

Optogenetic Inhibition of Lateral Hypothalamic Inputs into Ventral Pallidum Amplifies Aversive
'Disgust'

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Abstract

The ventral pallidum (VP) is a brain structure within the basal ganglia that is involved in the regulation of motivation, behavior, and emotions. VP has also been found to play a special role in generating the hedonic impact (‘liking’) of food and other rewards. However, this ability is anatomically restricted to a roughly cubic millimeter ‘hedonic hotspot’ in its posterior half, and has shown to be activated by opioid or orexin microinjections. Previous work has shown that selective optogenetic stimulation of lateral hypothalamic (presumably orexinergic) inputs into the VP hotspot amplifies ‘liking’ to sucrose solutions, demonstrating the sufficiency of this pathway to recruit the VP hotspot and alter ‘liking’. Here, we explored the necessity of this LH-VP pathway in hedonic processing by infusing an inhibitory optogenetic virus (hsyn-NpHr3.0-mcherry) and implanting optic fibers into the VP hotspot to selectively knock out this pathway during taste reactivity and food intake behaviors. It was found that inhibition of the LH-VP pathway caused an increase in the number of aversive ‘disgust’ reactions to a bitter quinine solution while leaving hedonic or aversive reactions to palatable sucrose unchanged. ‘Wanting’ for palatable M&M candies was also not affected by LH-VP inhibition. These results indicate that LH may be involved in mediating baseline hedonic tone, such that disruption of its inputs into the hotspot may unbalance how VP processes unpalatable and disgusting stimuli.

Keywords: lateral hypothalamus, ventral pallidum, optogenetics, hotspot, coldspot, disgust

Optogenetic Inhibition of Lateral Hypothalamus Inputs into Ventral Pallidum Causes Increased Aversion

Clinical Applications of 'Hedonia'

Chronic conditions such as depression and addiction have been found to involve aberrations in motivated and hedonic neural circuits, the word 'hedonia' meaning pleasure. "Food and sex are potent sensory pleasures with liked hedonic impact, and it is widely acknowledged that the 'liking' of food and sex carries important survival and reproductive benefits for humans and animals" (Berridge & Pecina, 2006). These benefits can sometimes exceed moderation and those foods with larger hedonic impact are consumed more than those with lower hedonic impact. This can lead to disease: "hedonic liking for sensory pleasures is an important aspect of reward and excessive 'liking' of particular rewards might contribute to excessive consumption and to disorders such as obesity" (Berridge & Pecina, 2006). If the pleasure circuits and systems in the brain can be understood, diseases such as obesity and depression can be minimized and potentially eliminated.

Introduction to Incentive Sensitization Theory

There are three components to reward: affective valence, incentive value, and learning (Berridge, Robinson, & Aldridge, 2009). Together, they modulate how individuals respond to rewarding and aversive stimuli. However, though these three components often occur in tandem, the neural mechanisms underlying each process can be dissociated (Robinson & Berridge, 1993). 'Liking', or the hedonic aspect of reward, is associated with pleasure generated by the brain (Berridge & Kringelbach, 2008). Areas responsible for taking sensory information and overlaying an affective gloss have been termed 'hedonic hotspots' (Berridge & Kringelbach, 2012). Two areas in the brain that contain hedonic hotspots are nucleus accumbens (NAc) and

ventral pallidum (VP). 'Wanting', or incentive salience, is the motivation to obtain the reward (Berridge & Kringelbach, 2012). In contrast to the very localized 'liking' systems found in the brain, the neural systems that underlie 'wanting' are generally more broad and extensive (Berridge & Kringelbach, 2012). Pleasure and reward are extremely relevant to our understanding of the brain and its relationship to thoughts, feelings, senses, and other processes. How exactly are we able identify these brain structures and study pleasure in rat models?

Taste reactivity testing is a laboratory technique used by affective neuroscientists to measure the palatability and hedonic impact of taste stimuli. This behavioral test can then be coupled with various brain manipulations to determine which brain areas are involved with generating affective valence (Berridge, 2000). The taste reactivity test measures and classifies ('liking' versus 'disgust' versus neutral) orofacial reactions to solutions such as sucrose and quinine. This test has very important implications for humans, for affective facial reactions made by non-human primates and/or rats are similar to ones made by human infants. "Such 'liking' enhancements can be measured via increases in positive affective orofacial reactions elicited by sweetness, which are homologous in rats, monkeys, apes, and humans" (Grill and Norgren, 1978; Berridge, 2000; Steiner et al., 2001; Ho & Berridge, 2013). Hedonic or positive reactions are considered to be a result of 'liking', while aversive or disgust reactions are a result of 'disgust'. This method has been used to functionally define which brain areas are involved in 'liking'; areas that are capable of amplifying hedonic reactions to a palatable taste stimulus are termed hedonic hotspots.

A prime example that dissociates the concepts of 'liking' and 'wanting' involves studies that compare manipulating the VP and NAc hotspots and surrounding areas. Pecina and Berridge (2005) injected a mu opioid receptor agonist (DAMGO) into the nucleus accumbens and found

that subjects (rats) had increased motivation ('wanting') to eat. This was true of all microinjection sites in NAc. However, the only site in which DAMGO microinjections likewise enhanced the hedonic impact of a sucrose taste was in the rostradorsal quadrant of the medial shell. "Opioid-generated 'liking' mechanisms are restricted to a single cubic millimeter site localized in the rostradorsal quarter of the medial shell of the nucleus accumbens" (Pecina & Berridge, 2005). This shows that 'liking' mechanisms do not have to activate in concurrence with mechanisms of 'wanting'. Outside this hotspot, DAMGO microinjections leave sucrose reactions unchanged or even suppressed, despite the effect of food intake (Pecina & Berridge, 2005).

Similarly, the ventral pallidum, a structure within the basal ganglia of the brain involved in conditioned place preference and drug self-administration, contains abundant mu opioid receptors in its posterior half (Smith & Berridge, 2005; Waraczynski & Demco, 2006;). "Increase in opioid transmission in the ventral pallidum is sufficient to enhance hedonic 'liking' reactions to sucrose as well as motivational 'wanting' to eat, but only in a restricted subregion of posterior ventral pallidum" (Smith et al., 2009). These findings are almost identical to ones found in NAc, and again the 'wanting' and 'liking' mechanisms can be separated.

In contrast to the hotspots, neuratomically localized hedonic 'coldspots' have also been found in NAc and VP. In these regions, intense suppression of hedonic reactions to sucrose occurs after opioid stimulation. The coldspots are found near the hotspots, but are qualitatively different anatomical zones. For example, the NAc hedonic hotspot is localized to the rostradorsal quadrant of the medial shell, whereas the NAc coldspot is located in the caudal half. Likewise, the ventral pallidum hedonic hotspot is contained in the posterior one-third, while the coldspot has been located in the anterior VP.

