA). A 2mm long 22 G stainless steel intracerebroventricular (i.c.v.) guide cannula (Plastics One, Roanoke, VA, USA) was implanted in the left lateral cerebroventricle (coordinates from Bregma in mm: AP –0.3, ML +1.0, DV –2.0). The patency of the cannula was maintained by insertion of a dummy cannula of equal length constructed from 28 G stainless steel tubing. Three stainless steel screws (1.52 mm O.D., 3.18 mm length, Small Parts, Miami Lakes, FL, USA) were placed in the surface of the skull to serve as an anchor. The cannula and three screws were fixed to the skull with dental cement (Bosworth, Skokie, IL, USA). To provide pain relief, carprofen (5mg/kg, s.c.) was injected prior to surgical incision and 24 h later. Hydration was ensured by injection of 0.4 ml of saline (s.c.) on the day of surgery. The antibiotic trimethoprim sulfamethoxazole was added to the home cage drinking water (trimethoprim 0.08mg/ml, sulfamethoxazole 0.4mg/ml) and administered for 5 days following surgery.

General test design

Eleven to twelve days after cannula surgeries, mice were retrained in the lickometer for an additional 8 days with the same parameters used during the initial 7 days of training (20% sucrose, 4-hour food deprivation). Testing in the lickometer for Experiments 1, 2, and 3 occurred sequentially, in 3 blocks of 5 days duration, with 2 non-test days between experiments. On all test days, mice were placed in the lickometer apparatus for 30 min and allowed continuous and free access to solutions. To re-establish baseline responding at the beginning of each 5 day testing block, mice were given a single test session on the 1st day of each block, using training conditions (20% sucrose, 4-h food deprivation), except for Experiment 3, where 0.01% sucralose was used with 4-h food deprivation.

Experiment 1: Effects of i.c.v. injection of NOC on licking for sucrose

During days 2–5 of the 5 day block, the effects of NOC on licking for 20% sucrose was tested under both sated (0 hrs food deprivation) and hungry (20 hrs food deprivation) states. Ten to 12 min prior to placement in the lickometer, VEH or 1 nmol of NOC was administered i.c.v. in 1 µl over 15 s, via a 2.2 mm long 28 G stainless steel blunt-ended cannula, inserted through the i.c.v. guide cannula. The injector was attached to a 10 µl Hamilton syringe via PFA tubing (0.40 mm I.D., 0.60 mm O.D.). Mice were sequentially

exposed to each of the following 4 test conditions, 1 per day, randomly, and in a counter-balanced order: sated-VEH, sated-NOC, hungry-VEH, hungry-NOC.

Experiment 2: Licking for sucrose/quinine

The purpose of Experiment 2 was to expand on previous studies from our lab, suggesting a role for endogenous NOC signaling in aversive taste reactivity to quinine solutions (Koizumi et al., 2009). Testing during days 2–4 of the 5 day block of Experiment 2 was identical to that described for Experiment 1 except that rather than testing the effects of i.c.v. NOC on licking for 20% sucrose, licking for 20% sucrose was compared with licking for 20% sucrose adulterated with 3 mM quinine, in the absence of any i.c.v. intervention. Mice were exposed to each of the following 4 test conditions, 1 per day, randomly, and in a counter-balanced order: sated-sucrose, sated-sucrose plus quinine, hungry-sucrose, hungry-sucrose plus quinine.

Experiment 3: Effects of i.c.v. injection of NOC on licking for sucralose

Testing during days 2–4 of the 5 day block of Experiment 3 was identical to that described for Experiment 1 except that 0.01% sucralose replaced 20% sucrose. Mice were exposed to each of the following 4 test conditions, 1 per day, randomly, and in a counter-balanced order: sated-VEH, sated-NOC, hungry-VEH, hungry-NOC.

Histological verification of probe and cannula placement

Following licking experiments, animals were anesthetized by intraperitoneal injection of pentobarbital (100 mg/kg) and then transcardially perfused with heparinized (0.02 mg/mL) 0.9% NaCl followed by 4% buffered formalin. Brains were removed, sectioned at 50 μ m, mounted onto gelatinized slides, and stained with cresyl violet for verification of the position of i.c.v. cannulae. Gross observations of cannula placements showed that all cannulae were correctly implanted into the left lateral cerebral ventricle of all mice.

