The Ventral Pallidum As a Limbic Pleasure Generator

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Psychology)
in The University of Michigan
2010

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Acknowledgements

The most important guidance of all projects in this dissertation comes from my advisor Dr. Kent Berridge. I would like to thank Kent for providing the most stimulating and pleasant working environment a graduate student could hope for, and for offering generous encouragement and feedback on occasions when projects went through low times. I would also like to acknowledge the other members on my dissertation committee: Dr. Jon-Kar Zubieta, Dr. J. Wayne Aldridge, and Dr. Brandon Aragona. They guided me to look at the projects with alternative perspectives and offered tremendous help in producing this dissertation.

Two important people who opened the door of the neuroscience/ biopsychology field for me were Dr. Chen- Tung Yen and Dr. Keng- Chen Liang at the National Taiwan University. The experience of working in Dr.Yen's neurobiology lab and taking Dr. Liang's Biopsychology course brought me onto the track of studying behavioral neuroscience. I have been enjoying this flourishing field ever since.

The projects in this dissertation could not have been accomplished without the help and support from my colleagues in the Berridge lab: Dr. Casey Cromwell, Dr. Susana Pecina, Dr. Kyle Smith, Dr. Stephen Mahler, Dr. Eric Jackson, Dr. Michael Robinson, Jocelyn Richard, Alex DiFeliceantonio, Rebecca Hsieh, Ryan Selleck, Michelle Dimondo, Gretchen Sprow, Victor Baron, Stephen Burwell, and Andrew Deneen. I sincerely appreciate their advice on my experimental designs, helpful conversations about different aspects of my dissertation, and technical support during the experiments.

Finally, I would like to thank my friends and family in Ann Arbor, in Taiwan, and in other places of the world. I would like to thank my parents Chen- Yang Ho and Shu-Chin Shih and my sister Pei-Yi Ho for always being there for me whenever I needed it. I am particularly thankful of Dr. Chun- Hui Chang and Jamie Pitts' great accompany in Ann Arbor. Also, to the Ho family, the Shih family, my high school friends, and my college friends, thank you all for inspiring me to be curious about the world and to have the courage of pursuing answers to the unknown.

Five years abroad has been quite a long journey, and I am thankful of having each of you in my life to share it with me.

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Chapter 1

Introduction

Psychological Components of Reward: 'Liking' and 'Wanting'

Reward consists of at least two psychological components: affective 'liking' and motivational 'wanting'. According to the theory developed by Berridge & Robinson, 'liking' is defined as the hedonic impact or pleasure immediately following the receipt of a reward, and 'wanting' is defined as incentive salience, or a motivational magnet quality that makes rewards or reward predictive cues desirable and attractive (Berridge, 2009b; Berridge & Robinson, 2003). It is widely acknowledged that 'liking' and 'wanting' of rewards carry important survival and reproductive benefits for humans and animals: 'liking' reinforces the behavior associated with reward, and 'wanting' directs attention and promotes approach towards reward. In the case of food reward, hedonic 'liking' of a food reward would cause cues that predict the availability of food, such as its sight and smell, and food itself desirable and attributed with incentive salience, or 'wanting'. Next time when the food or food-predictive cues are encountered, they would trigger animal's motivation to approach and consume. Thus, 'liking' and 'wanting' contribute separate, but equally critical, components to reward and to goal-directed behaviors like eating.

In this dissertation, we focus on the mechanisms for 'liking' of reward. It is crucial to identify brain mechanisms that generate 'liking' – both at normal levels and at enhanced of 'liking'.

Methods to Measure 'Liking' in the Brain

The hedonic 'liking' impact of food reward elicited orofacial reactions that are homologous across humans, primates, and even rats, and can be objectively measured (Berridge, 2000; Berridge & Robinson, 2003). Unlike human adult subjects that are capable of verbally describing their feelings, non-verbal animals, such as rats, are incapable of expressing their feelings through language. Therefore, the adoption of a method that objectively quantifies and interprets animals' 'liking' reactions towards rewards is particularly critical. In the current study, we exploit the taste reactivity paradigm (Figure 1-1), which has been shown to be useful for quantifying 'liking' and 'disliking' affective reactions to different tastes in humans and animals (Berridge, 2000; Steiner, Glaser, Hawilo, & Berridge, 2001). For example, a drop of sweet taste into human infants' or rats' mouths elicits hedonic 'liking' orofacial reactions, including rhythmic tongue protrusions, lateral tongue protrusions, and paw licks (Figure 1-1 top). In contrast, a drop of bitter taste elicits aversive 'disliking' orofacial reactions, such as gapes, headshakes, chin rubs, and frantic wiping of the mouth, to actively reject the bitter taste from the mouth (Berridge, 2000; Grill & Norgren, 1978a; Steiner, et al., 2001) (Figure 1-1 bottom). The characteristics of these 'liking' and 'disliking' reactions were evolutionarily conserved and appeared to be homologous across mammals in phenotypic appearance and timing, with body size being strongly negatively correlated with orofacial reaction speed (Berridge, 2000).

Importantly, these affective reactions fluctuate in similar ways to human subjective pleasure when circumstances change (e.g. food is more pleasant to us when hungry, sweet tastes are more 'liked' when rats are hungry) (Berridge, 1996). For example, a 3-times-concentrated salty seawater is not pleasant to humans or normal animals, and elicits mainly aversive 'disliking' reactions, such as gapes. However, if the animals are pharmacologically deprived of body salt by hormone

injections, a state of 'salt appetite' develops. When animals are in this state, the same concentrated salty solutions elicit hedonic 'liking' reactions, such as tongue protrusions, instead of aversive gapes (Berridge, Flynn, Schulkin, & Grill, 1984; Tindell, Smith, Pecina, Berridge, & Aldridge, 2006). Thus, orofacial reactions to tastes not simply reflect sensory properties of the taste, but rather an evolutionarily- conserved behavioral output of a hedonic evaluation system that is capable of incorporating physiological needs. Based on these observations, neuroscientists are able to exploit taste reactivity paradigm to quantify 'liking' and 'disliking' aspects of rewards experienced by rats, and to pharmacologically manipulate the brain to identify neural mechanisms responsible for 'liking' and 'disliking' of reward.

Methods to Measure 'Wanting' in the Brain

There are a variety of objective behavioral measurements for 'wanting' attributed to innate incentive stimuli (unconditioned stimuli, UCSs) or to learned stimuli that are originally neutral but gained incentive salience and become 'wanted' due to their reward-predictive characteristics (Berridge, 2004; Berridge & Robinson, 2003). Natural rewards that are 'liked' are usually also 'wanted', which refers to the ability of the rewards to attract attention, energize reward seeking behaviors, facilitate approach, or even consumption of the rewards.

To *measure UCS 'wanting'*, the most straightforward way is to measure the voluntary consumption of a reward (Berridge, 1996; Kelley & Berridge, 2002). Food and other natural rewards, with their sights, smells, and textures, are capable of enhancing an animal's motivation to approach the UCS reward and facilitate consumption. Thus, eating behavior and food intake measurement serve as useful indicators for 'wanting'; eating behavior is an objective measurement of cue-triggered approaches towards the reward, and food intake quantifies the actual consumption. Experiments interested in whether or not a neural mechanism mediates 'wanting' can therefore ask if change of this mechanism causally alters eating behavior or food consumption.

In this dissertation, food intake will be used as the principal measurement for reward 'wanting', and neural manipulations that increase/decrease food consumption will be contrasted to those that increase/decrease 'liking' or 'disliking', as measured by the taste reactivity test, for comparison of neural substrates.

Identification of Neural Substrates for 'Liking' and 'Wanting'

Just as it is important to parse reward into distinct psychological components, it is crucial to identify neural mechanisms underlying 'liking' and 'wanting' of rewards. It has been hypothesized that neural mechanisms mediating core affect, such as 'liking' or 'wanting' for food reward, can evolve for social functions (Chapman, Kim, Susskind, & Anderson, 2009; Rozin & Fallon, 1987; Rozin, Lowery, & Ebert, 1994). This implies that emotional (or even moral) pleasure or disgust may share the same facial expression and neural mechanisms as food 'liking' or 'disliking' (Calder, et al., 2007; Chapman, et al., 2009; Olatunji, Cisler, McKay, & Phillips, 2010; Phillips, et al., 1997). Thus, the 'liking' and 'wanting' mechanisms we identify for food reward can provide insights to a variety of psychopathology, including eating disorders, obsessive compulsive disorders (OCD), and depression (Adolphs, 2002; Adolphs, Tranel, & Damasio, 2003; Olatunji, et al., 2010; Phillips, et al., 1997; Sprengelmeyer, et al., 1996; Sprengelmeyer, et al., 1997; Surguladze, et al., 2010; Suzuki, Hoshino, Shigemasu, & Kawamura, 2006).

Causal relationships between brain substrate and function

There are three relationships that can exist between an identified neural mechanism and a reward function: necessary, sufficient, and coding. When a neural mechanism is *necessary* for a reward function, the function cannot be carried on without the normal functioning of the brain mechanism (e.g. caused by antagonist drugs, neurotoxin lesions, or genetic manipulations). A different relationship is the case in which a neural mechanism is a *sufficient* cause for a reward function; the functional enhancement of the neural mechanism (e.g. by agonist drugs, brain stimulation, or genetic manipulation) increases the expression of the reward function. The third type of relationship between a neural mechanism and a reward

function is *coding*. In this case, the activation of the neural mechanism occurs when the reward function is being processed or being expressed, and appears to *code* such function. However, the activation of the neural mechanism may be a parallel process - no clear causal relationships can be drawn between the mechanism and the function in the case of *coding*. The identification of the causal relationships between neural mechanisms and reward functions is critical for pinpointing the causes and effects of psychopathology.

Neuroscience tools for measuring reward roles

Several neuroscience tools have been developed for the identification of neural mechanisms of reward. To reveal the precise neural substrate where the neural signal activation *causes* the enhancement of reward function, two techniques are commonly used: drug microinjections that produce neural activation, and Fos mapping that pinpoints the precise neural substrate activated by drug microinjections. For example, in the case of 'liking', if microinjections of an agonist for X neurotransmission in Y site increase 'liking' reactions (e.g. tongue protrusions, lateral tongue protrusions, paw licks) to sucrose infusion, X in Y site is a *sufficient* cause for hedonic 'liking' of reward. The same logic applies to 'wanting' of reward. Additionally, in order to precisely map the functional boundaries of Y site, the Fos plume technique is exploited (Mahler, Smith, & Berridge, 2007; Pecina & Berridge, 2005; Reynolds & Berridge, 2008; K. S. Smith & Berridge, 2005). The reason is because drugs can diffuse from the site of microinjection and spread to the surrounding areas. This spread leads to the well-known caveats of the microinjection: the uncertainty about what subregion of the Y site contributes to the observed reward function and whether or not the functional effect is caused by the Y site or by the nearby structures. Fos plume technique, which labels Fos proteins translated from immediate early genes activated by drug microinjection, estimates the area and degree of neural activation around the microinjection site via Fos quantification. By comparing Fos plumes produced by X drug to the non-drug condition, we are able to estimate the averaged area and intensity of functional/neural activation caused by X drug in the Y substrate and address the

problems related to microinjections. To reveal the *necessary* site where loss of function disrupts reward processing, antagonist drugs or excitotoxin lesion are used to suppress neurotransmission, and neuronal counts are used to quantify extitotoxin lesions (Cromwell & Berridge, 1993, 1994; Shimura, Imaoka, & Yamamoto, 2006; K. S. Smith & Berridge, 2007). Similar to the logic of identifying *sufficient* cause for 'liking', antagonist drugs and excitotoxin lesion help us identify whether or not Y structure is *necessary* for 'liking', and neuronal count help us pinpoint the precise subregion within Y structure that is *necessary* for 'liking'.

In this dissertation, all of the approaches mentioned above are used to identify neural mechanisms that are *sufficient* or *necessary* for hedonic 'liking', aversive 'disliking', or food 'wanting'.

Ventral Pallidum Function for 'Liking' and 'Wanting'

Embedded in reward circuit

Limbic system, so named because of its special location on the rim of the cerebral cortex adjacent to evolutionarily older structures in the midbrain, plays an important role in mediating rewards for food, drugs, and sex. Within this system, nucleus accumbens shell (NAcSh) and ventral pallidum (VP) form the ventral striataopallidal pathway that is commonly described as a main pathway for reward information flow in the brain (de Olmos & Heimer, 1999; Haber & Knutson, 2010; Kalivas, Churchill, & Romanides, 1999; Kalivas & Volkow, 2005; Mogenson & Yang, 1991; Pecina, Smith, & Berridge, 2006; K. S. Smith, Tindell, Aldridge, & Berridge, 2009; Zahm, Williams, & Wohltmann, 1996). Between these two structures, VP was isolated from adjacent areas (including the globus pallidus, substantia innominata, extended amygdala, lateral preoptic area of hypothalamus, or the olfactory tubercle) and identified as a distinct anatomical structure by Heimer and Wilson only a few decades ago (Heimer & Wilson, 1975). Although VP has recently gained more attention for its role in reward (as opposed to its traditional role in motor functions), compared to NAc, relatively little is known about the functional contribution of VP in reward.

Anatomically, VP has been suggested as the 'final common path of reward'.