In summary, this past research has shown that injections of drugs that boost mu-opioid neurotransmission can dramatically increase consumption of palatable food, while opioid drugs also increase taste hedonic reactions to sucrose after injection into localized hedonic hotspots (Parker and other 1992; Peciña and Berridge 2006). These hedonic hotspots are only a cubic millimeter in size and behavioral techniques such as taste reactivity and food intake have revealed the affective value of tastes and have helped identify the existing aforementioned hotspots. If mu-opioid activity is subsequently blocked in these brain areas, consumption and incentive can be reduced.

The existence of multiple hotspots allows for the existence of a potential 'hedonic circuit'. To determine whether the NAc and VP hotspots work together to augment hedonic impact, Smith and Berridge utilized the above techniques to discover that VP and NAc do indeed interact with one another (Smith & Berridge, 2007). Stimulation of the VP hotspot can cause activation in the nucleus accumbens and vice versa (Smith & Berridge, 2007). Measuring this activation was accomplished by activating one hotspot (e.g. NAc), and quantifying c-Fos expression in the other hotspot (Smith & Berridge, 2007). C-Fos, a protein located in the cell nucleus, can be used as a marker of cell activation. "Expression of c-Fos, or other immediate early gene products, by individual neurons can be used a marker of cell activation, making staining of these proteins an extremely useful technique for functional anatomical mapping of neuroendocrine systems" (Hoffman, Smith, & Verbalis, 1993). They found that when one hotspot was activated, there was an increase in Fos expression in the other, indicating that these hotspots likely work together, or at the very least, recruit the other hotspot once activated (Smith & Berridge, 2007). Also, opioid blockade in either VP or NAc hotspot prevents DAMGO

enhancement of positive 'liking' reactions in the other hotspot (Smith & Berridge, 2007). These results show that the hotspots require unanimous activation in order to augment hedonic impact.

The hedonic hotspots of both ventral pallidum and nucleus accumbens contain receptors for the peptide neurotransmitter orexin, involved in regulating arousal, wakefulness, and appetite (Ho & Berridge, 2013). Due to this overlap between the opioid hotspot and orexin receptors, it is possible that orexin may amplify 'liking' reactions as well. Ho and Berridge (2013) tested this hypothesis by microinjecting orexin into caudal VP. They found that orexin stimulation enhanced hedonic reactions to sucrose in a similar way as opioids. Similarly, Terry, Castro, and Berridge (2014) showed that orexin microinjections into the NAc hotspot also enhances 'liking'. These findings implicate orexin in hedonic circuitry and affect.

Although the NAc and VP possess a functional relationship, they do not have any known direct reciprocal anatomical connections. The lateral hypothalamus (LH), part of the diencephalon involved in hunger and reward, has direct reciprocal connections with all of the hotspots – putting it in a good position to mediate their activity (Berridge, 2000). Additionally, the lateral hypothalamus is the only brain area containing neurons that release orexin, the same neurotransmitter found to increase 'liking' reactions to sucrose upon microinjection into VP and NAc hotspots. The LH/orexin system has also been shown to project to a number of brain areas, including VP and NAc (Peyron et al., 1998; Baldo et al., 2003). Due to the existence of the orexin hotspot in VP, it is likely that the VP receives hedonically relevant signals from the lateral hypothalamus.

Optogenetics

In order to manipulate brain structures, many neuroscientists employ the use of a relatively new technique termed optogenetics. Optogenetics is a neuromodulation technique that

controls the activities of individual neurons in living tissue. It can be used for point-to-point stimulations or inhibitions. This technique can even be employed within freely moving animals to alter behavior, and the effects of those manipulations can be measured in real-time (Feng & Miesenbock, 2006). This tool removes genes from specific types of algae and infects neurons with a viral vector. The neurons are then forced to express the genes coding for photoreceptors, which causes them to be sensitive to particular wavelengths of light. The genes are transcribed and the photoreceptors are inserted into the cell membranes. Once the photoreceptors are activated, it causes the opening of sodium/chloride channels, thereby making them excitatory or inhibitory in nature. In this experiment, we used AAV5-hsyn-NpHr3.0-mcherry virus (inhibitory) that contains RNA instructions for building photoreceptor molecules. Upon green laser light photostimulation (532nm), the proteins allowed chloride ions to enter the cell causing hyperpolarization (inhibition) of the neurons. Furthermore, this technique allows for instant transformations of behavior, which are useful for within session manipulations.

Ventral Pallidum Disgust

While excessive 'liking' is extremely important clinically, excessive disliking can have just as crucial implications medically, psychologically, and socially. "In the absence of positive hedonic impact, either anhedonia or even dysphoria as excessive negative affect can characterize certain clinical disorders ranging from major depression to obsessive compulsive disorder (OCD)" (Ho & Berridge, 2014). In particular, excessive 'disgust' has been suggested to be associated with human anxiety disorders, phobias, anorexia nervosa as well as with OCD (Sprengelmeyer et al., 1997; Cisler et al., 2009; Olatunji et al., 2010; Weygandt et al., 2012). The ventral pallidum has been consistently linked with aversion, for Cromwell and Berridge (1994) found that damage to the VP causes aphagia and aversion, while damage to LH only produces

aphasia. In a similar experiment done by Ho and Berridge (2014), results indicated that posterior ventral pallidum lesions and/or temporary inactivation caused intense disgust reactions to palatable solutions such as sucrose. Curiously, they also found that, “adjacent to ventral pallidum, neither lesions nor inactivations in lateral hypothalamus or extended amygdala regions induced disgust as long as the posterior ventral pallidum was spared” (Ho & Berridge, 2014).

These findings suggest that the integrity of the VP itself is necessary for ‘liking’. Others have sought to understand if neuronal inputs into VP can mediate ‘liking’ and aversive reactions. As previously mentioned, there exists an orexin hedonic hotspot in VP in its posterior half. The VP receives orexin projections from lateral hypothalamus and orexin terminals are extremely dense in its posterior half, curiously the same location as the previously identified opioid hedonic hotspot (Ho & Berridge, 2013). It was found that, “Orexin stimulation, especially in the VP hotspot, nearly doubled the magnitude of positive ‘liking’ reactions elicited by the taste of sucrose” (Ho & Berridge, 2013). Furthermore, Castro and Berridge (2015) found that optogenetic channelrhodopsin stimulation of intrinsic VP neurons selectively doubled sucrose ‘liking’ reactions alone but did not alter ‘wanting’. Stimulation of intrinsic LH neurons produced the opposite effect – alteration of ‘wanting’ but not ‘liking’. Stimulation of the LH-VP neuronal connections altered both ‘liking’ and ‘wanting’, showing that LH to VP projections are sufficient to alter hedonic impact (Castro & Berridge, 2015). The LH is recruiting the VP to hedonically enhance sweetness.