Drugs

For Experiments 1 and 3, 1 nmol (based on Micioni Di Bonaventura et al., 2013; Olszewski et al., 2002; Polidori et al., 2000a) of NOC (Cat# H-3036.001, Bachem, Torrance, CA, USA) was administered in 1 μ l of a 0.9% NaCl vehicle (VEH) over 15 s. For training and testing in Experiment 1, animals were allowed to lick for 20% (580 mM) sucrose. For Experiment 2, quinine (Cat# Q-1125, Sigma, St. Louis, MO, USA) was added to the 20% sucrose solution at a final concentration of 3 mM. For Experiment 3, licking for 0.01% (.251 mM) sucralose was assessed.

Statistical analyses

For training days, significant main effects and interactions of genotype and day on elements of licking microstructure were determined using repeated-measures ANOVA. For test days, significant main effects and interactions of genotype, treatment, and food deprivation were investigated using a linear mixed-model ANOVA and random intercepts, by mouse. For post-hoc analysis, tests of significant simple main effects were conducted. To minimize the

likelihood of a Type I error, obtained p-values were adjusted using the Bonferroni correction for multiple comparisons. A critical p-value of 0.05 was used for all analyses.

3. Results

Acquisition and retraining of sucrose licking behavior

Compared to WT littermates, NOP KO mice initiated fewer bouts of licking during initial acquisition of licking behavior (Figure 1B, days 1–7, prior to cannula implantation; $F_{(1,10)}=6.14$, $p<0.05$), while total licks and mean bout length were similar between the genotypes (Figure 1A, 1C). This effect persisted during the eight days of retraining post-cannulation (Figure 1B, days 8–15; $F_{(1,10)}=5.63$, $p<0.05$), there again being no difference between the genotypes for total licks or the mean bout length measure. These data suggest a role for endogenous NOC in supporting motivation for obtaining food rewards, potentially in the absence of an influence on hedonic aspects of sucrose consumption.

Experiment 1: Effects of i.c.v. infusions of NOC on licking for sucrose

The aim of Experiment 1 was to investigate the role of NOC (endogenous and exogenous) in the hedonic and motivational aspects of feeding behavior. Across test days, infusions of NOC induced increases in total licks in sated, but not hungry, wildtype mice (Figure 2A), suggesting that exogenous activation of NOPs can promote increases in the consumption of sucrose under sated conditions. A significant enhancing effect of elevated hunger state on total licks was observed, across treatments and genotypes, as expected ($F_{(1,27)}=4.75$, $p<0.05$). More importantly, significant two-way interactions between genotype and treatment ($F_{(1,27)}=11.46$, $p<0.01$), and genotype and hunger state ($F_{(1,27)}=4.70$, $p<0.05$), were observed on this measure. Post-hoc comparisons, which had all undergone Bonferroni correction, revealed that, under sated conditions, infusions of NOC caused a significant increase in total licks in wildtypes ($p<0.05$), but not NOP KOs ($p=11.56$). No significant effects of NOC were observed under hungry conditions (i.e., 20 h food deprivation), in wildtype ($p=0.79$) or NOP KOs ($p=0.92$). It should be noted that following vehicle infusions, there was a near significant hunger-induced increase in total licks in NOP KOs ($p=0.06$), but not wildtypes ($p=6.20$), suggesting that endogenous NOC signaling plays a role in suppressing the tendency for hunger to increase sucrose intake (see also Experiment 2).

The effect of exogenous NOC to promote licking under sated conditions in wildtype mice, described above, was accounted for entirely by an increase in the number of licking bouts (Figure 2B). For this measure, there was a significant main effect of genotype ($F_{(1,9)}=5.85$, $p<0.05$), and significant two-way interactions between genotype and treatment ($F_{(1,27)}=8.17$, $p<0.001$) and genotype and hunger ($F_{(1,27)}=4.27$, $p<0.05$), as well as a significant three-way interaction between genotype, treatment and hunger ($F_{(2,27)}=4.51$, $p<0.05$). Post-hoc comparisons revealed that, when tested sated, infusions of NOC caused a significant increase in total bout number in wildtypes (Figure 2B; $p=0.001$), but not NOP KOs ($p=11.78$). No significant effect of treatment was observed on total bout number under hungry conditions, for either wildtypes ($p=7.78$) or NOP KOs ($p=5.21$). Importantly, no significant main effects or interactions were observed on mean bout length (Figure 2C) when licking for sucrose.