It receives input from brain structures in the limbic system, such as nucleus accumbens, amygdala, ventral tegmental area, and lateral hypothalamus, as well as input from the cortical structures, such as orbitofrontal, prefrontal, and infralimbic cortex (Carnes, Fuller, & Price, 1990; Chrobak & Napier, 1993; Fuller, Russchen, & Price, 1987; Groenewegen & Berendse, 1990; Grove, 1988; Klitenick, Deutch, Churchill, & Kalivas, 1992; Maurice, Deniau, Menetrey, Glowinski, & Thierry, 1997; Napier, Muench, Maslowski, & Battaglia, 1991; Turner, Lavin, Grace, & Napier, 2001; Usuda, Tanaka, & Chiba, 1998a; Zaborszky, Gaykema, Swanson, & Cullinan, 1997; Zahm, Zaborszky, Alones, & Heimer, 1985) (Figure 1-2). Conversely, VP projects back to nearly all of its input structures, including the NAc, and further re-enter the cortocolimbic loop via direct projections to medial prefrontal cortex, or via indirect relay through mediodorsal nucleus of thalamus (Churchill & Kalivas, 1994; Churchill, Zahm, & Kalivas, 1996; Groenewegen, Berendse, & Haber, 1993; Haber, Groenewegen, Grove, & Nauta, 1985a; Haber, Lynd-Balta, & Mitchell, 1993; Heimer, Zaborszky, Zahm, & Alheid, 1987; Young, Alheid, & Heimer, 1984; Zahm, Zaborszky, Alheid, & Heimer, 1987) (Figure 1-2). Lastly, VP also projects to motor-related areas, such as subthalamic nucleus, substantia nigra, and peduncular pontine neuclues (Bell, Churchill, & Kalivas, 1995; Groenewegen & Berendse, 1990; Groenewegen, et al., 1993; Haber, Groenewegen, Grove, & Nauta, 1985b; Kincaid, Penney, Young, & Newman, 1991; Zahm, 1989). Together, the anatomical evidence suggests that VP is capable of converging information from the limbic system and the cortical area, sends reciprocal output to modulate neural signals in these areas, and/or transfer the signal to motor output (Figure 1-2). Thus, VP may serve as final common path of reward where reward signals travel to influence cognitive processes or motor expression (Kelley, Baldo, Pratt, & Will, 2005; Pecina, et al., 2006; K. S. Smith, et al., 2009; Waraczynski & Demco, 2006; Zahm, 2006).

Necessary for food 'liking' and eating

As for VP's functional role in reward, VP is not only *necessary* for food 'wanting', but may also be the only known structure necessary for hedonic 'liking' in the brain. Excitotoxin lesions to VP or/and the surrounding areas cause rats to develop aphagia, which is inability or refusal to eat, or loss of food 'wanting'

(Cromwell & Berridge, 1993; Schallert & Whishaw, 1978; Shimura, et al., 2006; Stellar, Brooks, & Mills, 1979; Teitelbaum & Epstein, 1962). In addition, VP lesions also cause suppression of 'liking' reactions to sweet sucrose taste, and replace them with aversive 'disliking' reactions, as if the sweet taste is nasty (Cromwell & Berridge, 1993). A second line of support comes from the studies of GABA inactivation in VP. Microinjections of the $GABA_A$ agonist muscimol in VP produce similar effects as lesion. Rats with GABA inactivation in VP showed decreased intake of all solutions, including sweet saccharine taste, bitter quinine taste, and water. Also, these rats appeared to be aversive, rather than hedonic, to sucrose sweet taste as measured by the taste reactivity test (Shimura, et al., 2006). In further support of the notion that VP is necessary for food reward processing, it has been shown that VP excitotoxin lesions block the acquisition of a sucrose place preference, and excessive GABAergic inhibition of VP blocks primed reinstatement of food seeking behavior (McAlonan, Robbins, & Everitt, 1993a; McFarland & Kalivas, 2001). Overall, it is clear that VP is necessary for food 'wanting' and 'liking'. However, prior to this dissertation, it is unclear in what specific subregion of VP these reported effects were produced, and whether the lesion aversion site is the same or different from the GABA-caused aversion site.

Similar to animal studies, two recent clinical studies suggest that the normal functioning of VP is critical for drug craving and hedonia (Miller, et al., 2006; Vijayaraghavan, Vaidya, Humphreys, Beglinger, & Paradiso, 2008). In one case, a long-term drug addicted human patient with bilateral lesions in VP (overlapping with globus pallidus) reported to "experience a depressed mood" and doctors noted as a general anhedonia. Also, after VP lesion, the patient reported the disappearance of all drug cravings and no longer experienced pleasure from drinking alcohol, suggesting that he had loss motivational 'wanting' for drug reward (Miller, et al., 2006). In the other case, a patient with bilateral damage to the globus pallidus (the lesion is by authors' definition likely to be extending to VP) reported an "inability to feel emotions", as well as a profound lack of motivation and loss of motivation to work for food (Vijayaraghavan et al., 2008).

Sufficient for food 'liking' and eating

Besides being necessary for food 'wanting' and 'liking', activation of specific neurotransmission signal in VP is also *sufficient* for increasing hedonic 'liking' and motivational 'wanting' of reward. The first evidence for VP's sufficient role in reward processing came from brain stimulation studies in the 1990s, which demonstrated that animals would repeatedly press a lever to self-stimulate through electrodes implanted in VP (Kretschmer, 2000; Panagis, Miliaressis, Anagnostakis, & Spyraki, 1995; Panagis, et al., 1997). The first evidence that suggested the activation of VP can sufficiently enhance food 'wanting' came from one GABA$_A$ antagonist microinjection study, which showed that the disinhibition of VP via removal of GABA inhibitory input caused the rats to consume more food rewards (Stratford, Kelley, & Simansky, 1999). In a separate study from our lab, it was shown that GABA$_A$ antagonist microinjections did not alter hedonic 'liking' reactions to sweet sucrose taste (K. S. Smith & Berridge, 2005). Thus, VP GABA disinhibition appears to be a mechanism of enhancing 'wanting' without enhancing 'liking'. So, are there neurotransmission signals in VP that enhance 'liking'? Prior to this dissertation, one study from our lab identified that μ-opioid in VP, as well as μ-opioid in NAc shell, can sufficiently enhance hedonic 'liking' along with food 'wanting' (Pecina & Berridge, 2005; K. S. Smith & Berridge, 2005). Microinjections of μ-opioid agonist DAMGO in a cubic millimeter hotspot in posterior VP, similar to DAMGO microinjections in a 'liking' hotspot in anterior medial shell of the NAc, caused the rat to express more 'liking' reactions to sucrose infusion, as well as more feeding-related behaviors and food intake. However, microinjections of DAMGO in anterior VP actually suppressed 'liking' of sucrose, despite the fact that food 'wanting' was enhanced. Thus, it appeared that μ-opioid neurotransmission signal in the posterior hedonic hotspot can sufficiently enhance 'liking' and 'wanting' of reward, while μ-opioid in anterior VP only enhanced 'wanting' but decreased 'liking'. It is important to note that the opioid 'liking' hotspot in posterior VP highly overlaps with the region where lesion caused 'liking' suppression and 'disliking' elevation, suggesting that the opioid 'liking' hotspot in VP may be both *sufficient* and *necessary* for 'liking'

of reward. However, prior to this dissertation, it was unknown whether the hedonic 'liking' hotspot in NAc shell is also necessary for 'liking' of reward. Also, prior to this dissertation, no other neurotransmission signal except for μ-opioid in NAc Shell and VP and benzodiazepines in the parabrachial nucleus in the brain stem had been shown to sufficiently enhance hedonic 'liking' of reward.

Coding for 'liking' and 'wanting' for reward

VP appears to *code* hedonic 'liking' and reward 'wanting' as demonstrated in electrophysiological recording studies (Tindell, Berridge, & Aldridge, 2004; Tindell, Berridge, Zhang, Pecina, & Aldridge, 2005; Tindell, Smith, Berridge, & Aldridge, 2009; Tindell, et al., 2006). In a normal state, an intense salty solution (3x seawater concentration) elicited mainly aversive reactions, while in a salt-depleted state, this salty solution flipped to become appetitive and elicited mainly hedonic 'liking' reactions. Caudal VP neurons were capable of tracking this aversive-to-hedonic change of the same concentrated salty solution without changing the firing pattern to a sweet taste, suggesting that VP codes hedonic 'liking' rather than the sensory identity of the taste or arousal (Tindell, et al., 2006). In a follow-up study, VP neurons were shown to code the flip of incentive 'wanting' for a CS cue that predicts the intense salty solution. Through Pavlovian learning, the CS cue was attributed to the incentive value of the intense salty solution and elicited negative motivation in the normal state. However, in the salt-depleted state, the intense salty solution became 'wanted' by the animal, as well as the salty-solution-predictive CS cue. VP neurons were shown to code this cue-triggered 'wanting' for the intense salty solution in the salt-depleted state (Tindell, et al., 2009). It is critical to note that in the above two experiments, the firing pattern of VP neurons change the first time the intense salty solution (in the case of 'liking') or the CS cue (in the case of 'wanting) were encountered, suggesting that the change of 'liking' or 'wanting' did not require further learning. Besides coding 'liking' and cue-triggered 'wanting' for natural tastes, VP neurons also code dopaminergic enhancement of incentive salience. Both repeated administration of amphetamine and acute amphetamine

resulted in psychomotor sensitization and the change of VP firing pattern towards incentive coding.

Human evidence for VP representation of 'liking' and 'wanting'

The notion that VP codes implicit affective and motivational aspects of reward is supported by several recent imaging studies in humans. VP have been shown to code rewards for drugs, sex, and money, even when the reward images were displayed with a short duration that did not cause conscious awareness (Calder, Keane, Manes, Antoun, & Young, 2000; A. R. Childress, et al., 2008; Pessiglione, et al., 2007; Schaefer, et al., 2003). Furthermore, VP neural response predicts the subjective positive ratings of the images of drug or sex in a visible manner, and the effort the subjects were willing to put in to gain the implicit money reward (A. R. Childress, et al., 2008; Pessiglione, et al., 2007). Together, these results demonstrated that human VP is able to code different forms of implicit rewards (sex, drug, money), and to transfer the implicit emotion/motivation associated with the reward into behavioral effort. Human VP has also been shown to code both positive and negative stimuli, which elicit positive/negative affection and/or motivation. For example, fMRI studies have detected heightened activity in caudal VP in response to images of appetizing food, which may correspond to the caudal opioid hedonic 'liking' hotspot in rats, and it is the rostral VP that showed heightened activity when the images displayed are disgusting food. And the degree of rostral VP response to disgusting food is correlated with individual sensitivity to disgust (including food aversion and social aversion), suggesting that emotional disgust may be rooted in food aversion (Calder, et al., 2007). In another experiment design specifically targeting emotional processing, VP together with nucleus accumbens were reported to code both 'hot' and 'cold' emotions in a schematic condition, in which metaphoric sentences elicited implicit emotional processing (Schaefer, et al., 2003). Together, these two studies support the notion that VP code positive and negative affects and/or motivation. The malfunction of VP has been linked to clinical diseases or disorders. For example, the deactivation of μ-opioid transmission in VP, reflected by higher μ-opioid receptor availability, is correlated

with increased negative affect ratings and decreased positive affect ratings, and patients with borderline personality disorders demonstrated greater activation of opioid system in VP in response to sustained sadness (Zubieta, et al., 2003). Obese subjects directed their visual attention to food images for a longer time, and obese women showed higher neural activity in VP to pictures of high-calorie foods (Castellanos, et al., 2009; Stoeckel, et al., 2008). The studies above indicated that human VP plays a significant role in reward. Future studies designed to further tease apart the coding of 'liking' and 'wanting' components in human VP are necessary.

Other Roles of VP in Reward

Disgust

While μ-opioid neurotransmission in VP was identified as a sufficient mechanism for hedonic 'liking', some evidence suggests that the GABA system in VP may mediate aversive 'disliking' of reward. Microinjections of $GABA_A$ agonist muscimol in VP elicited strong aversion not only to a bitter quinine taste but also to water and sucrose (Shimura, et al., 2006). Furthermore, GABA in VP mediates the palatability shift in a taste aversion paradigm; an increased of GABA neurotransmission is correlated with the pattern of hedonic-to-aversive change to sucrose taste (Inui, Yamamoto, & Shimura, 2009). Blockade of GABA transmission in VP disrupted the development of conditioned taste aversion (Inui, Shimura, & Yamamoto, 2007). In total, excessive GABA signal in VP appears to be tightly linked to the elevation of aversion. This dissertation is the first study so far to identify the precise subregion of VP where GABA mediates aversion.

The identification of brain mechanisms that generate gustatory 'disliking' may have broader impact on mechanisms of moral disgust and disgust-related disorders. It has been suggested that the emotion of disgust is evolved from a more phylogenetically older gustatory 'disliking' system, implicating that the orofacial rejections of disgusting food and emotional disgust (e.g. towards contaminated foods, dirty items, unfairness in social interactions, etc) may exploit the same facial expressions and neural mechanisms (Phillips, et al., 1997; Rozin & Fallon, 1987;

Rozin, et al., 1994). One recent study from Chapman et al supported this idea by showing that gustatory distaste, contaminant images-elicited disgust, and social unfairness-induced disgust indeed evoke the same pattern of orofacial response as measured by EMG recording (Chapman, et al., 2009). As for neural response to disgust, lesion studies and imaging studies have demonstrated the critical involvement of the basal ganglia. For example, in one clinical case, a patient with damage to the left basal ganglia (and left insula) was impaired in his ability to recognize facial expressions of disgust, as well as to experience disgust (Calder, et al., 2000). Some additional insights into the role of basal ganglia in disgust processes come from disgust-related psychopathology studies. Subjects with obsessive-compulsive disorder, Parkinson's disease, and Huntington's disease, which are diseases that preferentially damage basal ganglia, have all been reported to have deficits in disgust recognition or/and loss of disgust (Adolphs, 2002; Adolphs, et al., 2003; Olatunji, et al., 2010; Phillips, et al., 1997; Sprengelmeyer, et al., 1996; Sprengelmeyer, et al., 1997; Surguladze, et al., 2010; Suzuki, et al., 2006). One study particularly relevant to the current dissertation from Calder et al further pointed out that the anterior part of human VP code disgust sensitivity to nasty food (Calder, et al., 2007). Thus, it is particularly important to identify specific neural substrates and neurotransmission that mediate gustatory 'disliking' processes to advance our understanding of emotional disgust and its related psychopathology.

Drug Reward

VP also plays a significant role in drug reward. One key feature involved in the rewarding aspect of commonly abused drugs is a reduction of VP GABA transmission. For example, this phenomenon of decreased GABA in VP was observed in self-administration of heroin, or systemic administration of morphine, amphetamine, and a cannabinoid CB1 agonist (Bourdelais & Kalivas, 1990, 1992; Caille & Parsons, 2004). In addition, μ-opioid antagonist CTAP blocks cocaine-reinstatement and cocaine-induced reduction of GABA in VP, suggesting that μ-opioid and GABA interact in VP to mediate drug reward (X. C. Tang, K. McFarland, S.

Cagle, & P. W. Kalivas, 2005). Both μ- opioid and GABA have been shown to participate in food reward processes as mentioned in the previous section.