The question remains as to whether these projections are necessary for normal ‘liking’. When the VP is “offline”, we see excessive ‘disgust’, and we know that the LH seems to be able to modulate VP, so is the LH necessary to keep VP “online”? I studied what would happen if this connection were inhibited and how that would alter hedonic impact. We posit that the LH-VP

hotspot inhibition may potentially increase 'disgust' reactions to bitter and unpleasant foods, such as quinine. It is possible that turning off the pathway leaves only the coldspot available, which in turn may cause food and drink stimuli to taste worse than usual.

Methods

Subjects

Male and female Sprague Dawley rats (male $n = 7$, female $n = 2$) were pair-housed by sex at 21° C on a 12-hour light/dark cycle. Rats had *ad libitum* access to water and food. Males weighed between 300-600 g while females weighed between 240-400 g. All experimental procedures were approved by the University of Committee on the Use and Care of Animals at the University of Michigan.

Surgery

Virus injection. All animals were handled prior to surgery and experiments were run with 2-3 animals at a time. Each rat was anesthetized with ketamine hydrochloride (80mg/kg, i.p.) and xylazine (5mg/kg, i.p.) in the same syringe. The animal was also given atropine (.05mg/kg, i.p.) to prevent respiratory distress, and sodium chloride (2ml) to prevent dehydration. The rat was placed in a stereotaxic apparatus (David Kopf Instruments) and received bilateral microinfusions of either the inhibitory virus AAV5-hsyn-NpHr3.0-mcherry ($n = 5$) or a control virus lacking the photoreceptive opsin (AAV5-hsyn-mcherry; $n = 4$) into the mid-tuberal portion of lateral hypothalamus (AP, -3.0; ML, +/-1.8; DV, -8.4). Animals receiving virus microinjections were infused with .75ul/side at a rate of 0.075/1 m for 10 minutes, immediately followed by a 10-minute waiting period to allow for virus diffusion. Surgical staples were used to close the initial cut, and recovery time was 5 weeks. Rats were also given

subcutaneous injections of carprofen (5mg/kg) for analgesia and cefazolin (60mg/kg) as an antibiotic immediately after surgery.

Oral cannulae. A second surgery was done approximately 5 weeks after the first in order for the virus to have optimal expression. The animals were again anesthetized with ketamine hydrochloride (80mg/kg, i.p.) and xylazine (5mg/kg, i.p.), and atropine (0.05mg/kg, i.p.). Bilateral oral cannulae [polyethylene (PE)-100] were first implanted to allow direct infusions of sucrose or quinine for taste reactivity testing. The first maxillary molar is used as marker for horizontal implantation of the needle, followed by ascending beneath the zygomatic arch, and exiting the skin above the dorsal head cap. Excess tubing was cut and bilateral oral cannulae were placed and wrapped with wire.

Optic fibers and headcaps. Optic fibers had 230um diameter cores and were inserted into 9mm long zirconia ferrules. Fibers were tested before and after experimentation to ensure fiber integrity. After implantation of oral cannula, rats were again placed into a stereotaxic apparatus and received bilateral implants of 230um optic fibers into ventral pallidum hotspot (AP, -0.8; ML, +/-3.0; DV, -7.8). The surgical site was sealed by acrylic cement and a headcap was fashioned so that fibers and cannulae were held in place. Again, rats were given subcutaneous injections of carprofen (5mg/kg) and cefazolin (60mg/kg) immediately after surgery. Recovery time for the surgery was approximately 7-8 days and normal eating was not disrupted by oral cannulae implantation.

Taste Reactivity Testing

In order for the rats to grow accustomed to the testing conditions and chamber, 3 days of habituation were held. During all habituation and testing days, rats had *ad libitum* access to food and water, and fibers were hooked up to optic cables attached to a doric rotary joint (the laser

apparatus was not turned on unless otherwise noted). Each rat underwent two testing days, each with a different condition: laser and no laser. Laser inhibition of VP hotspot was administered at a constant illumination ($\sim 2\text{-}4\text{mW}$) for the entire taste reactivity test (LASERON) during laser test days. Testing occurred during 1-min infusions of sucrose or quinine administered during both test days. To infuse sucrose solution into the mouth, a syringe containing sucrose (1.0%, 0.029 M, 1ml per test) was placed into a syringe pump. Hollow tubing (PE-50 connected to a PE-10 delivery nozzle) was attached to the syringe by the PE-50 end and attached to the rat's oral cannulae by the PE-10 end. A 1 ml volume of sucrose was infused over a period of 1 minute and orofacial taste reactivity responses were video recorded via close-up lens and an angled mirror placed underneath the transparent floor for subsequent slow-motion video analysis. Infusing quinine ($3 \times 10^{-4}\text{M}$, 1ml per test) into the mouth was done in a similar manner five minutes after infusion of sucrose. Again, orofacial reactions were recorded and later scored.

Taste Reactivity Video Scoring

The recorded video sessions of taste reactivity testing were uploaded to Noldus Observer®. This program allows one to classify rat orofacial reactions into aversive, hedonic, or neutral categories. Hedonic behaviors include tongue protrusions, paw licking, and lateral tongue protrusions. Aversive behaviors are face washing, forelimb flailing, head shaking, gaping, and chin rubbing while doing nothing, mouth movements, passive dripping, and yawning are all neutral behaviors. The video speed was slowed down significantly in order for accuracy of classification. Behaviors were separated into an overall, overarching state that lasts more than a few seconds such as "Doing Nothing" or "Passive Dripping", and more specific, instantaneous behaviors such as "Gape" or "Head Shake".

A time-bin scoring procedure was used to ensure that hedonic and aversive responses that occur at different frequencies still contribute equally in the final affective response totals. Behaviors classified as states occurred for relatively long periods of time so they are separated into bouts. If the rat for example, showed tongue protrusions for 30 seconds, we would count it as 15 bouts rather than 30. Longer behaviors like rearing, mouth movements, passive dripping, and paw licking were all scored in 5 s time bins. Rhythmic midline tongue protrusions and chin rubs, which occur in shorter bouts, were scored in 2 s time bins. The remaining behaviors listed above were counted as is, for if a rat gaped 3 times it would count as 3 occurrences. Individual totals were then automatically calculated in order to compare the sum of hedonic reactions vs. aversive ones.