These data suggest that exogenous NOC increases the motivation for sucrose without affecting sucrose palatability.

Experiment 2: Licking for sucrose solution plus quinine

The aim of Experiment 2 was to investigate the role of endogenous NOC in licking responses for a semi-aversive stimulus. Significant main effects of elevated hunger state (Figure 3A; $F_{(1,27)}=12.46$, $p<0.01$) and suppressing effects of quinine adulteration ($F_{(1,27)}=43.42$, $p<0.001$) were observed on total licks, as expected. Additionally, a significant three-way interaction was observed ($F_{(2,27)}=6.89$, $p<0.01$). Post-hoc comparisons, following Bonferroni correction, reveal that in NOP KO mice, addition of quinine to sucrose significantly decreased total licks when hungry ($p<0.001$), but not when sated ($p=2.96$). No significant effects of quinine adulteration were observed in wildtype mice when tested either sated ($p=0.12$) or hungry ($p=0.13$). Consistent with the trend noted in Experiment 1, when licking for sucrose alone, hunger induced a significant increase in total licks in NOP KOs ($p<0.001$), but not wildtype mice ($p=3.62$).

A significant enhancing effect of elevated hunger state ($F_{(1,27)}=5.01$, $p<0.05$) and a suppressing effect of quinine adulteration ($F_{(1,27)}=77.11$, $p<0.001$) was observed on mean bout lengths (Figure 3C). A main effect of genotype was also observed on mean bout length ($F_{(1,9)}=10.40$, $p=0.01$), as well as a significant two-way interaction between genotype and treatment ($F_{(1,27)}=11.74$, $p<0.01$), and three-way interaction between genotype, treatment, and food deprivation ($F_{(2,27)}=3.49$, $p<0.05$). Post-hoc comparisons revealed that under sated conditions, adulteration of sucrose with quinine significantly decreased mean bout length in both wildtype ($p<0.05$) and NOP KOs ($p<0.01$). However, when tested hungry, quinine adulteration significantly decreased mean bout length in NOP KO mice ($p<0.001$), but not wildtypes ($p=0.30$). Additionally, post-hoc comparisons revealed significant effects of hunger on mean bout length when licking for sucrose alone, in NOP KO ($p<0.05$), but not wildtype mice ($p=10.76$), suggesting that endogenous NOC suppresses the tendency for hunger to increase sucrose intake by suppressing the hedonic impact of sucrose. While there was a significant enhancing effect of elevated hunger state ($F_{(1,27)}=6.90$, $p<0.05$) and a significant suppressing effect of quinine adulteration ($F_{(1,27)}=7.77$, $p<0.01$) on total bouts of licking (Figure 3B), no other significant effects were observed. The main finding of this experiment was that the attenuating effect of quinine on sucrose licking was not compromised by NOP deletion. While previous studies have reported altered aversive taste reactivity to quinine solutions in NOP KO mice following involuntary consumption, these data suggest that the ability of quinine to decrease voluntary sucrose licking is independent of NOC signaling.

Experiment 3: Effects of i.c.v. infusions of NOC on licking for sucralose

In an attempt to parse the role of NOC transmission in the hedonic versus metabolic components of feeding, we employed the non-caloric sweetener sucralose as the stimulus in this experiment. While there was a significant enhancing effect of elevated hunger state on total licks, as expected (Figure 4A; $F_{(1,24)}=13.16$, $p<0.001$), there was no significant main effect of treatment or treatment by state interaction for this measure.