Social affiliation

VP is also critically involved in sex and social affiliation, which is also important natural reward for survival. Monogamous prairie voles that form pair bonds have higher vasopressin V1a receptors in VP compared to non-monogamous species, and V1a receptor up-regulation in the VP elevates pair-bonding (Pitkow, et al., 2001; Wang & Aragona, 2004) In the monogamous Callicebus Titi monkey, the activity of VP was shown to be involved in the formation and maintenance of pair-bonding (Bales, Mason, Catana, Cherry, & Mendoza, 2007).In humans, VP/ventral globus pallidus has been reported to increase activity during male sexual arousal and while viewing subliminal images of sex (Rauch, et al., 1999). However, it is unclear whether VP is involved in 'liking' or 'wanting' aspect of social affiliation.

Nucleus Accumbens and Reward

Research on NAc reward functions has revealed several neurotransmitter subsystems that cause elevations in 'liking' and 'wanting'. 'Liking' has been demonstrated in opioid neurotransmission and endocannabinoid (anandamide) neurotransmission in the NAc shell, and perhaps also GABA signal (Mahler, et al., 2007; Pecina & Berridge, 2005; Reynolds & Berridge, 2002). 'Wanting' has been most intensely studied for dopamine in NAc (Berridge, 2007; Wyvell & Berridge, 2000, 2001), and also a wider range of neurotransmission signals that can simulate eating behavior, including GABA, glutamate, opioids, and endocannabinoids (Baldo & Kelley, 2007; Berridge, 1996; Berthoud, 2002; Levine & Billington, 2004; Reynolds & Berridge, 2001, 2003).

NAc opioid signal and reward 'liking'

Opioid neurotransmission in dorsal medial shell of the nucleus accumbens has been shown to enhance food intake, as well as 'liking' aspect of the reward (Berthoud, 2002; Kelley, et al., 2002b; Levine & Billington, 2004; Pecina & Berridge, 2005; Pecina, et al., 2006). Microinjections of μ-opioid agonist DAMGO in a cubic

millimeter hedonic hotspot facilitate 'liking' reactions to a sweet sucrose taste by up to quadruple the usual number. DAMGO microinjections also simultaneously reduce 'disliking' reactions to quinine, both in the hedonic hotspot and the surrounding area. In an even larger site, which encompasses the hedonic hotspot and the 'disliking' reduction site, DAMGO increased eating behavior and food intake (Pecina & Berridge, 2005; K. S. Smith & Berridge, 2007). Similar to opioid neurotransmission, endocannabinoid transmission in dorsal medial shell of NAc also enhanced both 'liking' and 'wanting'. The endocannabinoid hedonic 'liking' hotspot highly overlaps with the opioid 'liking' hotspot, but is larger in size (Mahler, et al., 2007).

NAc dopamine signal and reward 'wanting'

Dopamine transmission has long been studied for its role in reward, but it was not until recently that it was shown to mediate 'wanting' without 'liking'. For example, dopamine in NAc increases eating, especially in the shell region, suggesting that dopamine is involved in 'wanting' of reward (Sills & Vaccarino, 1996; Swanson, Heath, Stratford, & Kelley, 1997). A second line of evidence comes from studies that demonstrate dopamine in NAc mediates incentive salience. Acute intra-NAc injection of amphetamine, as well as NAc amphetamine-induced sensitization, trigger incentive 'wanting' for Pavlovian CS+ cue in a PIT paradigm (Wyvell & Berridge, 2000, 2001). However, intra-NAc injection of amphetamine did not alter 'liking' reactions to sucrose, supporting the notion that dopamine mediates 'wanting' but not 'liking'. Lastly, dopamine interacts with glutamate circuit in NAc, and is necessary for disruptions of corticolimbic glutamate signals in shell to generate positive or negative incentive salience in the shell (based on anatomical site and other conditions) (Faure, Reynolds, Richard, & Berridge, 2008). Overall, these results support the notion that dopamine transmission in NAc either directly mediates 'wanting' of reward or indirectly modulates 'wanting' of reward via interactions with other neurotransmitter systems. However, dopamine transmission in NAc does not alter 'liking' of reward.

NAc GABA signal and reward

GABA signals in the NAc shell and disruption of glutamate signals are both able to generate intense motivated behaviors corresponding to desire (food eating) and/or dread (defensive paw treads) along a rostro-caudal gradient (Kelley & Berridge, 2002; Reynolds & Berridge, 2001, 2002, 2003; Stratford, 2005; Stratford & Kelley, 1999). However, only GABA neurotransmission elicits affective 'liking' and 'disliking' in correspondence to the motivational 'desire', measured by eating, and 'dread', measured by defensive treading (Faure, Richard, & Berridge, 2010).

NAc lesion does not suppress food 'wanting'

Several NAc lesion studies indicate that the NAc does not function as a necessary cause for reward 'liking'. For example, animals with NAc lesion do not show impairments in food intake, learning Pavlovian or instrumental conditioning for food reward, or responding for food reward (Albertin, Mulder, Tabuchi, Zugaro, & Wiener, 2000; Dworkin, Guerin, Goeders, & Smith, 1988; Meredith, Baldo, Andrezjewski, & Kelley, 2008; Parkinson, Olmstead, Burns, Robbins, & Everitt, 1999). Thus, while evidence suggests that VP may be both necessary and sufficient for reward 'liking', dorsal medial NAc shell may only be sufficient but not necessary for reward 'liking'. In this dissertation, I aim to compare the effect of disrupting NAc shell and VP to assess their relative necessities in normal hedonic reactions.

Human NAc plays a critical role in reward processes

Several human imaging studies have supported the idea that NAc is involved in reward processing. Both PET studies and fMRI studies have shown that neural activity in NAc (or ventral striatum) increased in response to primary reward (taste, smells, sound, etc) (Berridge & Kringelbach, 2008; Anna Rose Childress, et al., 2008; Goldstone, et al., 2009; Kringelbach, 2004; Kringelbach & Berridge, 2009; Stoeckel, et al., 2008) and secondary reward (money, music, humor) (Blood & Zatorre, 2001; Knutson, Bhanji, Cooney, Atlas, & Gotlib, 2008; McClure, York, & Montague, 2004; Mobbs, et al., 2009; Singer, et al., 2006). Additionally, human NAc has also been reported to decrease neural activity in response to different forms of aversion, including loss of money, social inequity, and facial expression of aversion (Beaver, Lawrence, Passamonti, & Calder, 2008; Tom, Fox, Trepel, & Poldrack, 2007; Tricomi,

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Rangel, Camerer, & O'Doherty, 2010; Wagner, et al., 2007). Together, these results suggest that NAc play a bivalent role in reward processing.

Comparison between the opioid hedonic hotspots in NAc and in VP

The opioid hedonic 'liking' hotspot in NAc shell, together with the opioid 'liking' hotspot in VP, form a functional circuit that control 'liking' and 'wanting' processes (K. S. Smith & Berridge, 2007). Opioid activation at either hotspot recruits the other hotspot, as measured by Fos expression, while blockade of either site disrupts 'liking' elevation induced by opioid activation in the other site, suggesting that both sites are needed for 'liking' enhancement. In contrast, elevation of food 'wanting' induced by opioid neurotransmission in NAc shell can function even without an opioid signal in VP, which is produced by microinjections of an opioid antagonist. Thus, for *sufficient* mechanisms, hedonic hotspot in NAc shell and hedonic hotspot in VP contribute equally to 'liking' processes but differently to 'wanting' processes via opioid neurotransmission. How about their *necessary* role in reward processes? In other words, are they both needed for the normal functioning for 'liking' and/or 'wanting'? There is evidence that suggests VP hotspot may be necessary for normal 'liking' and normal 'wanting'. For example, microinjections of excitotoxins, which produced neuronal lesion, in a location that encompasses VP hedonic hotspot and the surrounding area cause the animals to develop aphagia, or loss of food appetite, along with suppression of hedonic 'liking' to sweet tastes (Cromwell & Berridge, 1993; Schallert & Whishaw, 1978; Shimura, et al., 2006; Stellar, et al., 1979; Teitelbaum & Epstein, 1962). Some earlier studies suggested that microinjections of excitotoxin in NAc shell did not suppress food appetite. However, no study prior to this dissertation examined whether or not NAc shell is *necessary* for normal 'liking' (Albertin, et al., 2000; Dworkin, et al., 1988; Meredith, et al., 2008; Parkinson, et al., 1999).

Other Potential 'Liking'- Enhancing Neurotransmitters

Orexin

One potential 'liking'-enhancing neurotransmitter is orexin. Orexin neurons in the lateral hypothalamus (separate from arousal-related orexin neurons, which

are located more laterally) can monitor changes of nutrients and appetite hormones in the system (Acuna-Goycolea & van den Pol, 2004; Asakawa, et al., 2002; Burdakov & Alexopoulos, 2005; Cai, et al., 1999; Diano, Horvath, Urbanski, Sotonyi, & Horvath, 2003; Dube, Horvath, Kalra, & Kalra, 2000; Ganjavi & Shapiro, 2007; Griffond, Risold, Jacquemard, Colard, & Fellmann, 1999; Lopez, et al., 2000; Niimi, Sato, & Taminato, 2001; Perello, et al., 2009; Shiraishi, Oomura, Sasaki, & Wayner, 2000; Takenoya, et al., 2005; Toshinai, et al., 2003; Yamanaka, et al., 2000) and at the same time send wide projections to reward- associated areas (Baldo, Daniel, Berridge, & Kelley, 2003; Cutler, et al., 1999; Marcus, et al., 2001; Nixon & Smale, 2007; Peyron, et al., 1998; Trivedi, Yu, MacNeil, Van der Ploeg, & Guan, 1998). Although traditional studies focused more on the role of orexin in homeostasis, studies on orexin's role in food and drug rewards in the last decade have produced remarkable results and opened a new direction of research in the field. The first report of a possible role of orexin in food reward appeared in 1998 (Sakurai, et al., 1998). This study started a new line of researches that characterize oreinx's functional role in food reward. For example, it has been shown that microinjections of orexin in lateral hypothalamus facilitated eating and enhanced operant responding for food reward (Alberto, Trask, Quinlan, & Hirasawa, 2006; Clegg, Air, Woods, & Seeley, 2002; Harris & Aston-Jones, 2006; Kotz, Teske, Levine, & Wang, 2002; Rodgers, et al., 2000; Sharf, Sarhan, Brayton, et al., 2010; Thorpe, Mullett, Wang, & Kotz, 2003), while orexin knockout caused hypophagia and showed decreased operant responding for food (Akiyama, et al., 2004; Hara, et al., 2001; Haynes, et al., 2000; Ishii, Blundell, Halford, Upton, Porter, Johns, Jeffrey, et al., 2005; Ishii, Blundell, Halford, Upton, Porter, Johns, & Rodgers, 2005; Rodgers, et al., 2001; Sharf, Sarhan, Brayton, et al., 2010). Furthermore, one single dose of orexin antagonist sufficiently reduced food intake and blocked orexin-induced hyperphagia. Together, the results suggest that orexin may mediate motivation to seek and consume food rewards.

The first study that identifies orexin's possible role in drug reward happened in 2003 (Georgescu, et al., 2003), with findings that orexin neurons are involved in opiate withdrawal. From then on, a series of studies, particularly those from the Aston-Jones group, provide new insights on orexin's prominent role in reward

processing (Aston-Jones, Smith, Moorman, & Richardson, 2009; Aston-Jones, et al., 2010; Harris & Aston-Jones, 2006; Harris, Wimmer, & Aston-Jones, 2005; Harris, Wimmer, Randall-Thompson, & Aston-Jones, 2007; Kane, et al., 2000; Lawrence, 2010; Moorman & Aston-Jones, 2009; Sharf, Sarhan, & Dileone, 2008, 2010; R. J. Smith, See, & Aston-Jones, 2009; von der Goltz, et al., 2009; Zhou, et al., 2006). For example, orexin neurons were activated by reward- associated cues (food, morphine, cocaine), and this activation varies in proportion to animals' preference of the reward (Harris, et al., 2005). The blockade of orexin neurotransmission disrupts cue-induced reinstatement of cocaine-seeking behavior in self-administrating rats (Aston-Jones, et al., 2009; Harris & Aston-Jones, 2006; Harris, et al., 2007). One study particularly relevant to the current dissertation showed that orexin neurons may be able to track hedonic processing during protracted withdrawal; Fos expression of LH orexin neurons were higher when morphine withdrawn animals were tested with drug preference (compared to normal animals) and lower when these same animals were tested with food or novelty preference, which correspond with these animals' behavior (Aston-Jones, et al., 2010; Harris, et al., 2005).

So, is it possible that orexin signal can sufficiently enhance 'liking'? Two other pieces of information led us to hypothesize that VP might be involved in orexin-mediated hedonic processes. Firstly, LH orexin neurons send dense projections to VP, with particular focus on the caudal part where the opioid hedonic hotspot is located (Baldo, et al., 2003; Peyron, et al., 1998). Corresponding to the dense orexin projections, VP contains significant level of OrexinA and OrexinB receptors (Cutler, et al., 1999; Marcus, et al., 2001). Together, the anatomical findings suggest that VP has a tight anatomical connection with LH orexin neurons. Secondly, orexin and opioid show strong interaction when processing food reward. For example, functional opioid pathways are necessary for Orexin A –induced eating, and orexin A singal in ventral tegmental area is required for high fat appetite induced by orexin injections in nucleus accumbens (Clegg, et al., 2002; Sweet, Levine, & Kotz, 2004; Zheng, Patterson, & Berthoud, 2007). Thus, it is likely that the opioid

'liking' hotspot in the caudal VP interacts with orexin to mediate hedonic aspect of reward, which leads to the second study of this dissertation.