Food Intake Testing

Following taste reactivity testing, rats remained in the chamber with fibers hooked up to laser cables (laser only on during laser test day). The rats were given a pre-weighted amount of palatable chocolates (M&Ms, ~25g) and were able to voluntarily eat as much as desired for an hour. All activity was video recorded via close-up lens and an angled mirror placed underneath the floor, as in taste reactivity. During the laser ON day, the setting used was 10 mins ON, 10 mins OFF to prevent over-activating the photoreceptive opsins. After the 1-hour period expired, the remaining M&Ms were weighted and amount eaten was calculated. All behavior was recorded during the 60 min test, and scored later offline for video analysis by a researcher blind to the drug microinjection condition. Videos were scored for eating behavior (duration in seconds), water-drinking behavior (in seconds), grooming behavior (in seconds), and for number of bouts of food sniffs, food carrying (grasping and transport of food by 2 or more steps), cage crosses, and rears.

Histology

Following the two test days, animals were returned to the original testing chamber and given a final 30-minute training session in the presence of laser stimulation. After this 30-minute period, rats were deeply anesthetized with an overdose of sodium pentobarbital and perfused. Brains were stored in 4% paraformaldehyde, cryoprotected in 30% sucrose, and then sliced at 40 micrometers. Sections were mounted, air-dried, and coverslipped with ProLong Gold antifade reagent (Invitrogen). In order to assess viral spread and location, and fiber tip placement, relevant sections were examined using a Leica microscope and results were marked on a coronal schematic in Adobe Illustrator using the rat brain atlas (Paxinos & Waxton, 2006).

Statistical Analysis

A comparison between all the data in order to determine an overall affect was accomplished by using a Freedman's ANOVA test. A Wilcoxon Signed-Rank Test compared hedonic, aversive, and food intake across test days, in a fashion analogous to a paired T-test. Finally, the Mann-Whitney U test determined if there is significance in data between control and experimental subjects (rats). The significance level for all data was set at 0.10.

Results

Sucrose

The Freedman's ANOVA showed no overall effect of the virus ($X = 1.667, p = 0.197$). This is expected, for the proposed effect was limited to one variable (quinine) and was masked amongst the remaining data. To determine whether such a masking effect occurred, we investigated whether the laser had an effect on hedonic or aversive reactions to sucrose or quinine. Hedonic reactions to sucrose in halorhodopsin injected animals remained consistent

across both laser and non-laser test days ($Z = -1.604, p = 0.109$). Negative reactions to sucrose also did not change ($Z = -1.342, p = 0.18$). Furthermore, the laser did not have any effect on control animals in comparison to experimental animals in either hedonic or aversive reactions to sucrose ($Z = -0.124, p = 0.905$; $Z = -1.073, p = 0.413$). This statistical analysis was calculated by comparing the net gains or losses in sucrose reactions across the two groups. It was done separately for hedonic reactions to sucrose and aversive reactions to sucrose. This finding suggests that optogenetic inhibition of the lateral hypothalamus to ventral pallidum is not directly involved in stimulating orofacial reactions to sucrose. Rats therefore did not 'like' the sucrose any less or more when this pathway was inhibited.

Quinine

Positive reactions to quinine were non-existent during both laser and non-laser test days ($Z = 0.00, p = 1.00$). Mann-Whitney U tests between NpHr and control animals confirmed this finding ($Z = -0.0, p = 1.0$). Negative reactions to quinine did elicit a statistically significant effect after comparing laser and non-laser test days ($Z = -1.753, p = 0.080$). During laser test days, 'disgust' to quinine was increased almost 2 fold and we believe that the p-value would have been considerably lower if the sample contained greater than 5 subjects. Comparison of aversive reactions to quinine between control and halo animals also indicated a statistically significant effect ($Z = -1.845, p = 0.063$). This was calculated by taking the number of aversive reactions to quinine on the non-laser test day and subtracting that value from the number of aversive reactions to quinine on the laser test day. This gave us a net gain or loss in aversive reactions, which we compared across control and halo animals. Therefore, halo and control are not different in terms of effect (using difference scores), except for reactions to 'disgust'. Halo animals significantly dislike quinine more relative to controls. This indicates that laser inhibition

of LH terminals in the VP hotspot is sufficient to increase negative orofacial reactions prompted by the bitter taste of quinine. More specifically, all aversive behaviors such as gaping and chin rubbing increased in response to photo inhibition. By inhibiting the LH-VP pathway, it is indeed increasing 'disliking' and not causing a reflexive or stereotyped motor effect.

Food Intake

Statistical analysis of food intake data showed no change in eating over both test days ($Z = -0.674, p = 0.500$). In addition, comparisons between halo and control animals indicated that virus did not have an effect on food intake ($Z = -0.735, p = 0.556$). Overall, food intake was not affected by laser photoinhibition of the LH-VP pathway. Therefore, the 'wanting' aspect of reward was unaffected and rats were not motivated to eat in excessive amounts, even though stimulation of this same pathway is sufficient to enhance food intake.

Discussion

Increased Quinine Aversion

We found that optogenetic inhibition of lateral hypothalamic projections to the VP hotspot increased quinine 'disgust'. These findings were similar across both male and female rats. The virus immunofluorescence was localized to mid-tuberal lateral hypothalamus, and fiber placements were found in posterior ventral pallidum. This provides evidence that the LH-VP connection is involved in mediating affective reactions to aversive stimuli.

What mechanism might account for the selective increase in aversive 'disgust' reactions? By inhibiting the LH to VP pathway, neuronal inputs from the LH into the VP are eliminated. However, the VP hotspot is still intact and functional, and the mechanisms that mediate 'liking' are not directly being affected. Therefore, rather than directly affecting hedonic processing, we

may be more subtly biasing the hedonic system towards aversion by making bad stimuli worse. Our results, in conjunction with the LH-VP stimulated enhancement of hedonic 'liking' described above, potentially implicates the LH-VP connection in modulating affective responses.

One endogenous mechanism for this pathway would include hunger alliesthesia, in which food tastes better when an individual is hungry. Orexin is upregulated during food deprived states, and down regulated upon food administration. Our results in particular may be tapping into the opposite phenomenon, satiety. The idea that LH may be important for flexibly altering affective responses to homeostatic states stems from studies of decerebrate rats in the late 1970's, in which cutting of the brain at the brainstem leaves only the brainstem alive (Grill & Norgren, 1978). These decerebrate animals also show normal 'liking', although they do not show hunger alliesthesia or learned taste aversions. The flexibility of pleasure is lost with decerebrate animals and this is likely because both the hot and coldspots were gone in VP. In our study, the hot and coldspots remained intact. It is therefore possible that the VP coldspot may be playing a crucial role in this increased 'disgust'. The coldspot, located in anterior ventral pallidum is in a perfect position to mediate this response. "Negative suppression of 'liking' and 'wanting' is produced if the same DAMGO microinjections are made in a more anterior coldspot" (Kringelbach & Berridge, 2009). Further, Calder and colleagues found that appetizing pictures of foods activated posterior ventral pallidum while repulsive images activated the anterior coldspot (Calder et al., 2007). The VP coldspot and hotspot may therefore be interacting in such a way that 'liking' and 'disgust' are tempered and balanced by LH inputs into the hot and coldspots. Inhibition of the LH-VP connection is removing the tempering of aversion that is normally intact.