For total bouts of licking (Figure 4B), significant main effects of elevated hunger state ($F_{(1,24)}=7.87$, $p=0.01$) and treatment ($F_{(1,24)}=7.87$, $p=0.01$) were observed. No interactions were observed for total bouts of licking. Interestingly, following Bonferroni correction, post-hoc comparisons show that, under hungry conditions (i.e., 20-h food deprivation), NOC infusion significantly increased total bouts of licking in wildtypes ($p<0.01$), but not NOP KOs ($p=2.84$). No significant effect of NOC infusion was observed when testing was conducted under sated conditions in wildtypes ($p=3.24$) or NOP KOs ($p=7.89$). Notably, there was no significant effect of hunger alone on this measure in either wildtype ($p=7.10$) or NOP KO ($p=2.59$) mice. When looking at mean bout length (Figure 4C), a significant enhancing effect of elevated hunger state was observed ($F_{(1,24)}=4.65$, $p<0.05$). No other significant effects of NOC infusion were observed on mean licking bout length, when licking for sucralose. In striking contrast to Experiment 1, where sucrose was the stimulus, NOC had no effect on any aspect of licking for sucralose under sated conditions. In an additional contrast to the sucrose experiment, NOC significantly increased lick bout number in the hungry state. Thus, it appears that, while hunger alone is insufficient to significantly increase consumption of a non-caloric sweet tastant, the addition of exogenous NOC to this condition will promote such behavior.

Discussion

In addition to meeting caloric and nutritional needs, feeding can be a highly rewarding event. A role for NOC in feeding behavior and associated psychological processes has long been postulated (see Witkin et al., 2014). Early studies noted that NOC has potent orexigenic effects when administered throughout the brain (Pomonis et al., 1996), or into discrete nuclei, such as the arcuate nucleus of the hypothalamus and the nucleus accumbens shell (Polidori et al., 2000b; Stratford et al., 1997). Considerable discussion has focused on the ability of NOC to stimulate feeding behavior as a result of NOC-induced changes in the incentive motivational or hedonic properties of food (see Sakoori and Murphy, 2008), as is the case for other opioid peptides, such as those acting through mu opioid receptors (Mendez et al., 2015; Ostlund et al., 2013; Pecina and Smith, 2010). Therefore, the purpose of the present experiments was to compare the effects of NOC i.c.v. infusions on licking microstructure in wildtype and NOP KO mice, under both sated and hungry conditions.

Analysis of licking microstructure is commonly used in pharmacological studies to evaluate feeding behavior, and specific licking characteristics have been suggested to represent different psychobiological aspects of feeding (D'Aquila, 2010; Davis and Smith, 1988; Frisina and Sclafani, 2002; Higgs and Cooper, 1998). Specifically, the total bouts of licking that an animal voluntarily engages in during a licking session is suggested to reflect the incentive motivational properties of the stimulus, whereas the average length of time an animal maintains a bout once engaged is suggested to reflect the hedonic properties (i.e. orosensory pleasure) of a food stimulus. For example, it has been argued that once a licking bout is initiated, the immediate hedonic impact of the solution (i.e. palatability) determines the amount of time devoted to the current licking bout, particularly over a short timescale and in the absence of homeostatic modulation (for review see Dwyer, 2012). The use of licking bout length as an index of palatability is supported by studies demonstrating that licking bout sizes, but not licking bout numbers, increase with increases in the concentration

of palatable solutions such as sucrose, and decreases with increases in the concentration of unpalatable solutions such as quinine (Davis, 1989; Davis and Perez, 1993; Davis and Smith, 1992; Spector and St John, 1998). Researchers have argued that the incentive properties of food stimuli and its associated cues (such as the spout) can provoke food-seeking behavior, suggesting that the number of times an animal initiates a new bout of licking reflects motivational components of feeding (see D'Aquila, 2010 for further discussion). This notion is supported by studies showing that the licking bout number, but not licking bout length, is sensitive to drugs known to decrease motivation (Higgs and Cooper, 1998), as well as studies showing that bout number is less sensitive to the immediate hedonic impact of the cue and more sensitive to other cues, such as post-ingestive states (Davis, 1973; Davis and Smith, 1992). Interestingly, research from our own lab has recently used licking microstructure analysis to conduct indepth investigations of the roles of other opioid signaling pathways (enkephalin, beta-endorphin, mu opioid receptors). These studies suggest dissociable roles of enkephalin and beta-endorphin in driving licking bout numbers and bout lengths, respectively (Mendez et al., 2015; Ostlund et al., 2013). It should be noted that the incentive motivational and hedonic properties of stimuli are likely to be closely linked under most circumstances, and large changes in hedonic value may permeate to changes in incentive value, as “liked” stimuli are typically “wanted” stimuli. However, the connection between hedonic impact and motivation can indeed be dissociated, such as in pathological states like addiction or under specifically engineered experimental conditions (Berridge, 2009; Nogueiras et al., 2012; Pecina and Smith, 2010).