Endocannabinoids

Another potential hedonic neurotransmitter is cannabinoid. Cannabinoid drugs (e.g. THC extracted from marijuana plants) and endocannabinoids (anandamide and 2-AG) have been shown to increase appetite (e.g. marijuana 'munchies'), especially for sweet food (DiPatrizio & Simansky, 2008; Mahler, et al., 2007; Shinohara, Inui, Yamamoto, & Shimura, 2009; Solinas, Goldberg, & Piomelli, 2008; Williams & Kirkham, 2002a; Williams, Rogers, & Kirkham, 1998), and are associated with pleasure in both humans and animals (Higgs, Williams, & Kirkham, 2003; Jarrett, Limebeer, & Parker, 2005; Mahler, et al., 2007; Solinas, et al., 2008). In humans, anecdotal observations from more than 30 years ago described that marijuana induced not only hyperphagia but also the desire to consume highly palatable food in humans (so called as 'marijuana munchies'). In animals, microinjections of cannabinoid drugs and endocannabinoids stimulate eating not only in normal animals but also in sated animals (Williams, et al., 1998). In addition, cannabinoid appeared to enhance hedonic impact of reward, as measured by taste reactivity paradigm or voluntary licking bouts at a sucrose spout (Higgs, et al., 2003; Jarrett, et al., 2005). Prior to this dissertation, only one study had examined the specific location where this cannabinoid- induced elevation of hedonics happens, and identified dorsal medial shell as a critical site where microinjections of anandamide produced >150% of hedonic reactions than normal (Mahler, et al., 2007). Interestingly, this cannabinoid hedonic hotspot highly overlaps with the opioid liking hotspot in the NAc shell. Ventral pallidum, the only other structure we know so far that also contains an opioid hedonic hotspot, is anatomically and functionally linked to NAc in mediating 'liking' of reward. In addition, VP contains a high level of CB1 receptors (Glass, Dragunow, & Faull, 1997; Mailleux, Parmentier, & Vanderhaeghen, 1992; Mailleux & Vanderhaeghen, 1992). Thus, we speculate that VP may contain an endocannaibinoid hedonic hotspot where anandamide induces elevation of 'liking'.
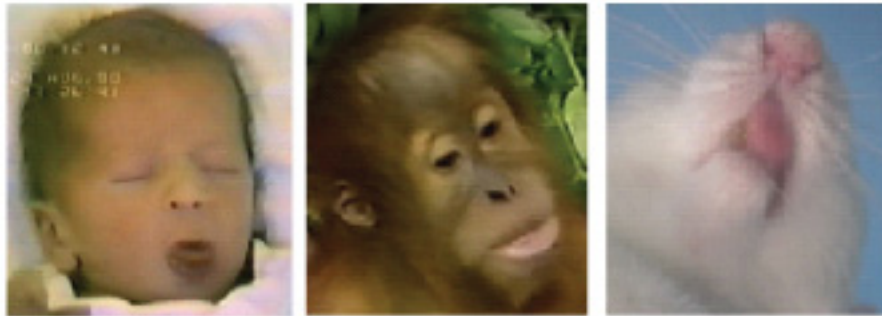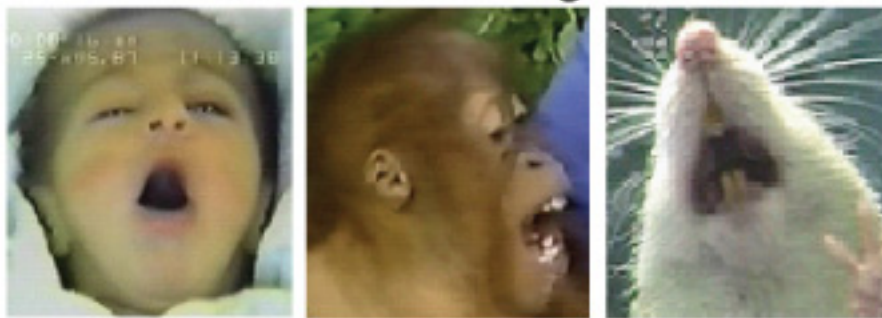
Figure1-1 Taste reactivity reactions across species. The top row shows an example of hedonic 'liking' reactions to a sweet taste in a rat, primate, and human infant (homologous rhythmic tongue protrusions). The bottom row shows an example of aversive 'disliking' reactions to an unpleasant bitter taste (homologous gapes). Orofacial expressions such as these provide an objective index of 'liking' and 'disliking' reactions to the hedonic impact of tastes.
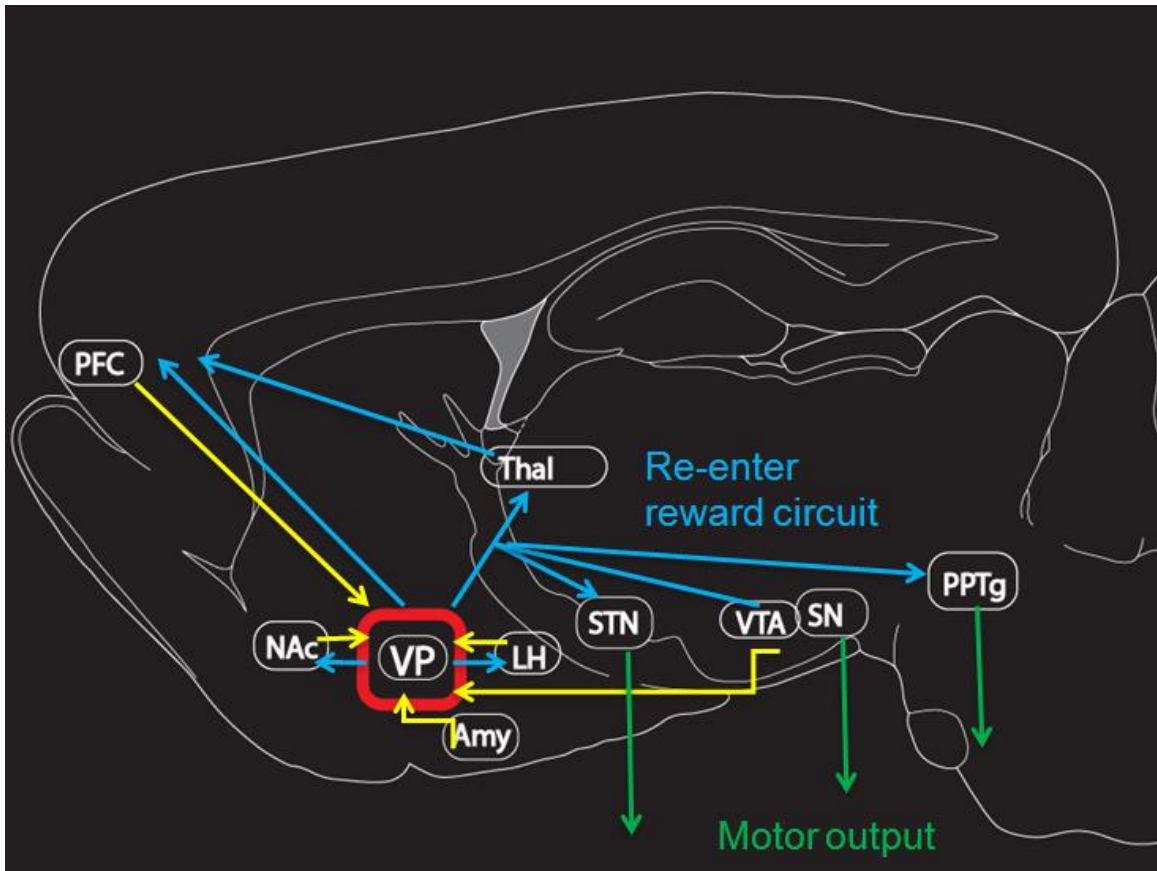
Figure 1-2 Sagittal view of major ventral pallidum circuits. The ventral pallidum (VP) receives input (yellow) from the amygdale (Amy), nucleus accumbens (NAc), prefrontal cortex (PFC), ventral tegmental area (VTA), lateral hypothalamus (LH), etc. In turn, VP projects reciprocally to most of its input structures (blue) for feedback of reward-related information. In addition, VP projects to the mediodorsal thalamus (Thal) for relay to cortex and also projects to cortex directly. Separately, VP projects to motor-related areas, including subthalamic nucleus (STN), substantia nigra (SN), and pedunculopotine nucleus (PPTN) to translate reward signals into behavioral action.

Chapter 2

Where in the VP does GABA Inhibition or Lesion Cause Aversive 'Disliking'?

Introduction

How the brain determines if a sensory stimulus is positively 'liked' or negatively 'disliked' is crucial for decision- making and for survival (Berridge & Robinson, 2003; Kelley, Schiltz, & Landry, 2005; Kringelbach, 2005). The immediate pleasure, or 'liking', followed by the receipt of a positive reward reinforces reward-associated behavior and increases the motivation to pursue the reward. In contrast, aversive 'disliking' followed by the receipt of a sensory stimulus promotes avoidance and decreases animals' motivation to approach the stimulus and thus protects the animal from objects that can be harmful for survival. In affective psychopathology, misattributions of 'liking' and 'disliking' of rewards are likely to be the causes of some clinical problems, including obesity, drug addiction, depression, etc (Aston-Jones, et al., 2010; Berridge, 2007; Berridge, Ho, Richard, & Difeliceantonio, 2010; Berridge, Robinson, & Aldridge, 2009; Berthoud, 2004, 2007; Berthoud & Morrison, 2008; Blundell & Finlayson, 2004; Borsook, et al., 2006; Everitt & Robbins, 2005; Finlayson, King, & Blundell, 2007; Hoebel, 1985; Hyman, 2005; Kelley, 2004; Kelley, et al., 2002a; Kelley, Schiltz, et al., 2005; Koob, 2000; Koob & Le Moal, 2001; Mela, 2006; Napier & Mickiewicz, 2010; Pecina, et al., 2006; Robinson & Berridge, 1993, 2008; Small, 2009; Volkow, Wang, Fowler, & Telang,

2008; Zheng & Berthoud, 2007; Zubieta, et al., 2003). Therefore, it is important to understand the mechanisms underlying the generation of 'liking' and 'disliking' of reward.

The ventral pallidum might be one of the best neural substrates to study 'liking' and 'disliking' of reward. This is due to its unique anatomical location in mesocorticolimbic reward circuit and its dichotomy role in processing both positive and negative rewards (Calder, et al., 2007; Johnson, Stellar, & Paul, 1993; Kretschmer, 2000; K. S. Smith & Berridge, 2005). Anatomically, VP receives integrated GABA, glutamate, opioid, and dopamine inputs from nucleus accumbens (NAc), striatum (STR), amygadala (Amy), and prefrontal cortex (PFC) (Carnes, et al., 1990; Chrobak & Napier, 1993; Fuller, et al., 1987; Groenewegen & Berendse, 1990; Grove, 1988; Klitenick, et al., 1992; Maurice, et al., 1997; Napier, et al., 1991; Turner, et al., 2001; Usuda, et al., 1998a; Zaborszky, et al., 1997; Zahm, et al., 1985). In return, VP projects reciprocally to most of these reward-related areas, upwards to cortical areas via the medial dorsal nucleus in the thalamus, and downwards to motor-related areas, such as subthalamic nucleus (Churchill & Kalivas, 1994; Churchill, et al., 1996; Groenewegen, et al., 1993; Haber, et al., 1985a; Haber, et al., 1993; Heimer, et al., 1987; Young, et al., 1984; Zahm, et al., 1987). The critical location of VP in reward circuit has led to the hypothesis that VP serves as a 'final common path for mesocorticolimbic pathway' (Kelley, Baldo, Pratt, et al., 2005; Pecina, et al., 2006; K. S. Smith, et al., 2009; Waraczynski & Demco, 2006; Zahm, 2006).

Functionally, VP has been shown to be heterogeneously involved in reward processing depending on the anatomical subregions and the neurochemical systems being involved. Microinjections of μ-opioid agonist (DAMGO) in the posterior VP and in the anterior VP differentially increase and decrease intracranial self-stimulation (ICSS)-induced reward (Johnson, et al., 1993; Kretschmer, 2000). The result suggests that different subregions of the VP are distinctively involved in reward processing. Furthermore, Smith & Berridge showed that μ opioid agonist (DAMGO) in a cubic-millimeter 'hedonic hotspot' in the posterior VP sufficiently

enhances rats' orofacial 'liking' reactions to sucrose, while the same manipulation in anterior VP almost completely abolishes 'liking' to the sweet sucrose taste(K. S. Smith & Berridge, 2005, 2007). Thus, similar to μ-opioid modulation of ICSS-induced rewards, μ-opioid causes a gradient of increased 'liking' to decreased 'liking' in VP along the posterior to anterior axis.

At the same time, posterior VP damage or inhibition has also been shown to be involved in aversive 'disliking' aspect of rewards (Cromwell & Berridge, 1993; Schallert & Whishaw, 1978; Shimura, et al., 2006; Stellar, et al., 1979; Teitelbaum & Epstein, 1962). Permanent neuronal loss caused by excitotoxin lesions in a small area that involves posterior VP almost completely abolished hedonic 'liking' responses (e.g. rhythmic tongue protrusions, lateral tongue protrusions) to sucrose infusion and replaced them with 'disliking' responses (e.g. gape, forelimb flail, headshake) (Cromwell & Berridge, 1993). Consistent with the VP lesion result, temporary inhibition of VP by GABA (A) agonist muscimol microinjections also caused decreased 'liking' reactions and increased 'disliking' reactions to sucrose, along with general decreases of fluid intake regardless of tastes (Shimura, et al., 2006). However, the anatomical recognition of VP morphology has changed dramatically in brain atlases in the past decade, and it is unclear where in the VP GABA inhibition caused an increase of aversion to sucrose, or whether the GABA-induced aversive site and the lesion-induced aversive site are essentially the same site in VP.   That is the chief question I am setting out to answer in this dissertation.

The NAc is a structure that has reciprocal connections with the VP and also contains a cubic millimeter opioid hedonic hotspot in the rostrodorsal medial shell (Churchill & Kalivas, 1994; Groenewegen, et al., 1993; Hakan, Berg, & Henriksen, 1992; Mogenson & Yang, 1991; Pecina & Berridge, 2005; K. S. Smith & Berridge, 2005; Zahm, 2000; Zahm & Heimer, 1993). Similar to the opioid hedonic hotspot in caudal VP, microinjections of a μ-opioid agonist amplify hedonic 'liking' reactions to sucrose, as well as 'wanting' for food (Pecina & Berridge, 2005). Also, previous studies suggest the two opioid hedonic hotspots interact to mediate reward 'liking'. For example, microinjections of μ- opioid agonist in one of the two hotspots recruit

the other hotspot as measured by c-Fos activation. The blockade of opioid signal in either of the two hotspots disrupts the effect of hedonic 'liking' enhancement induced by opioid microinjections in the other hotspot (K. S. Smith & Berridge, 2007). Together, the results suggest that NAc opioid hedonic hotspot and VP opioid hedonic hotspot interact to mediate hedonic 'liking' and are required for the opioid-induced amplification of 'liking' to sucrose. However, several NAc lesion studies indicate that the NAc hotspot may function as a necessary cause for reward 'liking'. For example, animals with NAc lesions do not show impairments in food 'wanting', learning Pavlovian or instrumental conditioning for food reward, or in responding to food reward (Albertin, et al., 2000; Dworkin, et al., 1988; Meredith, et al., 2008; Parkinson, et al., 1999). Thus, while evidence suggests that VP may be both *necessary* and *sufficient* for reward 'liking', dorsal medial NAc shell may only be *sufficient* but not *necessary* for reward 'liking'. I aim to compare the effect of disrupting nucleus accumbens shell and VP to assess their relative necessities in normal hedonic reactions.