The data indicates that the ventral pallidum plays a crucial role in this phenomenon, which is reinforced by previous studies that have implicated VP in disgust. In the paper by Ho and Berridge (2014), localized dysfunction (lesions or neural inactivation) was found to induce intense orofacial ‘disgust’ reactions to a once pleasurable stimulus. Other well-known hedonia related brain areas were considered as well: the nucleus accumbens shell, lateral hypothalamus, and adjacent, extended amygdala (Ho & Berridge, 2014). They found that temporary GABA inactivation of the hotspot in the caudal half of the medial shell generated sensory disgust, but lesions never did at any site. Inactivation of the rostral half of the shell failed to induce disgust as well (Ho & Berridge, 2014). Neither lesions nor inactivation of the lateral hypothalamus and amygdala induced disgust, indicating that the ventral pallidum is unique in its contribution to hedonic/disgust processing (Ho & Berridge, 2014).

Altogether, we suggest that the LH-VP pathway is important for mediating shifts in affective evaluations. Inhibition of the LH-VP hotspot pathway, as done in our study, begins to bias responding towards aversion. However, because the hotspot itself remains intact, our manipulation leaves hedonic reactions to sucrose unchanged.

Behaviors Due to Actual Increases in ‘Disgust’

How can we be sure that our results are due to changes in affect, and not simply a result of motor defectiveness? An alternative explanation to this finding may be that optogenetic inhibition of the LH-VP pathway could be causing a psychomotor effect. A psychomotor explanation would advocate that the enhanced orofacial reactions to quinine were caused by a reflexive motor syntax. This could be interceded by orexin neurons, which have been found to be involved in wakefulness, arousal, and general motor activity (Anaclet et al., 2009). However, general changes in locomotor activity seems to be an unlikely explanation, as laser

photoinhibition did not broadly increase taste reactivity responses, but specifically increased aversive ‘disgust’ reactions to quinine, leaving hedonic or neutral reactions unchanged; gapes, forelimb flails, and chin rubs all increased while hedonic behaviors such as tongue protrusions and lateral tongue protrusions were stable across the tests. It is important to note that aversive reactions *generally* increased and we did not elicit a motor effect in which animals only gaped. Animals increased aversive responses in a way unique to each individual animal. In addition, if the optogenetically induced increases in aversive reactions was eliciting some sort of fixed syntactical action pattern, then we ought to have observed an increase in aversive ‘disgust’ reactions to sucrose as well. Given that sucrose palatability was left untouched during laser photoinhibition, it seems unlikely that our observed effects were the result of abnormally behaving motor patterns.

Orofacial Reactions to Sucrose Not Affected By Inhibition

As mentioned above, orofacial reactions to sucrose were not affected by laser photoinhibition. Since there was an increase in ‘disgust’ reactions to quinine, it seems plausible that one could expect a corresponding decrease in hedonic reactions to sucrose: in other words, a shift towards aversion. Why would positive ‘liking’ reactions remain untouched, even while inhibition of the LH-VP pathway increased aversive ‘disgust’ reactions to quinine in the same animals? One explanation involves the integrity of the VP hotspot itself. So far, only manipulations that directly inhibit the hotspot have been shown similar increases in ‘disgust’ reactions to palatable stimuli, such as GABA stimulation via muscimol microinjections or excitotoxin lesions of caudal VP (Ho & Berridge, 2014). However, the behavioral profiles of these manipulations differ in an important way from the results of our LH-VP inhibition: direct inhibition/lesion of VP causes an intense flip in affective reactions to sucrose, whereas our

results only saw an increase in aversion to an already aversive stimulus. Therefore, we would expect direct optogenetic photoinhibition to likewise increase aversion to both sucrose and quinine.

Why does inhibition of the LH input, which ought to alter neural firing in VP, not affect ‘liking’? One reason might be because removal of this pathway is insufficient to cause any real change in hedonic signaling, given that ‘liking’ appears to be signaled by increases in neural activity, and neurons in the VP hotspot are easily excitable. In a study by Smith et al. (2011), the authors sought to distinguish the hedonic impact, motivation, and learned associative predictions for a particular reward). An electrode was placed into the VP hotspot and animals were given sucrose while monitoring neuronal firing (Smith, Aldridge, & Berridge, 2011). A gradual increase in firing was observed in VP as soon as animals were given a palatable sucrose solution. This electrophysiological signal was amplified after DAMGO administration into the NAc hotspot (Smith et al., 2011). This means that increases in ‘liking’ reactions to sucrose is controlled by increases in neuronal firing in the VP. They also found that if the natural input neurons are removed, the VP is significantly less excited (Smith et al., 2011). Incentive salience signals were also increased by this stimulation but firing signals to the learned prediction value of a reward were left unaffected (Smith et al., 2011). Therefore, the increased neuronal firing in caudal VP seems to code for increased hedonic impact.

With this in mind, it is interesting to note that Kupchik and Kalivas (2012) have shown that caudal VP is composed predominantly of neurons that are easily excited. “We show that more lateral levels of the subcommissural VP are homogenous but that a more medial slice contains two types of neurons” (Kupchik & Kalivas, 2012). In order to show this, they placed recording electrodes into different slices of VP along the rostrocaudal extent. One type of neuron

located more caudally, had primarily GABAergic input and was easily excitable due to a depolarized membrane potential (Kupchik & Kalivas, 2012). The second type located mostly in rostral subcommisural VP contains major glutamatergic input and no spontaneously firing neurons due to a hyperpolarized membrane potential (Kupchik & Kalivas, 2012). As they moved along the VP rostrocaudally, they found the population of neurons to change proportionally. The profile of rostrocaudal VP is different based on specific location. Between rostral and caudal VP, there is an almost equal mix of the two types (Kupchik & Kalivas, 2012). This first type located more caudally is of particular interest to us, for it is found in the VP hedonic hotspot (also located in caudal VP). These neurons as previously mentioned, are able to elicit an action potential with greater speed, indicating that very little input is necessary for their excitability. Therefore, because caudal VP neurons are easily excitable, the removal of a potentially excitatory input (those of LH into VP) may actually have less of an effect than one would expect, again leaving hedonia untouched.