Findings from this study show that the role of endogenous NOC signaling in supporting the motivation to engage in new bouts of licking is statistically evident over several days of analysis (as demonstrated by lower bout numbers in NOP KO mice during training with sucrose, Figure 1B), but is less readily apparent when assessed at single time points (as demonstrated by non-significant decreases in bout number in NOP KO mice during rebaseline and test days, Figures 2B, 3B, 4B). Additionally, the observed attenuated NOP KO licking bout numbers have been observed in other studies from our lab (unpublished results), supporting the consistency of these effects. When NOPs are activated by experimenter administered NOC, significantly more bouts of licking during single test sessions are induced (as demonstrated in wildtypes when licking for sucrose when sated, Figure 2B, and sucralose following 20 h of food deprivation, Figure 4B). As mentioned, a general hyperphagic effect of exogenous NOC administration has been reported many times within the literature (Micioni Di Bonaventura et al., 2013; Polidori et al., 2000b); however, we believe this is the first time the effects of exogenous NOC on specific licking microstructures have been reported. Together, these findings reveal a role for NOC in initiating new bouts of licking for sweet solutions and suggest that NOP activation promotes motivational aspects of appetitive behavior.

Results from our study also suggest that the role of NOC in motivation may depend on the energy content of the food stimulus. This is concluded because, when tested sated, NOC infusions significantly increased total bouts of licking for calorie-dense sucrose (Figure 2B), but not the non-caloric sweetener sucralose (Figure 4B). Artificial sweeteners, such as sucralose, are often weak instigators of behavior, and the inability of NOC to elicit statistically significant increases in licking measures when sated may stem, at least in part, from the fact that sucralose is inherently of meager incentive motivational and hedonic

value. However, it may also be argued that the relative lack of effect observed during sated licking for sucralose occurs because the concentrations of sucrose and sucralose used in these experiments are not equally optimal for inducing licking behavior in mice. Notably, a previous study using a home cage, two-bottle preference test showed that while both concentrations are preferred over water in C57BL/6J mice, 20% sucrose induces maximal preference and fluid intake, whereas 0.01% sucralose does not (Bachmanov et al., 2001). That said, previous findings from our laboratory, suggest that the 0.01% sucralose concentration used in present study does indeed induce maximal licking behavior in mice (Ostlund et al., 2013).

Interestingly, however, when our mice were hungry and licking for sucralose, bouts of licking were significantly higher following NOC infusions compared to VEH infusions (Figures 4B). Overall, these data suggest that while the orexigenic effect of exogenously applied NOC is insufficient to induce notable increases in the number of licking bouts for the non-caloric sweetener sucralose, it is when combined with it is combined with the orexigenic effect of hunger. Future, more detailed, studies could assess licking behavior in these mice over a range of tastant concentrations.

Regarding the role of NOC signaling in the hedonic aspects of feeding, activation of NOPs following administration of exogenous NOC infusions did not appear to affect bout lengths when licking for sucrose or sucralose (Figure 2C and 4C, respectively). However, when looking at the effects of hunger following vehicle i.c.v. infusions, licking bout lengths were increased in NOP KO mice relative to wildtypes, when licking for sucrose (Figure 3C). While this hunger-induced increase in bout length (but not number of bouts) in NOP KOs has been observed in other studies in our lab (unpublished data), suggesting that this is a robust effect, the mechanisms and functionality of this phenomenon remain to be investigated. Based on the proposal that bout lengths reflect hedonic aspects of feeding, this observation would suggest that during states of high caloric need, endogenous NOC acts to attenuate hunger-induced increases in the hedonic impact of food rewards. Conversely, however, one recent study using NOP KO mice found no evidence of a role of endogenous NOC in affective (i.e. hedonic) reactions to forced administration of sucrose (Koizumi et al., 2009). Additionally, while some researchers have found little evidence that exogenous NOC regulates intake of preferred diets or macronutrients (Olszewski et al., 2002), others have observed decreases in the consumption of specific preferred diets in hungry NOP KO mice, suggesting that endogenous NOC is actually involved in increasing the hedonic impact of food (Koizumi et al., 2009).