The identification of neural substrates involved in 'disliking', or aversion, processes is particularly important for understanding the perception of facial expression of disgust in humans and disorders associated impairments in disgust perception, such as obsessive-compulsive disorders (OCD), Huntington's disease, anxiety disorders, etc (Adolphs, 2002; Adolphs, et al., 2003; Olatunji, et al., 2010; Phillips, et al., 1997; Sprengelmeyer, et al., 1996; Sprengelmeyer, et al., 1997; Surguladze, et al., 2010; Suzuki, et al., 2006). Since the time of Darwin, it has been argued that the emotion of disgust has its evolutionary origin in the rejection of harmful substances and contaminants. Some investigators of studying moral disgust have suggested that such abstract emotions are generated out of the same brain mechanisms that originally evolved to generate gustatory disgust or 'disliking' (Phillips, et al., 1997; Rozin & Fallon, 1987; Rozin, et al., 1994). Indeed, several studies have demonstrated that taste disgust and emotional disgust or moral disgust share the same facial expression and neuronal substrates (Calder, et al., 2007; Chapman, et al., 2009; Olatunji, et al., 2010; Phillips, et al., 1997). Lesion studies and imaging studies of human subjects that showed impairments in disgust recognition

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further identified the most crucial neural substrates, including basal ganglia in which VP is one of the main structures, in the processes of taste disgust and emotional disgust(Adolphs, 2002; Calder, et al., 2000; Phillips, et al., 1997; Sprengelmeyer, et al., 1996; Suzuki, et al., 2006). Thus, the identification of brain mechanisms that generate gustatory 'disliking' may carry implications that extend into more abstract forms of psychological disgust.

Goals of the study

The goals of the present study were to functionally map where local GABA inhibition or excitotoxin lesions cause an increase of aversive 'disliking' reactions to a natural sweet taste (sucrose), and to compare the localizations of the two aversion sites. To measure GABA-induced and lesion-induced changes in aversion to sucrose, we used taste reactivity paradigm (a measurement based on homologous orofacial expressions of affective 'liking' and 'disliking' reactions elicited by tastes in human infants, apes, monkeys, and rats) to quantify changes in aversive 'disliking' reactions to sucrose. Drug-induced changes in aversive 'disliking' reactions to a bitter quinine taste were also quantified as comparison and control. To estimate the intensity and area of GABA-induced neuronal inhibition, we used the Fos plume technique (a measurement based on drug-induced Fos activation in tissues surrounding microinjection tips) to label and measure Fos protein expression caused by GABA microinjections and compare it to the ACSF/vehicle condition. Similarly, a lesion cell-count mapping technique was used to estimate the intensity and area of excitotoxin- induced neuronal loss. Our results indicate that both GABA inhibition and lesion significantly increased aversive 'disliking' to sucrose in sites that are overlapped but non- identical in the caudal VP.

Materials and Methods

General design

Two groups of rats were used to construct maps of 'disliking' response to sucrose and maps of 'disliking' response to quinine (Figure 3-1). In the first group of rats, within-subject design was used to test GABA and lesion effects over vehicle (Figure 3-1, top row). Rats in this group were implanted with both cranial cannulae

and oral cannulae, tested with taste reactivity test to quantify the intensity of 'disliking' responses to sucrose or quinine after drug microinjections (ACSF or GABA agonists) or lesion, and euthanized for microinjection center identification and lesion quantification. Rats in the second group (Figure 3-1, middle row) were used to construct GABA Fos plume maps, which quantify the intensity and area of neuronal activation/inhibition caused by GABA microinjections. Rats in this group were implanted with cranial cannulae, handled, and microinjected with drugs, and euthanized for Fos activation quantification. Fos protein activation in neurons around the microinjection center was compared to that in the non-surgical tissues in order to estimate the area and intensity of neuronal inhibition caused by GABA microinjections.

For maps about GABA effects, the results from both groups are combined to construct maps of 'liking' response to sucrose or 'disliking' response to quinine by color coding the behavioral effects (from the first group) onto the Fos plume symbols (from the second group) placed onto the microinjection centers identified by histology (first group) (Figure 3-1, bottom row). To precisely locate the most intense GABA- /lesion- induced 'disliking' effect in the ventral pallidum, we combine results from the taste reactivity test and GABA Fos plume/ lesion plume measurement, and present the combined results with color symbols. The size of the symbols represents the averaged intensity and area of GABA-induced Fos inhibition/lesion-induced neuronal loss in VP, while the color of the symbols represents the increases or decreases of 'disliking'. To precisely locate the most intense GABA- /lesion- induced 'disliking' effect in ventral pallidum, we combine results from taste reactivity and GABA Fos plume/ lesion plume measurement, and present the combined results with color symbols. The size of the symbols represents the averaged intensity and area of GABA-induced Fos inhibition/lesion-induced neuronal loss in VP, while the colors of the symbols represent the increases or decreases of 'disliking' (Figure 3-1). To further localize the site where lesions caused aversion, the behavioral results and the histology results of neuronal counts from the first group are combined.

Surgery

Under anesthesia (80mg/kg ketamine HCL, 10 mg/kg xylazine, and 0.2 ml atropine sulfate), male Sprague-Dawley rats (GABA n=62, lesion n=53, 250-400g) were bilaterally implanted with guide cannulae (23 gauge) positioned 2 mm above the ventral pallidum (VP), or surrounding structures, for microinjection of drugs. Coordinates for implantation were: anteroposterior (AP), 0 to -0.8 mm; mediolateral (ML), ± 3 mm; dorsoventral (DV), -5.5 mm. The cannulae were anchored to the skull with stainless steel screws and dental cement. Obturators were inserted in guide cannulae to prevent clogging. Rats were given at least 7 days to recover before behavioral testing. In the same surgery, all rats were bilaterally implanted with intraoral cannulae (PE-100 tubing) for delivery of the taste solutions. Oral cannulae enter the mouth lateral to the first maxillary molar, travel beneath the zygomatic arch, and exit the dorsal head near the skull screws. Oral cannulae did not disrupt normal eating. All rats were given at least 7 days to recover prior to the first test day.

Drugs and microinjections

GABA (A) agonist muscimol (0.1μg) or the combination of GABA(A) agonist muscimol (0.1μg) and GABA(B) agonist baclofen (0.1μg) was dissolved in ACSF (0.2μl). No behavioral difference was found between microinjections of GABA (A) agonist and the cocktail of GABA (A) agonist and GABA (B) agonist, and results from the two groups were thus combined.

As for the order of microinjections, rats always received vehicle (ACSF) on the first day and GABA agonists on the second test day (n=62). The reason for GABA to be tested after vehicle was because GABA microinjections usually caused aversion in rats, which could lead to rats' association of aversion with the testing environment and contaminate results for the subsequent test days. In order to make sure results from ACSF microinjections are not being contaminated by the lingering aversion caused by GABA microinjections, ACSF was always tested first and served as control in the within-subject design. Tests were spaced at least 48 h apart. On a particular test day, each rat received bilateral microinjections of 0.2 μl per side of

vehicle or GABA agonists. Microinjections were made through a stainless-steel injector cannula (29 gauge) connected to PE-20 tubing and a syringe pump at a rate of 0.2 μl /min. Microinjector tips were held in place for an additional 1 minute after infusion to allow drug to diffuse away from the cannula tip and to avoid backflow. After microinjections, stylets were inserted back to the cranial cannulae to prevent clotting or infection, and rats were placed into the transparent taste reactivity chamber with intraoral fluid delivery tubing attached (described in behavioral taste reactivity testing).

Lesion

Behavioral effects caused by excitotoxin lesions were always tested on the last test day, for lesion-induced irreversible neuronal loss and permanent changes of cellular composition would confound later testing results. To lesion VP and surrounding areas, rats were injected with excitotoxin (ibotenic acid, 15 μg in 1 μl 0.1M PB, or quinolinic acid 10 μg in 1 μl 0.1M PB) (n=54) intended to destroy neurons via microinjection setup. Rats were first anesthetized with halothane (within a desiccator) and then methoxy-fluorane given through an air anesthesia system for small animals. Excitotoxin (1 μl) was injected over a 3-min period. Injectors were left in place for an additional 5 min. Rats were maintained under halothane anesthesia for an additional 25 min before being placed back to their home cages. Diazepam (8mp/kg) was given 5 min and 35 min after excitotoxin injection to prevent seizure. All rats were given cereal mash, food pellets, and water in their home cages. Food intake was monitored by the approximate amount of mash eaten. Rats were considered aphagia if neither chow pellets nor cereal mash were eaten. Rats with severe aphagia were incubated with 6 to 12 ml sweetened milk solution, up to 3 incubations per day. Taste reactivity tests and food intake tests of lesion effects were conducted at least 24 hours after lesion to allow neuronal loss to happen.

Behavioral taste reactivity testing

Rats were handled and habituated in the taste reactivity chamber for 4 consecutive days for 30 minutes each before behavioral testing, and received a mock injection of vehicle on the final habituation day. On each test day, hollow fluid

delivery tubing (PE-50 connected to a PE-10 delivery nozzle) was attached to the intraoral cannula to deliver solutions. The other end of the tubing was attached to a syringe pump, which infuses solutions at a stable rate of 1 ml/min. Rats received sucrose solution (1%, 1 minute duration) 30 minutes after vehicle/GABA microinjections or 24 hours after lesion. Oral facial 'liking' and 'disliking' responses were video recorded via an angled mirror placed underneath the transparent floor during the one- minute test. Some rats (n=29) received additional quinine infusions (3x10-4M, 1 minute duration) 10 minutes after sucrose infusions. Quinine was always infused and tested after sucrose because hedonic 'liking' responses to sucrose are generally more vulnerable to contamination from the prior taste compared to aversive 'disliking' responses to quinine.

Taste reactivity video scoring

Hedonic, aversive, and neutral response patterns were scored offline using frame-by-frame slow-motion videotape analysis developed to assess hedonic *vs.* aversive taste valuations (Berridge, 2000). Hedonic responses included rhythmic tongue protrusions, lateral tongue protrusions, and paw licks. Aversive responses included gapes, headshakes, face washes, forelimb flails, and chin rubs. Neutral responses, which are less consistently linked to hedonic/aversive taste valuation, included grooming, passive dripping of solution out of the mouth, rhythmic mouth movement, and doing nothing. All video analyses were conducted blind to the microinjection contents and cannula placements using Observer software (Noldus, Netherlands).

A time-bin scoring procedure was used to ensure that taste reactivity components of different relative frequency still contributed equally to the final affective hedonic/aversive totals (Berridge, 2000). For example, rhythmic mouth movements, passive dripping of solution, paw licking, and grooming typically occur in long bouts and were thus scored in 5 s time bins (5 s equals one bout occurrence). Tongue protrusions, which occur in shorter bouts, were scored in 2 s time bins (2 s equals one bout occurrence). The other behavioral components (lateral tongue protrusions, gapes, forelimb flails, head shakes, and chin rubs) typically occur in discrete events and were thus scored as single occurrences each time they occurred

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(e.g., one gape equals one occurrence). Finally, individual totals were calculated for hedonic versus aversive categories by adding all response scores within an affective category for each rat. A hedonic 'liking' reaction total was the sum of occurrences for lateral tongue protrusions, rhythmic tongue protrusions, and paw licks. Similarly, an aversive 'disliking' reaction total was the sum of occurrences of gapes, headshakes, face washes, forelimb flails, and chin rubs.

Food intake test

To further confirm that aversion induction after potent excitotoxin lesions or GABA inhibition (temporary lesions) translated into suppression of appetitive behavior, and also to probe the ability of lesions to suppress food 'wanting' without necessarily inducing 'disliking', I assessed the drug effects and lesion effects on food intake and general behavior by placing rats in clear cages with food, bedding, and water immediately after taste reactivity test. Chow pellets and water were premeasured and freely available for 1 hour. Rats were videotaped during the test for subsequent offline scoring of eating, locomotion, and other behaviors. Food intake was measured after the test.

Histology

After completion of behavioral experiments, tissues were processed in two different ways based on weather or not the animals developed lesion-induced aphagia (loss of appetite to eat). For rats that did not develop aphagia, the tissues were processed only for identification of placements for microinjector centers. The rats received an overdose of sodium pentobarbital (0.2g/kg). Rats' brains were extracted and post-fixed with 10% formalin in 0.1 M phosphate buffer (PB) for a few days and then transferred to 30% sucrose in 0.1M PB. Brains were sectioned with a freezing microtome into 60 μm, stained with cresyl violet, and mapped for the centers of the microinjections according to Paxinos and Watson atlas (2008). For rats that developed aphagia, the tissues were processed both for identification of microinjection centers and quantification of neuronal loss. Rats were perfused transcardially with 0.9% saline, followed by 4% paraformaldehyde solution. Their brains were removed, post-fixed with 4% paraformaldehyde, transferred to 30% sucrose in 0.1MPB, sectioned (40 μm), mounted on slides, stained with cresyl violet,

and mapped for the centers of the microinjection tips. Images of brain slices with microinjection centers were also taken under the microscope and processed offline to assess the extent of neuronal loss.

General procedures for Fos plume measurement

Images of Fos activation, stained by c-Fos immunohistochemistry, were taken under the microscope and processed offline to assess the extent of Fos activation (Figure 2-2). To quantify the area and the intensity of GABA-induced neuronal inhibition, Fos plumes caused by microinjections of GABA agonists were compared to Fos plumes caused by vehicle microinjections and non-surgical tissues (Figure 2-2, top). GABA-induced Fos plumes were thus constructed and further divided into different intensity zones. The radii for each intensity zone were calculated and averaged. Assuming a spherical shape of functional drug diffusion, the averaged radii were applied to calculate the volumes of different Fos intensity zones and to construct the averaged Fos plume symbol of different intensities on the 2-D plane (Figure 2-2, bottom).