Food Intake Not Affected by Inhibition

LH-VP photoinhibition did not alter food intake of palatable M&M candies. It was therefore concluded that the lateral hypothalamus to ventral pallidum pathway cannot directly decrease the 'wanting' aspect of reward. This is somewhat surprising, for when this pathway is activated, an increase in eating is observed (Castro & Berridge, 2014). We believe that a decrease in eating during inhibition is not shown because the hedonic system is remaining unaffected (food palatability is therefore intact). Previous work has shown that 'liking' and 'wanting' mechanisms can be dissociated, and we now extend the dissociation between affect and motivation to include increased 'disgust' without reductions in 'wanting'.

Hedonic Interpretation

Based on our results, we believe that the lateral hypothalamus to ventral pallidum connection is involved only during changes in hedonic interpretation. Further support of this ‘change in hedonic interpretation’ theory is the concept of alliesthesia mentioned above. As previously mentioned, previous research has shown that exogenous stimulation of the posterior VP by orexin microinjections amplifies hedonic impact of sucrose (Castro & Berridge, 2014). Based on this, we believe that orexin elevations in this orexin hedonic hotspot in VP might make food taste better during hunger (Ho & Berridge, 2013). This would explain the commonly known phenomenon of ‘food tasting better when hungry than when satisfied’. Increased sensory pleasure of food in hunger compared with satiety has been termed ‘alliesthesia’, and it is LH alliesthesia and the role of orexin neurons that should be further investigated. Alliesthesia along with this study will represent a more cohesive explanation of the LH-VP connection and if it is indeed involved in changes rather than direct stimulations and inhibitions.

The Role of Neurotransmitters in the LH-VP Pathway

In this experiment, we used a viral vector that used a neuron specific promoter (allowing us to target neurons, but not glial cells). However, one disadvantage to the adeno-associated vector is that we cannot target particular types of neurons. Despite this, we believe that the LH orexin population is playing an important role in the results described above. Orexin neurons project to posterior ventral pallidum and when activated, the terminals release the neuropeptide orexin (hypocretin). As previously mentioned, the posterior VP hotspot contains a dense population of orexin receptors (Marcus et al., 2001). Microinjection of exogenous orexin-A into this hotspot amplifies “liking” reactions to sucrose, whereas injection of orexin into other areas such as adjacent LH and amygdala produces no effect (Ho & Berridge, 2013). Glutamate and dynorphin may also be playing an important role in this aversion. Dynorphin is a peptide that

highly co-localizes with orexin, and preferentially actions on kappa opioid receptors. Although typically considered to be related to stress/aversion (Land & Chavkin, 2007), recent studies have indicated that the kappa/dynorphin system may be involved in "liking" (Castro & Berridge, 2014). Since orexin has also been shown to amplify hedonic impact in NAc it may be possible that orexin/dynorphin signals from LH might also act in VP as well. In addition, increases in general neural activity are seen when animals get sucrose (or are in salt depleted states) (Tindell, Smith, Berridge, & Aldridge, 2009). This indicates that glutamate, a neurotransmitter that holds a principal role in neural activation, may be imperative here as well. However, it may not be one or the other contributing to this effect; it is entirely possible that combinations of these three neurotransmitters are playing a vital role in modulating hedonic/aversive experience.

Limitations and future directions

Our study is limited due to the small sample size. In addition, we were unable to directly target orexin neurons and had to assume their involvement. Although this experiment presents a strong case for their participation, acquiring a virus with a promoter large enough to pinpoint orexin-releasing neurons would be ideal. In addition, we don't know which neurochemical system is mediating 'disgust', as mentioned above. In order to account for this discrepancy, one could inhibit the LH-VP pathway and inject exogenous orexin and see if the aversion is eliminated. If it is, then orexin must be involved in keeping excessive 'disgust' at bay. Furthermore, based on the limited information available to us, we are unsure of how to quantify laser inhibition in terms of how far the laser light is spreading. The laser light could be infiltrating other untargeted brain areas, which may have unknown effects on neural activity. Future projects could focus on using the same laser parameters and placing fibers into rostral VP rather than caudal VP. Based on our predictions, the increased aversion to quinine should not be

observed, for we believe it to be limited to LH inputs into the caudal VP hedonic hotspot. Because the rostral VP is outside the hedonic hotspot, any observed increased aversion should indicate that the laser parameters were most likely too widespread and would need to be adjusted. In addition, one could directly inhibit VP with optogenetics. Hedonic reactions only flip after lesions and/or drug injections to the VP hotspot and it would be interesting to see the effects of laser inhibition of the VP alone. We suspect that a similar flip in affective responding would occur.

Conclusion

Here we show that selective inhibition of lateral hypothalamic inputs into the ventral pallidum hotspot causes an increase in the number of aversive ‘disgust’ reactions to bitter quinine. This finding is specific to quinine and does not include reactions to sucrose or decrease food intake. While previous research has shown that optogenetic stimulation of the pathway recruits the hotspot and can directly alter ‘liking’ reactions to sucrose, it is not necessary to keep ‘liking’ reactions intact (Castro & Berridge, 2014). We believe this is because the neuronal makeup of caudal VP contains distinct, easily excitable neurons that are unaffected by the missing stimulation from LH when this pathway is turned off. The hotspot is therefore still “online” and can mediate ‘liking’ and ‘liking’ reactions.