Indeed, these conflicting findings prompt consideration of alternative theories to that of NOC affecting hedonic processing of food rewards. Interestingly, studies elsewhere have suggested that NOC may modulate systems controlling meal cessation (Bomberg et al., 2006; Farhang et al., 2010). For example, NOC signaling is increasingly becoming implicated in the inhibition of hypothalamic arcuate nucleus neurons expressing α -melanocyte-stimulating hormone, beta-endorphin and the anorexigenic precursor polypeptide, pro-opiomelanocortin (POMC), all of which have been implicated in signaling meal pattern, and particularly, meal termination (Farhang et al., 2010; Wagner et al., 1998).

Thus, prolonged consummatory episodes (e.g. bouts of licking) in NOP KO mice may indeed occur as a result of lost NOC regulation of meal cessation signals.

In addition to examining the role of NOC signaling in licking for rewarding food stimuli, this study also examined the role of endogenous NOC signaling in processing of aversive food stimuli, by assessing licking behavior for sucrose adulterated with the bitter tastant quinine. Our previous studies using affective taste reactions, where mice were exposed to quinine involuntarily, found increased aversive reactions to quinine alone when presented at 0.3 mM concentration and reduced reactions when presented at 3 mM, in NOP KO mice relative to wildtypes (Koizumi et al., 2009), suggesting a role for endogenous NOC signaling in the processing of aversive stimuli. Findings here, using a voluntary lick assay, show that wildtype and NOP KO mice show similar downshifts in lick responding for sucrose when adulterated with 3 mM quinine. This effect was reflected particularly in bout length, suggesting that, as expected, quinine adulteration reduces the hedonic impact of sucrose. Taken together, these findings suggest that while endogenous NOC signaling may be important for appropriate taste reactions following involuntary exposure to aversive solutions, NOC signaling plays little role in mediating voluntary licking responses to aversive solutions.

NOC neurotransmission is observed in brain regions implicated in consummatory behavior, and several lines of evidence support a role for NOC signaling (either directly or indirectly) in both the physiological and psychological processes driving feeding. For instance, studies have found that both food-restricted rats and those consuming less due to experimentally-induced conditioned taste aversion, display reduced mRNA expression for NOP in the hypothalamus, the primary brain region regulating food intake driven by caloric and nutritional need (Olszewski et al., 2010; Rodi et al., 2002; see Witkin et al., 2014 for further discussion). Similarly, it has been shown that in food deprived rats, NOP is down regulated in the dorsal raphe nucleus, a region sensitive to the “hunger hormone” ghrelin (Carlini, 2004; Przydzial et al., 2010). Regarding the psychological aspects of feeding, studies have identified relevant NOC signaling in limbic regions associated with conditioned responding for food (e.g. amygdala, hippocampus), as well as in areas involved in the “liking” and “wanting” of food (e.g. dorsal and ventral striatum) (Caputi, 2014; Goeldner, 2008; Redrobe, 2000; Rodi et al., 2002). Interestingly, Caputi et al (2014) demonstrated that NOC and NOP gene expression are changed in the caudate putamen and nucleus accumbens following repeated exposure to a drug reward. Specifically, in the ventral striatum, an area implicated in the incentive motivation for food rewards (DiFeliceantonio et al., 2012; Palmiter, 2008), it was showed that both NOC and NOP gene expression were decreased following repeated reward exposure (Caputi, 2014).

In addition to the role of NOC in central systems, NOC has been also been found to affect feeding mechanisms in the periphery. For example, NOC is found in the gastrointestinal tract, where it contributes to bowel homeostasis by affecting secretion and motility (Agostini and Petrella, 2014; Sobczak et al., 2013). Gut alterations resulting from NOP deletion may indeed lead to intestinal pathologies that suppress feeding behavior, including voluntary drinking of sweet solutions. Similarly, NOC has been shown to increase body weight gain by altering white adipose tissue storage and energy expenditure, as well as plasma levels of

leptin, insulin, and cholesterol (Matsushita et al., 2009). Thus, it is possible that increases in licking bout lengths observed in NOP KO mice when hungry, results from impaired energy balance or peripheral signaling following food deprivation.