Fos-like protein immunohistochemistry

Rats in the Fos plume group (n=10) were handled gently for 3 days for 10 min each, similar to the behavioral groups, and then microinjected bilaterally in the VP with either cocktail of GABA agonists (muscimol 0.1 μg, baclofen 0.1 μg, in 0.2 μl ACSF) or vehicle (ACSF). A separate group of non-surgical rats went through the same procedures but were held by the experimenter for 5 minutes instead of having microinjections. Ninety minutes later, rats were overdosed with sodium pentobarbital before transcardial perfusion. After perfusion, brains were removed and placed in 4% paraformaldehyde for 2 hours, transferred to 30% sucrose overnight, sectioned at 40 μm, and stored in 0.1M NaPb, pH 7.4. Alternate series of sectioned slices were processed for immunofluorescence.

For immunofluorescence processing, we used the avidin-biotin procedure (Hsu et al, 1981). In brief, brain sections were rinsed (0.1MNaPB+ 0.2% Triton), pre-blocked (5% Norrmal Donkey Serum (NDS) in 0.1M NaPB and 0.2% Triton), and incubated in primary antibody (goat anti- Fos 1:500) and 5%NDS overnight at 4'C.

After primary incubation, the slices were once again rinsed, pre-blocked, and incubated in secondary antibody (4% donkey-anti-goat Alexa 488) and 5% NDS for 2 hours followed by final rinses. The slices were mounted in 0.01M NaPB, let dried, and cover slipped with anti-fade.

Fos plume maps of GABA-Induced neuronal inhibition spread.

The procedure for measuring drug-induced Fos plumes immediately surrounding a local microinjection site was adopted from previous studies from our lab (Mahler, et al., 2007; K. S. Smith & Berridge, 2005).

To visualize and quantify drug-induced Fos activities, Fos-labeled cells on tissue surface were visualized with 5x–40x magnification under the microscope followed by image processing (Figure 2-2, top). Fos count and Fos plume construction were done post-hoc by an observer that was blind to the drug conditions. On the Fos images, sampling blocks (50 x 50 µm) were placed along seven radial arms emanating from the center of the microinjection site (45, 90, 135, 180, 225, 270, 315°) at 50 µm intervals (Figure 2-2, top). The 360° or 0° arm was not sampled because it was occupied by the microinjection cannulae track. Fos-labeled cells in each individual sampling box was counted individually (Figure 2-2, insets). In order to establish baseline of Fos activation, two control conditions were measured: (1) in normal VP tissue of intact brains without damages from surgical intrusion or gliosis (2) around the site of vehicle microinjections in the VP to assess Fos activation caused by microinjector tips and by the pressure from vehicle microinjections. GABA-induced Fos inhibition was also measured and compared to normal and vehicle-induced Fos at the same site (Figure 2-2, insets).

GABA microinjections significantly decreased Fos activation compared to vehicle microinjection tissues. However, GABA microinjections did not significantly decreased Fos activation compared to normal tissue due to floor effect of Fos activation on normal tissues. As a result, in this study, we only mapped GABA inhibitory Fos plumes contrasted to vehicle and divided the map into zones of 'intense', 'moderate', or 'mild' inhibitions (Figure 2-2). To determine the averaged radii of intense, moderate, and mild Fos inhibition zones around the microinjection center, the distances from the microinjection center to the outer most sampling box

of 65%, 40%, and 25% Fos decreases were measured and averaged across all seven radial arms in all animals. The averaged radii were applied to determine the size of the hexagon map symbol, which represents the averaged sizes of 65%, 40%, and 25% Fos inhibition zones from the microinjection center (Figure 2-2, bottom).

Lesion plume maps of excitotoxin-induced neuronal inhibition spread

Similar to the construction of GABA plume maps, images of Nissl-stained brain slices (lesion or normal) were taken under the microscope, sampling blocks (125 x 125 μm) were placed onto the image along seven radial arms (with no interval), and the number of neurons in each sampling box was counted individually (Figure3-3). In order to establish baseline value for different subareas at different anterior-posterior (AP) levels, normal rat brains were divided into 6 sections along the AP axis (AP 0.3~0 mm; AP 0~-0.3 mm; AP -0.3~ -0.65 mm; AP-0.65~-1; AP -1~ -1.6 mm; AP after – 1.6 mm) and each section was divided into 5-7 subareas around ventral striatum based on results of neuronal density from previous studies (Cromwell & Berridge, 1993, 1994) (Figure 3-3, top). To quantify the baseline value for each subarea, at least 3 sampling boxes were placed onto each image, and at least 4 images were used to calculate the averaged number of neuron for each subarea. In order to quantify the level of neuronal loss, the number of neurons in each sampling box in lesioned tissue was calculated and compared to the baseline number of neurons in the corresponding subarea in normal tissue (Figure 3-3, top insets). And then we mapped lesion plume maps over normal and divided the map into zone of 'intense' (80%), 'moderate' (50%), or mild (30%) neuronal loss. In order to decide the critical level of neuronal loss which lesion caused aversion, we compared the site and the extent of neuronal loss between two groups of animals: animals that are 'aphagic and aversive' (n=5) and animals that are 'aphagic but not aversive' (n=4). For animals that are aphagic and aversive, the lesion sites focus in posterior VP and surrounding area, with an average of 74 ± 0.03 % neuronal loss in the unanimous lesion site. For animals that are aphagic but not aversive, the lesion sites focus in a similar region as aversive animals. However, the extent of neuronal loss is milder compared to the aversive animals and only reached a level of

53±0.03% neuronal loss. Most importantly, among the four 'aphagic but not aversive' animals, two of these animals had the lesion sites exactly located at the center of the unanimous lesion aversive site. However, excitotoxin only caused 50~70% of neuronal loss, which was the only difference between aversive animals and non-aversive animals. Thus, to conservatively map the most critical area where neuronal loss caused aversion to sucrose, we chose to map areas of 80% neuronal loss, which was slightly above the averaged 74% neuronal loss of aversive animals. To broadly capture areas where neuronal loss could potentially cause aversion, we mapped areas of 50% neuronal loss, which was slightly below the averaged 53% neuronal loss of non-aversive animals.

The averaged radii of the zones were measured and averaged across 5 animals, and were later applied to determine the size of the hexagon map symbol, which represents the averaged sizes of 80% and 50% neuronal loss from the microinjection center.

Functional mapping for localization of behavioral effect

The averaged Fos plumes (both GABA and lesion) and the behavioral data were recombined to map the localization of function (e.g. 'liking'). Microinjection sites from rats in the behavioral group were identified and plotted onto the atlas to represent the center of microinjections. The plume-derived hexagon symbols were superimposed onto the microinjection centers to represent estimated zones of intense, moderate, mild inhibition or neuronal loss caused by GABA agonists or excitotoxin lesion. The magnitudes of the behavioral effects of each microinjection were color coded onto the hexagons to represent the functional zones in which the behavioral effects were induced by drug (Figure 1, bottom row). Therefore, each hexagon symbol on the map illustrates three pieces of information: (1) the location of the microinjection in a particular rat tested for behavior, (2) the sizes of zones of intense, moderate, or mild neuronal inhibition/neuronal loss caused by the GABA agonists (based on averaged Fos-plume radii) or lesion (based on averaged lesion-plume radii), and (3) the magnitude of behavioral effect (e.g. 'liking' responses) when drug is injected at this specific site, which is represented by the color (based

on taste reactivity test). The bilateral cannulae for each rat were collapsed into one unilateral map and plotted separately (two sites per rat).

Identification of critical site for lesion-elevated aversion

In order to identify the most critical neural substrate where neuronal loss enhanced aversion to sucrose, only brain tissues from rats that developed aphagia and showed increased aversion to sucrose were analyzed (n=6). Similar to the methodology described previously in 'lesion plume maps', lesion brain tissues were imaged, sampling boxes were placed onto the images along the 7 arms, and the number of neurons was counted and compared to the baseline value (from normal tissues) to quantify the extent of lesion (Figure 3). Approximate brain slices of the 7 AP zones were picked for left or right side of the brain from each rat. Three zones of neuronal loss were mapped (80%, 50%, 30%) onto each image. The location and boundaries of the crucial aversion site (defined as the site which must be damaged in order to produce aversion to sucrose) were mapped by subtracting unshared regions of damages from the area of overlap among lesions that induced aversion. The rationale is that only a site that was damaged in every rat that showed elevated aversion could be a necessary site for aversion elevation. For sites that were spared in any aversive rat could not be regarded as a necessary site for aversion. In this study, the unanimous site of 80% neuronal loss across all rats was mapped and identified as the most crucial site where neuronal loss caused enhanced aversion.

Statistical analyses

All behavioral analyses were two-tailed and α was always set at $p<0.05$. Paired samples t-tests or one-way ANOVAS with bonferonni post-hoc test were used to test GABA and lesion effects on different behaviors. Between-subjects ANOVAs were used to determine anatomical location effects of microinjection sites (e.g. hotspot vs. non-hotspot).

Results

Fos plume mapping: identifying zones of local neuronal activation/inhibition

Fos plume maps help to identify the radius of spheres of modulation and the intensity of neuronal transcription activation or inhibition caused by microinjection of GABA drugs. In traditional microinjection studies, only the microinjection centers are identified and plotted to represent the location where the drug could cause behavioral effects. However, it is necessary to know how far the drug modulates local tissue extending away from the microinjection site in order to map the substrates responsible for drug-induced effects on behavior.

Fos plume mapping, a methodology that measures IEG activation in neurons surrounding a microinjection site, has been shown to be a useful tool to identify specific areas responsible for the drug-induced behavioral effects, as described in previous studies(Mahler, et al., 2007; Reynolds & Berridge, 2008; K. S. Smith & Berridge, 2007). In this study, GABA agonist microinjections mostly suppressed Fos expression either directly through acting on the same neurons or indirectly through modulating nearby neurons, which in turn suppressed adjacent neurons that express Fos. In either case, a Fos plume reflects spheres of local functional modulation induced by the drug and provides quantitative information on sphere intensity and size. Most importantly, the boundaries of the plume reveal the functional zones likely to be responsible for GABA-induced behavioral effects, even if drug molecules spread further beyond the plume and are insufficient to trigger gene transcription or have functional effects.

Microinjections of GABA agonists (0.1µg muscimol, 0.1µg baclofen) produced roughly spherical and concentric zones of Fos suppression when compared to microinjections of vehicle. Different functional zones are defined based on the intensity of Fos inhibition. The inner intense zone/middle moderate zone/outer mild zone (from the microinjection center) was defined as the zone in which Fos expression was suppressed by 65%/ 40%/ 25% over VP tissues injected with vehicle (Figure 2-2). The averaged radius of each zone, gathered from 6 to 10 Fos plumes, was applied to calculate the volume of drug-induced functional spheres and

to determine the size of hexagon symbol we used to represent functional drug effect on the 2-D plane (Figure 2-2 bottom).

The size of the Fos plume depends on the dosage and the speed of microinjection. Microinjection of GABA agonists (muscimol 0.1 μg and baclofen 0.1 μg in 0.2 μl ACSF) over one minute followed by maintaining the microinjectors in place for another minute produced an average Fos plume with a mean radius of 0.12 ± 0.01 mm for the inner intense zone, a mean radius of 0.24 ± 0.02 mm for the middle moderate zone, and a mean radius of 0.35 ± 0.15 mm for outer mild zone (Figure 2-2, bottom).

Fos plume maps were later generated by combining results from the Fos group (averaged Fos plume) and from the behavioral group (changes of 'liking' or 'disliking' caused by drug microinjections at a specific site).

Lesion plume mapping: identifying zones of neuronal loss

Similar to the reason for GABA Fos plume maps, it is necessary to know how far the excitotoxin spreads from the microinjection site and the extent of neuronal loss it causes in order to map the crucial neural substrates where neuronal loss caused the changes of behaviors. In this case, a lesion plume reflects spheres of local neuronal loss (caused by excitotoxin lesion) and provides quantitative information on intensity and size of lesion. Also, the boundaries of the plume reveal the crucial zones where neuronal loss caused behavioral changes, such as elevation of aversion to sucrose.

Excitotoxin microinjections produced roughly spherical and concentric zones of neuronal loss when compared to non-surgical tissues. The inner intense zone/middle moderate zone/outer mild zone was defined as the zone in which the level of neuronal loss reached 80%/50%/30% over normal VP tissues. The averaged radius of each zone, gathered from 8 lesion plumes, was applied to determine the size of hexagon symbols, which represented functional lesion effect on the 2-D plane. The mean radius for the inner, middle, and outer zones are 433 ±40 mm, 733 ± 41 mm, and 816 ± 29 mm. Note that the averaged size of the lesion symbol is significantly larger than the size of the GABA symbol. The reason was

because a high dosage and a high volume of excitotoxin was chosen to create intense neuronal loss, which was required to destroy a high level of neuronal loss and to cause elevation of aversion to sucrose. This large dosage and large volume of drug unavoidably spread further away, produced broader impact on surrounding tissues, and thus led to larger plumes, which indicated larger areas of loss of function.

Lesion plume maps were later generated by combining the results from the averaged lesion plume and from the results from the behavioral group (changes of 'liking' or 'disliking' caused by neuronal loss at a specific site).

Lesion and GABA inhibition in the VP elicited loss of food 'wanting' and decreased water intake

Both lesion and GABA inhibition in the VP elicited loss of food 'wanting' and decreased water intake at the same site, including the posterior VP and the surrounding areas (Figure 2-4). Lesion and VP GABA rats refused all foods, including normal rat chow, mashed cereal, sweetened mashed cereal, and even M&M chocolate. Food 'wanting', measured by the amount of food consumed in a one-hour food intake test, was suppressed almost completely by lesion or microinjections of GABA agonists in VP and surrounding brain structures (Figure 2-4, bottom; $F_{2,177}=22.91$, $p<0.01$). These same rats would not even eat or sniff M&M chocolate placed in the food intake test cage or their home cage, while normal rats would consume all (about 3-5 pieces) M&M chocolate in less than an hour. The results suggested that the animals lost their appetite not only for blend rat chows but also highly palatable (high fat and high sugar) rewards.