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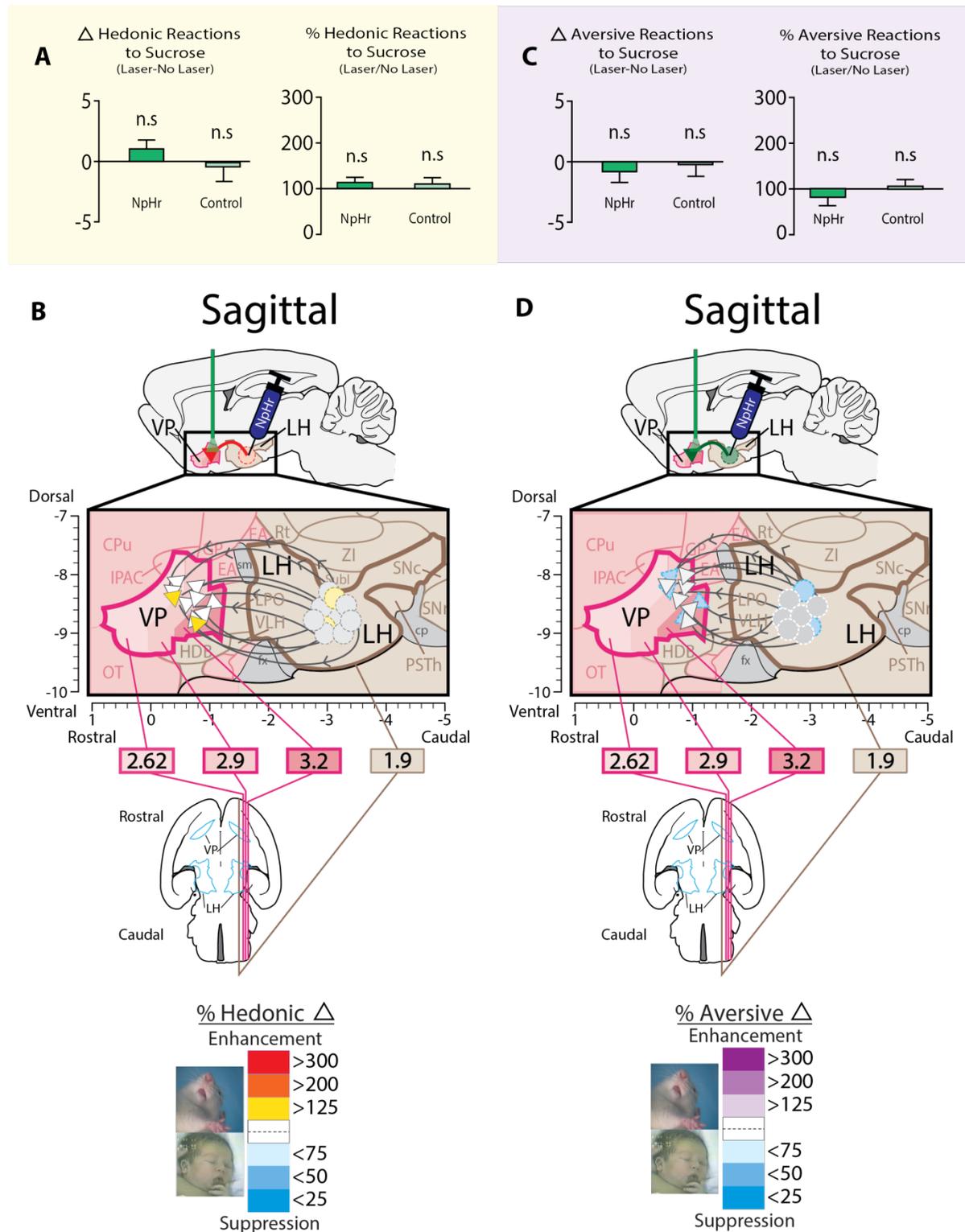


Figure 1. Effects of photoinhibition of the LH-VP hotspot pathway on hedonic or aversive

reactions to sucrose. A. The first graph depicts the net gains or losses in hedonic reactions to

sucrose. The second represents the percent change in hedonic reactions to sucrose compared to non-laser test days. The bar labeled "NpHr" represents animals infused with a halorhodopsin containing virus while the "control" represents animals given a control virus lacking the photoreceptive opsin. *B.* A sagittal map of ventral pallidum and lateral hypothalamus showing microinjection sites in mid-tuberal LH and fiber placements in posterior VP. Individual circles represent distinct microinjection sites and the yellow-red gradient shows 'liking' enhancement, while the white-blue gradient indicates 'liking' suppression. *C.* The first graph shows the net gains or losses in aversive reactions to sucrose, while the second shows the percent change compared to non-laser test days. *D.* This sagittal map of LH to VP shows microinjection sites in mid-tuberal LH and fiber placements in posterior VP. The white-purple gradient denotes increase in 'disgust', while the white-blue gradient indicates suppression of 'disgust'.