There are numerous other factors that should be considered in the interpretation of the results of this study. For example, while i.c.v. infusions can quickly deliver agents directly into the central nervous system, it is unknown how far, how quickly, and in what amount such agents penetrate (see Murphy, 2015 for discussion). A second factor that should be considered is a possible role of NOC in neophobia. Studies using both NOP KO mice and NOP antagonists have pointed to such a role, particularly in hungry states (Farhang et al., 2010; Hadjimarkou et al., 2004; Olszewski et al., 2010; Statnick et al., 2014; Witkin et al., 2014). Indeed, we too have observed that if initial exposure to 10% sucrose occurs following high food deprivation (20 hrs), NOP KO mice emit vastly fewer (approximately 80%) total licks across a 30-minute session relative to wildtype mice (unpublished). Therefore, the reduced licking responses for sucrose observed during training under mild food deprivation here (4 h) may have been driven, at least in part, by the absence of an endogenous NOC tone that normally acts to overcome neophobic responses.

There are numerous other factors to be considered in the interpretation of this, and other studies, relating to the role of NOC in appetitive and aversive behaviors (see Chiou et al., 2007; Witkin et al., 2014 for review). These include known roles of endogenous NOC in anxiety (Blakley et al., 2004; Jenck et al., 1997; Koster et al., 1999; Zhang et al., 2015), stress-reactivity (Ciccocioppo et al., 2014; Zhang et al., 2015), pain sensitivity (Chen et al., 2002; Suaudeau et al., 1998), maintenance of body temperature (Blakley et al., 2004; Yakimova and Pierau, 1999), water balance (Blakley et al., 2004; Burmeister and Kapusta, 2007), and growth (Farhang et al., 2010). While a detailed discussion of how NOC signaling in central and peripheral systems affects these factors is beyond the scope of this paper, it is indeed evident that the role of NOC in regulating feeding behavior is vast and intricate, meriting further investigation.

For decades, the family of opioid-like peptides and receptors has been implicated in psychobiological mechanisms driving reward, and particularly, feeding behavior. Despite this well-established role, detailed information about which peptides control which processes are lacking. In this study we investigated the role of the opioid receptor NOP and its selective endogenous agonist, NOC. Despite the complexity of the effects of NOC, the experiments reported here demonstrate that NOC signaling supports the incentive motivational properties of palatable tastants, and likely has ramifications in many types of appetitive and consummatory behaviors. Additionally, while observed increases in the sucrose licking bout lengths of hungry NOP KO mice suggest that endogenous NOC is involved in decreasing the hedonic properties of food, a role for endogenous NOC in meal cessation signaling may also underlie our observed decreases in NOP KOs licking bout lengths. These findings, and the complexities of the relationship between incentive motivational and hedonic processes, encourage further study of the role of NOC neurotransmission in the psychobiological aspects of feeding behavior. Considering the remarkable increase in obesity and diet related disease over the last 50 years, continued clarification of the role of NOC neurotransmission (and opioid signaling in general) in

feeding behavior and energy regulation will be particularly pertinent for developing therapies aimed at controlling aberrant eating disorders and obesity in general.

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Figure 1.

Acquisition of licking behavior, before and after cannula implantation surgery, for a 20% sucrose concentration solution, following 4 hours of food deprivation. Measures include A) total licks, B) total bouts of licking, and C) mean bout length. Data are presented as mean $\pm$ S.E.M. Plus sign (+) indicates significant main effect of genotype.

Figure 2.

Effects of i.c.v. infusions of vehicle or 1 nM of nociceptin on licking for 20% sucrose, following 0 hr or 20 hr of food deprivation. Measures include A) total licks, B) total bouts of licking, and C) mean bout length. Data are presented as mean + S.E.M. Asterisk (*) indicates significant post-hoc comparison.

A) Sucrose + Quinine **B) Sucrose + Quinine** **C) Sucrose + Quinine** **Figure 3.**

Licking for 20% sucrose or 20% sucrose plus 3 mM of quinine, following 0 hr or 20 hr of food deprivation. Measures include A) total licks, B) total bouts of licking, and C) mean bout length. Data are presented as mean + S.E.M. Asterisk (*) indicates significant post-hoc comparison.

Figure 4.

Effects of i.c.v. infusions of vehicle or 1 nM of nociceptin on licking for .01% sucralose, following 0 hr or 20 hr of food deprivation. Measures include A) total licks, B) total bouts of licking, and C) mean bout length. Data are presented as mean + S.E.M. Asterisk (*) indicates significant post-hoc comparison.