A large area of basal forebrain supported severe food intake suppression extended from the posterior VP (AP -0.8), where VP is ventral to the posterior part of the anterior commissure, lateral to the central part of the extended amygdala, and medial to the interstitial nucleus of the posterior limb of the anterior commissure, all the way forward to middle VP (AP +0.1), where the VP is ventral to the anterior part of the anterior commissure, lateral to the substantia innominata and the lateral preoptic area, and medial to the interstitial nucleus of the posterior limb of the anterior commissure, and downward to the posterior lateral hypothalamus (AP -2.5).The GABA caused loss of 'wanting' site is about 2.5 mm^3 in volume (Figure 2-4, top left), and the

lesion caused loss of 'wanting' site is about 4.8 mm^3 in volume (Figure 2-4, top right). These two sites highly overlap, with the lesion- caused loss of 'wanting' site covers some additional areas, including the ventral part of the caudate putamen, lateral part of the substantia innominata, and a larger portion of the anterior lateral hypothalamus. We think this size difference may have been caused by the large collateral damage excitotoxins produced in the surrounding areas, which led to broader impact areas. However, the intensity of lesion-caused inactivation may not be as strong as GABA inhibition, and lesions do not always accomplish the structural and functional damage they are assumed to do, as discussed in earlier papers (Waraczynski & Demco, 2006). Thus, while GABA and lesion both caused suppression of food 'wanting', the GABA 'wanting' suppression site we mapped here may be a more critical neural substrate that is necessary for food appetite. Overall, the result suggested that lesion and GABA inhibition suppressed food intake in a broader area surrounding the posterior VP, including the sublenticular extended amygdala (SLEA), the substantia innominata (SI), the ventral part of the caudate putamen (CPu), and the lateral hypothalamus (LH).

One-hour water intake was also suppressed by lesion or microinjections of GABA agonists in the posterior VP, a similar site where the two manipulations caused loss of food 'wanting'.

Lesion and GABA inhibition in VP caused decreased 'liking' to sucrose

Lesion and GABA inhibition in VP caused decreased 'liking' reactions to sucrose at a site that highly overlaps with the area where lesion and GABA inhibition suppressed food 'wanting' (Figure 2-5, top right; Figure 2-6 top right). The core area of decreased 'liking' focused at posterior VP and extended to the surrounding area, including EA, SI, ventral CPu, and LH (Figure 2-5, top right; 2-6 top right). Both lesions and microinjections of GABA agonists in VP and surrounding areas caused an overall decrease of hedonic 'liking' reactions (sum of rhythmic tongue protrusion, lateral tongue protrusion, and paw lick scores) elicited by sucrose infusion compared to control levels after vehicle microinjections. Sucrose infusion elicited mainly hedonic 'liking' reactions and reached the average of 27.6 ± 0.82 hedonic reactions over one minute in the control condition. In contrast, sucrose infusion hardly elicited hedonic 'liking' reactions in the GABA and lesion condition, and

reached the average of 2.14 ± 0.86 ($F_{1, 63}$= 131.761, p=0) and 7.43 ± 2.61 ($F_{1, 106}$= 44.374, p=0) hedonic reactions over one minute respectively.

The most dramatic switch in pleasure generation to sweetness would be the replacement of hedonic 'liking' reactions with aversive 'disliking' reactions

Both GABA inhibition and lesion could lead to a decrease of appetitive reward consumption, or loss of food 'wanting', along with replacing hedonic reward 'liking' with aversive reward 'disliking'. However, it is noteworthy that although GABA aversive site and lesion aversive site overlap, they encompass slightly different subareas, with the lesion aversive site being medial and posterior to the GABA aversive site (Figure 2-7).

Both of the aversive 'disliking' sites are smaller than the 'wanting' suppression site or the 'liking' suppression site: the aversive 'disliking' sites encompassed only posterior VP and may touch the anterior part of the sublenticular extended amygdala (SLEA) (0.8 mm^3 for GABA 'disliking' site; 3.2 mm^3 for lesion 'disliking' site) (Figure 2-7), while the area for loss of food 'wanting' or the area for hedonic suppression encompassed a larger area that also includes caudal part of the SLEA and lateral hypothalamus other than posterior VP (2.5 mm^3 for GABA 'wanting' suppression site; 4.8 mm^3 for lesion 'wanting' suppression site) (Figure 2-4, 5, 6). The precise locations of GABA aversive site and lesion aversive site are separately discussed below.

Microinjections of GABA agonists in the posterior VP and surrounding areas caused strong increases in the number of aversive 'disliking' reactions to sucrose (28.2 ± 3.8 disliking reactions/one minute) compared to microinjections of vehicle (4.1 ± 0.5 disliking reaction/one minute). On average, GABA inhibition caused >10X (11.5 ± 3.24 X) elevation of aversive 'disliking' reactions to sucrose ($F_{1, 62}$=32.141, p=0). In other words, these animals showed mainly aversive 'disliking' reactions to sucrose and very few hedonic 'liking' reactions. The center of the most critical GABA-induced aversive 'disliking' site in response to sucrose infusion focused at posterior VP (AP -0.7~ -1.4 mm), extended anterior to middle VP (AP -0.1- -0.6 mm), and posterior to the part where the most caudal part of the VP meets with the most

rostral part of the sublenticular extended amygdala (AP -1.2~ -1.5 mm) (Figure 2-7). The volume of this GABA aversive site is about 0.8 mm^3, which encompasses about 1/3 of the total volume of VP and only about ¼ of the total volume of the GABA 'wanting' suppression site. GABA microinjections at this aversive site caused >20X elevation of aversive 'disliking' reactions to sucrose, which was significantly higher than GABA in anterior VP or GABA in LH ($F_{2,30}$= 9.97, p<0.05 for both). It is important to note that GABA inhibition in LH did not cause an increase of aversive 'disliking' to sucrose stimuli despite the fact that GABA inhibition of the same region caused a decrease of food 'wanting' and a decrease of hedonic 'liking' to sucrose.

In order to identify neural substrates where lesion caused loss of food 'wanting' along with an increase of reward 'disliking', only data from rats that consumed no food or less than half of the given moistened cereal mash food over the time period of 24h after lesion were analyzed (n= 62 in total). Among these rats, lesion significantly increased >5x the number aversive 'disliking' reactions elicited by sucrose infusion over the one-minute test in the lesion aversive site ($F_{1,60}$=4.16, p<0.05). The most critical 'disliking' site where lesion caused increased aversion to sucrose stimuli was located at the border of posterior VP (AP -0.7 ~ -1.2 mm), extended anterior to middle VP (AP +0.3~ 0.0 mm) and posterior to the SLEA (AP -1.4 ~ -1.8 mm), which is posterior to VP and anterior to the. Similar to the case of GABA inhibition, lesion-induced aversive 'disliking' site to sucrose stimuli appeared to be more localized (3.2 mm^3) compared to the site where lesion caused loss of food 'wanting' (4.8 mm^3) (Figure 2-7). Further comparing the anatomical locations of the two 'disliking' sites caused by lesion and by GABA inhibition, we found that the two sites are highly overlapped in posterior VP, but the lesion 'disliking' site extended further posterior and medial (than the GABA 'disliking' site) and touched the most anterior part of the lateral hypothalamus (Figure 2-11). Similar to the reasons for 'wanting' suppression sites (collateral damage, incomplete functional and anatomical damage), the GABA 'disliking' site may represent the most critical site where loss of function caused aversion.

The unanimous area shared by the GABA 'disliking' site and the lesion 'disliking' site is located completely in posterior VP, perhaps also touching the

anterior part of the SLEA, with the center located at -0.7 mm posterior to bregma and 2.5 mm lateral to the midline (Figure 2-11).

Lesion plume methodology provided an efficient way to map the general area crucial for lesion-induced behavioral effects, while lesion maps, which were generated by quantifying neuronal loss, can further increase the precision of pinpointing the true necessary site for lesion-induced aversive 'disliking' effects to sucrose stimuli. The size of the brain substrate where loss of neurons caused aversion to sucrose may be smaller than the size of a lesion plume, which occupied about one quarter of the total volume of VP. In other words, the coarse anatomical resolution of the lesion plume methodology limited our ability to identify the true necessary site for lesion-induced aversion. Furthermore, a brain area is only considered to be necessary for a behavioral function when neuronal loss in that area completely destroys the behavioral function. Therefore, only a site of significant neuronal loss that was shared by all rats that showed increased aversion could be considered as the necessary site for lesion-induced aversion. For lesion sites that were spared in any rat that showed elevated aversion to sucrose could not be regarded as a necessary site for aversion.

The most crucial site necessary for aversive 'disliking'

Eight out of the 63 rats that were aphagic and adipsic were used to trace the recovery timeline of aphagic effects and aversion (Figure 2-8). On the second day after lesion, all rats were aphagic and showed aversion to sucrose. From the second day to the 5th day after lesion, six out of the eight rats were aphagic and showed aversion. After day 5, only two out of the eight rats were still aphagic and showed aversion to sucrose. After day 10, only one rat was still aphagic. All rats were fully recovered and started to eat normal rat chow by day 15. The recovery of lesion-induced aversion and lesion-induced aphagia proceeded roughly together (Figure 2-8).

To map a unanimous neuronal loss site shared by all rats (n= 6) that showed both aphagia and increased aversion, we quantified the level of neuronal loss in each sampling box and mapped out an unanimous area that consistently showed >80% neuronal loss in all 6 rats (details see materials and methods). This lesion

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aversive site was localized mainly in posterior ventral pallidum and extended to SLEA, but ends before the anterior tip of the LH (Figure 2-7). The center of this unanimous site was located at the level of 1.3 mm posterior to bregma, 2.4 mm lateral from the midline, and 8.2 ventral to the skull. This site overlaps with the GABA aversive site, but is more medial and lateral (Figure 2-11). The lesion aversive site has a volume of 0.81 mm^3 (radius: 0.58 mm), which is about the size of GABA aversive site. Animals with lesions in this unanimous lesion aversive showed significant more aversive reactions to sucrose than animals with lesions outside of the lesion aversive site ($F_{1, 60}$=4.16, $p<0.05$).

Comparison of sites for increased aversive 'disliking', suppression of hedonic 'liking', and loss of food 'wanting'

The three sites where GABA- or lesion- caused aversive 'disliking' to sucrose, suppression of hedonic 'liking' to sucrose, and loss of food 'wanting' highly overlapped in posterior VP as shown in figure 2-11. The areas these three sites occupied differ in sizes and appear roughly concentric, with the 'wanting' suppression zone and the 'liking' suppression zone to be most widely- distributed, and the aversive 'disliking' zone to be most localized. In other words, the loss of food 'wanting' zone encompassed the broadest area, including the posterior VP, the SLEA, and the anterior lateral hypothalamus. Within this large zone of loss of food 'wanting', GABA 'disliking' zone was localized in the medial and posterior VP without touching the SLEA or the lateral hypothalamus, and lesion 'disliking' zone occupied a similar area but non-identical to GABA 'disliking' area. This increased 'disliking' zone occupied about 15% (for lesion)~ 65% (for GABA) of the decreased 'liking' zone and localized only in posterior VP. It is noteworthy that in lateral hypothalamus, which has long been known as the homeostasis center, GABA inhibition and lesion caused loss of food 'wanting' without affecting affective 'liking' or 'disliking'. Also, the negative 'disliking' zone appeared to be anatomically more constrained compared to the positive 'liking' zone (Figure 2-11). The pattern of loss of 'wanting' and 'liking' of reward without increase of 'disliking' suggests that (1) loss of food 'wanting' can exist without an increase of aversive 'disliking' (2) a decrease of reward 'liking' can exist without an increase of aversive 'disliking'. In the

case of posterior VP inhibition, loss of appetite to eat was linked to decreased hedonic 'liking' of reward and increased of aversive 'disliking' of reward. However, in the case of LH or medial VP inhibition, loss of appetite to eat was linked solely to decreased food 'wanting' and decreased hedonic 'liking', aversion was not attributed to the loss of food appetite. Together with previous studies from our lab, in which reward 'wanting' and reward 'liking' were dissociated via opioid manipulation in middle VP (K. S. Smith & Berridge, 2005), the results suggested that 'wanting', 'liking', and 'disliking' can be dissociable through chemical manipulations at different subareas in VP.

Lesion and GABA inhibition in VP did not change aversion to quinine

In contrast to increased aversive 'disliking' reactions to sucrose, microinjections of GABA agonists or lesion in posterior VP had no significant effect on aversive 'disliking' reactions to quinine (gapes, head shacks, forelimb flails, chin rubs) (GABA: $F_{1,72}= 1.038$, lesion: $F_{1,62}= 1.07$, $p<0.05$ for both). Microinjections of GABA agonists or lesion in posterior VP did not change hedonic 'liking' reactions to quinine either although the two manipulations suppressed hedonic 'liking' reactions to sucrose.

Other behaviors elicited by microinjection of GABA agonists or lesion in VP

In some rats, other than elevation of aversion, both GABA and lesion in VP could cause forepaw treading, a behavior that resembled defensive-type or fearful treading ordinarily shown by squirrels to rattlesnakes in the wild or by lab rats to shock pods and other threatening stimuli. Treading was characterized by coordinated forward thrusting of forepaws and spraying of bedding material towards particular objects in the environment (e.g. cage corners). In the current study, as soon as quinine was infused into those rats' mouths, the rats would start making rounds and pushing their forelimbs toward the cylinder taste reactivity chamber, as if they were doing treading without bedding. Furthermore, when the rats were in the food intake test cage, they vigorously pushed beddings toward the side- walls of the cage, particularly toward the corners. However, GABA- and lesion- elicited forepaw treading was not seen in all rats. In some rats, GABA or lesion only caused severe aversion even without any quinine infusion. The rats would chin rub

and passively drip through the whole taste reactivity test, and the effect would last throughout the one- hour food intake test. In some rats, a mix of both aversion and treading was elicited by microinjections of GABA agonists and lesion. It is unclear what anatomical or neurochemical differences are accounted for the differentiation between rats with 'aversion and tread' and rats with 'aversion but no tread'.

Lesions in some rats also caused sensorimotor and regulatory changes in behavior: widespread hind-legs, swollen eyes surrounded by brown and red secretion, slightly hunched postures, or spiking untidy fur (failure to groom properly). Most aphagic rats showed some, but not all, of the above characteristics. The correlations between the abovementioned characteristics and aversion require further studies to clarify.