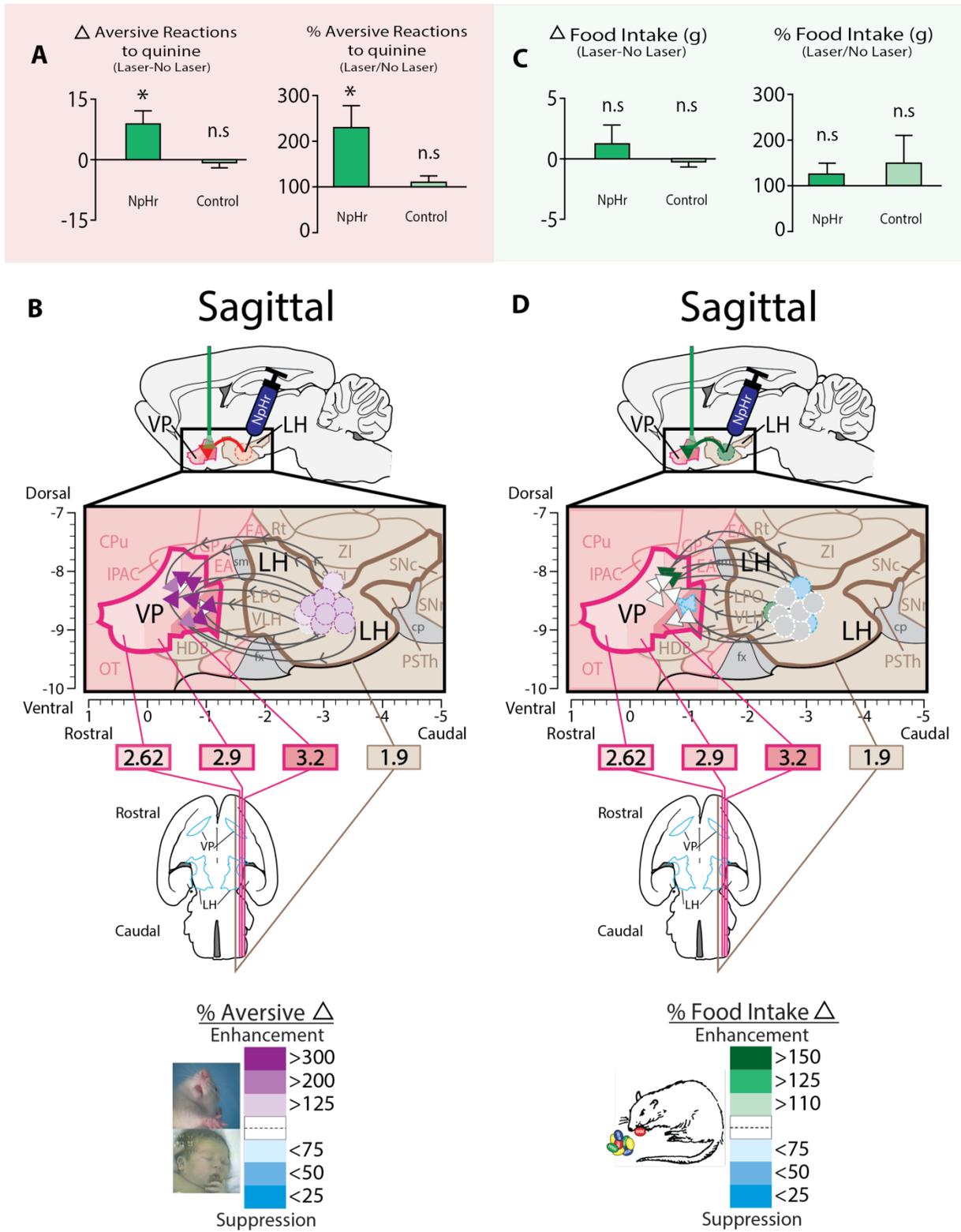


Figure 2. Effects of photoinhibition of the LH-VP hotspot pathway on aversive reactions to quinine, and laser effects on food intake. A. The first graph depicts the net gains or losses in

aversive reactions to quinine. The second represents the percent change in aversive reactions to quinine compared to non-laser test days. The bar labeled "NpHr" represents animals infused with a halorhodopsin containing while the "control" represents animals given control virus lacking the photoreceptive opsin. B. A sagittal map of ventral pallidum and lateral hypothalamus showing microinjection sites in mid-tuberal LH and fiber placements in posterior VP. Individual circles represent distinct microinjection sites and the white-purple gradient shows 'disgust' enhancement, while the white-blue gradient indicates 'disgust' suppression. C. The first graph shows the net gains or losses in food intake, while the second shows the percent change compared to non-laser test days. D. This sagittal map of LH to VP shows microinjection sites in mid-tuberal LH and fiber placements in posterior VP. The white-purple gradient denotes increase in 'disgust', while the white-blue gradient indicates suppression of 'disgust'.

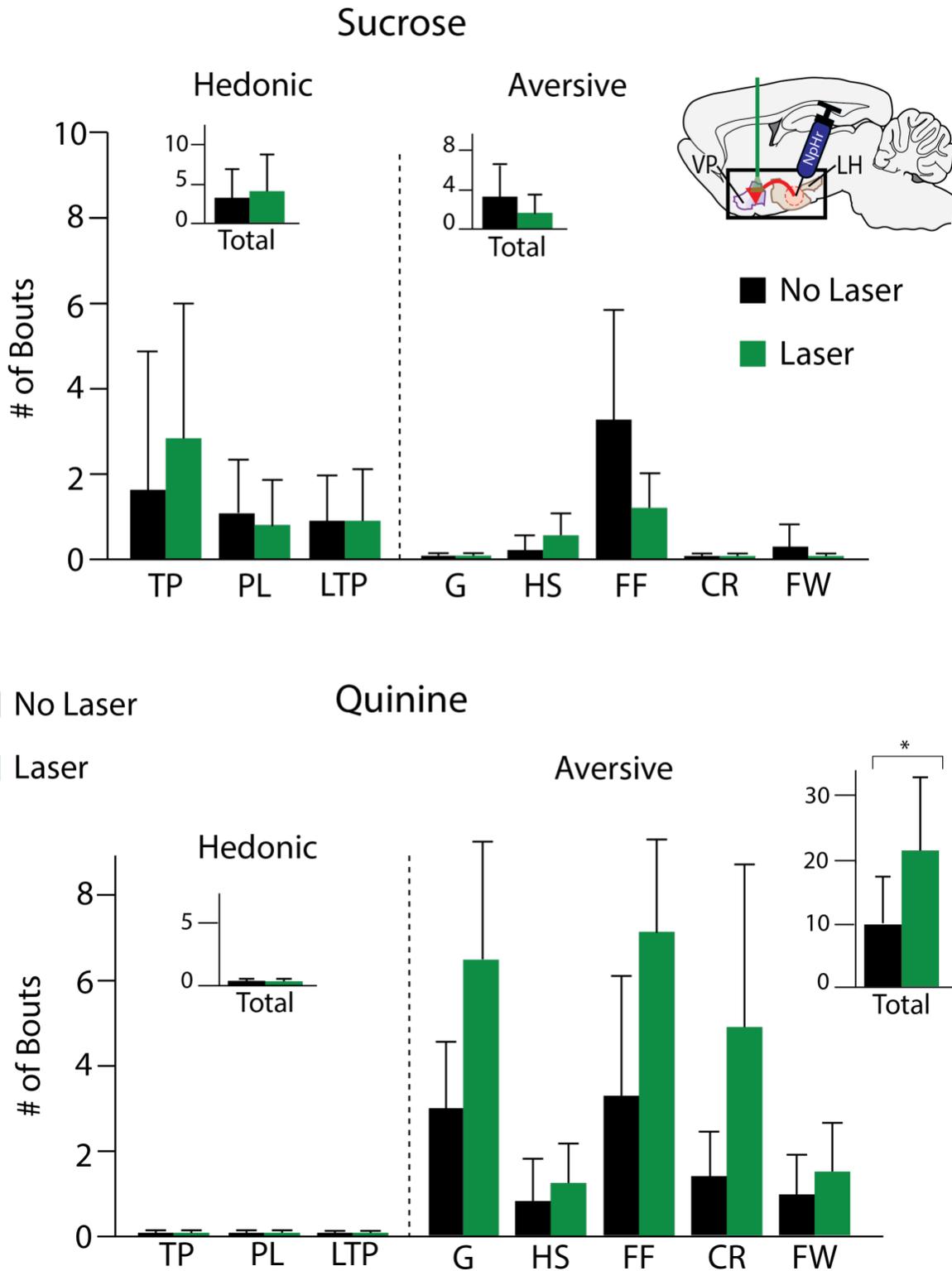


Figure 3. Breakdown of LH-VP inhibitory effects on taste reactivity behaviors. This graph shows the breakdown of individual taste reactivity behaviors included in the data analysis.

Hedonic reactions are tongue protrusions (TP), paw licking (PL), and lateral tongue protrusions (LTP). Aversive reactions are gapes (G), head shakes (HS), forelimb flails (FF), chin rubs (CR), and face washing (FW). The top half shows a non-significant change in individual hedonic and aversive reactions to sucrose. The bottom half clearly indicates a substantial increase in aversive reactions to quinine. The smaller graphs titled "Hedonic" are a sum of hedonic reactions to see an overall effect. Similarly, the smaller graphs titled "Aversive" are a sum of aversive reactions to see an overall effect.