GABA inhibition, but not lesion, in NAc shell suppressed 'liking' to sucrose

Only microinjections of GABA agonists, but not lesion, in NAcSh caused an overall decrease of hedonic 'liking' reactions (sum of rhythmic tongue protrusion, lateral tongue protrusion, and paw lick scores) elicited by sucrose infusion compared to control levels after vehicle microinjections. Sucrose infusion elicited mainly hedonic 'liking' reactions and reached the average of 27.6 ± 0.82 hedonic reactions over one minute in the control condition. In contrast, sucrose infusion hardly elicited hedonic 'liking' reactions in the condition where nucleus NAcSh was inhibited by GABA agonists and reached the average of 13 hedonic reactions over one minute respectively ($F_{1, 8}$= 9.232, p=0.016). However, unlike excitotoxoin lesion in VP in which strong 'liking' suppression was produced, excitotoxin lesion in NAcSh did not change the level of hedonic 'liking' reactions to sucrose ($F_{1, 28}$= 0.152, p>0.05). The core area of decreased 'liking' focused at dorsal medial NAc shell and extended anterior to the level of +2.0mm to bregma and posterior to the level of +0.6mm to bregma. The result is consistent with previous findings from our lab. However, in the current study, we do not have rats with microinjection sites in the most rostral part of the medial NAc shell, where muscimol microinjections have been shown to enhance hedonic 'liking' reactions to sucrose.

GABA inhibition, but not lesion, in NAc shell increased 'disliking' to sucrose

GABA inhibition, but not lesion, in posterior part of the medial NAc shell significantly increased the number aversive 'disliking' reactions elicited by sucrose infusion (Figure 2-10) over the one-minute test. The 'disliking' site where GABA inactivation caused increased aversion to sucrose stimuli was located at the most caudal part of the medial NAc shell at the level of +1.2mm~ +0.6mm to bregma. Although only four animals with NAc shell inhibition were tested for their 'disliking' reactions to sucrose, the pattern of increased 'disliking' to sucrose in caudal NAc shell was consistent with previous studies from our lab (Faure, et al., 2010; Reynolds & Berridge, 2002). Also, compared to the area where GABA inhibition caused decreased 'liking' to sucrose, it appeared that the 'disliking' zone was more localized and constrained in the caudal part of the NAc shell.

Discussion

GABA inhibition and excitotoxin lesion replace 'liking' reactions with 'disliking' reactions along with loss of food 'wanting' in the posterior VP

Our results demonstrated that both GABA inhibition and excitotoxin lesion are capable of suppressing hedonic 'liking' reactions (Figure 2-6) and replacing them with aversive 'disliking' reactions to sucrose (Figure 2-7) in the posterior VP, along with loss of food 'wanting' (Figure 2-4). The GABA 'wanting' suppression site and the lesion 'wanting' suppression site occupied a broad area in the posterior VP and the surrounding areas, including the sublenticular extended amygdala, ventral globus pallidus, ventral caudate putamen, and the lateral hypothalamus (Figure 2-4). The volumes of lesion 'wanting' suppression site and GABA 'wanting' suppression site are 4.8 mm^3 and 2.5 mm^3 respectively. The GABA 'liking' suppression site and the lesion 'liking' suppression site correspondingly occupied a similarly broad area as the GABA 'wanting' suppression site and the lesion 'wanting' suppression site. It is important to note that none of our microinjection sites land in the most anterior part of the VP (before +0.3mm to bregma). It requires future studies to clarify whether GABA inhibition or lesion in the most anterior part of the VP caused decreased 'liking' as well. From this study, it is safe to conclude that loss

of function in the medial VP, posterior VP, the SLEA, and the LH all cause hedonic 'liking' suppression.

In contrast to the widely- distributed 'wanting' suppression sites and the 'liking' suppression sites, GABA 'disliking' site and lesion 'disliking' site are both more localized (GABA: 0.8 mm^3; lesion: 3.2 mm^3) and are surrounded by the larger 'liking' suppression/ 'wanting' suppression areas (GABA: 2.5 mm^3; Lesion: 4.8 mm^3) (Figure 2-11). The most crucial aversive site, as defined by the site where GABA inactivation and excitotoxin lesion both cause aversive 'disliking' elevation, is located solely in the posterior VP and might touch the most anterior part of the SLEA. In this crucial 'disliking' site, GABA microinjections increased more than 20 times of the number of aversive 'disliking' reactions elicited by sweet sucrose, while excitotoxin lesion increased more than 5 times the level of aversion to sucrose compared to vehicle microinjections. In other words, in this crucial 'disliking' site, lesions abolished positive hedonic or 'liking' reactions to the taste of sucrose and replaced them with 'disliking' reactions such as gapes to sweetness that are normally reserved for bitter or similarly aversive tastes.

The most critical site for lesion-induced aversion to sucrose

The result of the lesion-induced aversive site from the current study further confirmed results from one previous study(Cromwell & Berridge, 1993), in which they showed that lesion in posterior VP caused aversive reactions to sucrose. With more advanced mapping techniques and with better-defined VP anatomical boundaries, we were able to more precisely map the critical site where neuronal loss caused aversion to sucrose. The lesion- induced aversive site we identified is located mainly in the most posterior part of the VP with a center at posterior 1.3 $\pm$ 0.3 mm to bregma, 2.4 $\pm$ 0.5 mm lateral to bregma, and -7.8 $\pm$ 0.5 mm ventral to the skull (Figure 2-7). The site extends posterior to the most caudal part of the sublenticular extended amygdala and stops at the anterior tip of lateral hypothalamus (AP -1.8~ -1.9mm), anterior to the posterior part of the anterior commissure, medial to the nucleus of stria medullaris, and lateral to the medial part of the VP. Compared to the previously identified lesion-induced aversion site, which

was centered at 1.3 mm posterior to bregma, 1.9± 0.5mm lateral to bregma, and 7.3±0.25mm ventral to the skull, the current lesion-induced aversion site overlaps with the previous site.   In sum, it is safe for us to conclude that the posterior part of the VP, together with possibly the anterior part of the sublenticular extended amygdala, is the most critical site for lesion-induced aversion to sucrose. But the lesion map may overestimate the site by including collateral damage or creating incomplete anatomical or functional damages that is not needed for aversion (Waraczynski & Demco, 2006). Thus, the improvement of lesion techniques and more future studies are required to improve the anatomical precision of this lesion aversive site.

The most critical site for GABA-induced aversion to sucrose

Our results of GABA-induced aversion to sucrose is consistent with findings from (Shimura, et al., 2006) but much more precise anatomically. They showed that microinjections of GABA (A) agonist in VP increased aversive 'disliking' reactions to a saccharin sweet taste. However, they treated the VP as a whole, and it was unclear in their study as whether or where in the VP GABA inhibition elevated aversion to sucrose. With the Fos plume technique, we are able to quantify the functional effect (the extent of neuronal inhibition) of GABA spread and more precisely locate the most critical site where GABA inhibition caused aversive 'disliking' to sucrose. The crucial anatomical substrate for GABA-induced aversion is located in posterior portion of the VP (center at AP -0.7 mm, ML ±2.5 mm, DV -8.1 mm) that is ventral to anterior commissure and globus pallidus, caudal to olfactory tubercle and interventricular foramen, and anterior to the rostral tip of the sublenticular extended amygdala.

Comparison of GABA-induced aversion and lesion-induced aversion

The aversive 'disliking' sites identified by GABA inhibition and by excitotoxin lesion are highly overlapped in the posterior VP (AP -0.7~ -1.3 mm) but not exactly the same: the lesion aversion site extends more medial to the outer edge of lateral preoptic area and the nucleus of stria medullaris, and more posterior to the caudal part of the sublenticular extended amygdala.

But why do we compare results from GABA and lesion manipulations of the brain? Traditionally, the most direct way to assess a brain area's role in a function is to impair or remove that area by lesion and examine the functional consequences. Despite its success in identifying brain structures critical for rewards, lesion effects can be ineffective or inconsistent across different studies due to compensations of function and plasticity in the brain. Transient inactivation, including GABA inhibition, provides a different way to challenge the functional integrity of a neural circuit and locate brain substrates where loss of function or suppression leads to changes in functional processes (Waraczynski & Demco, 2006). In fact, transient inhibition may present stronger functional challenge than lesion due to its intense and immediate suppression of a brain substrate without the effects of functional compensation or plasticity. For example, inactivation of ventral posterior suprasylvian cortex with cooling impairs the recall of previously learned 3-D object discrimination, but ablation does not (Lomber, Payne, & Cornwell, 1996).

We think GABA aversive site might be a more precise site (compared to lesion aversive site) where loss of function causes aversion. One crucial reason is that GABA inhibition induces stronger anatomical and functional inhibition (Lomber, et al., 1996; Waraczynski & Demco, 2006), and the level of inhibition is crucial for the generation of aversion (Cromwell & Berridge, 1993, 1994). To support the notion of critical level of loss of function, results from the current study showed that one major difference between aversive and non-aversive animals is the extent of neuronal loss in caudal VP. For example, the lesion sites for animals that are aphagic and aversive contained an average of 74% neuronal loss, while lesion sites for animals that are aphagic but not aversive contained only 53% neuronal loss, suggesting that the level of neuronal loss, or loss of function, might set the threshold for the generation of aversive 'disliking' to sucrose. Indeed, among the four 'aphagic but not aversive' animals, two of these animals had the lesion sites located exactly at the center of the unanimous lesion aversive site. But the lower level of neuronal loss (50~70%) prevents them from developing aversive 'disliking' to sucrose. There are anatomical evidence and behavioral evidence from the current study that suggest that GABA inhibition serves as a stronger anatomical and

functional challenge. Anatomically, GABA Fos plume maps showed that GABA microinjections in VP is capable of causing >65% of neuronal inhibition in an area with a radius of 0.12 ± 0.01 mm, suggesting that GABA inactivates neuronal activity to the extent that can cause aversion within a concentric area. Functionally, GABA inhibition in posterior VP induced stronger aversive 'disliking' to sucrose (>20X) compared to the lesion in posterior VP, which only caused >5x increase of aversion, suggesting that GABA inhibition indeed causes stronger functional effect (elevation of aversion) in the current study. Because of the more localized anatomical effect and stronger functional effect of GABA, we speculate that the GABA aversive site is a more crucial site (compared to lesion aversive site) where loss of function causes aversion to a sweet taste.

Other control sites outside of VP

While GABA inhibition and excitotoxin lesion caused loss of food 'wanting' in a broad area around the posterior VP, GABA and lesion only increased aversive 'disliking' in the posterior VP, but not in the anterior VP, which is rostral to the anterior limb of the anterior commissure and to the rostral tip of the globus pallidus, the SLEA (except for the most rostral part), or the LH. This result suggests that neuronal substrate identified as critical for 'active rejection of food' in the anterior LH or the GP might actually touch the caudal VP and caused aversion (Morgane, 1961; Schallert & Whishaw, 1978; Stellar, et al., 1979; Teitelbaum & Epstein, 1962).

The fact that the posterior VP, but not the anterior VP, is involved in aversive 'disliking' processes suggests that VP is heterogeneously involved in processing aversive 'disliking' of rewards. Depending on the location GABA inhibition occurs, different subareas within VP can be differentially involved in the same functional processes, which is consistent with previous findings (Johnson, et al., 1993; Kretschmer, 2000; K. S. Smith & Berridge, 2005; Waraczynski & Demco, 2006).

'Disliking' reactions to quinine is unchanged by GABA or lesion

GABA inhibition and excitotoxin lesion did not change 'disliking' reactions to a bitter quinine taste in any site in VP, even at the site where 'disliking' reactions to

sucrose were increased. The selective elevation pattern suggests that inhibition or loss of function of posterior VP only affects aversive 'disliking' value of natural sweet rewards without affecting the aversive 'disliking' value of natural bitter tastes. In other words, GABA and lesion made a sweet taste nasty and more 'disliked', but did not make a bitter taste even nastier or more 'disliked'. Also, the selective elevation of aversion to sucrose but not to quinine suggests that the behavioral effects we collect from taste reactivity paradigm are not due to general arousal or increased locomotion.

Dissociation of 'disliking' and loss of food 'wanting'

Despite the fact that lesion and GABA inhibition only increased aversive 'disliking' of sweet rewards in posterior VP, food 'wanting' is broadly abolished by lesion and GABA inhibition in all compartments of the VP and the surrounding areas. In other words, GABA inhibition and lesion-induced neuronal loss in posterior VP significantly increased aversive 'disliking' reactions to sucrose along with decreased food 'wanting', while the same manipulations in anterior ventral pallidum almost completely abolished food 'wanting' without altering aversive 'disliking' reactions to sucrose. This pattern of loss of food 'wanting' without 'disliking' of rewards was observed in anterior VP, ventral globus pallidus, ventral putamen, and lateral hypothalamus. The dissociation between 'disliking' and loss of food 'wanting' indicates that GABA inhibition and lesion suppress animals' motivation to consume food reward, or food 'wanting', via a non-aversive mechanism, which seems quite different from the loss of food 'wanting' produced in the posterior VP that involves increased aversive 'disliking' of rewards.

Another study from our lab similarly suggests that 'wanting' and 'liking' can be dissociated via μ- opioid signals in different subareas in the VP. While μ-opioid agonist in the posterior VP increases both 'liking' and 'wanting', μ-opioid agonist in the anterior VP only enhances 'wanting' and actually decreases 'liking' (K. S. Smith & Berridge, 2005, 2007), suggesting that μ-opioid agonist in the anterior VP enhanced food consumption, or food 'wanting', via a non-hedonic mechanism.

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Together, it appears that the changes of neuronal activity in the posterior VP can simultaneously alter affective and motivational aspect of reward, while changes in the anterior VP only affects motivational 'wanting' but not affective 'liking'.

GABA aversive site and lesion aversive site highly overlap with opioid hedonic 'liking' hotspot in the posterior VP

The GABA aversive site and the lesion aversive site identified by the current study highly overlap with the previously found opioid hedonic 'liking' hotspot, where microinjections of μ-opioid agonist caused significant increases in hedonic 'liking' reactions to sucrose (K. S. Smith & Berridge, 2005). The two aversive sites and the opioid hedonic hotspot are all localized in posterior part of the VP, which is caudal to interventricular foramen and the olfactory tubercle, and rostral to lateral hypothalamus. Together, the results suggest that μ-opioid signal in posterior VP activates affective 'liking' circuit and paints hedonic 'liking' gloss onto sensations; in contrast, disruption of the 'liking' circuit with GABA inhibition or excitotoxin lesion removes the hedonic gloss and turns a sweet taste that is originally 'liked' into a nasty taste that is 'disliked'. Interestingly, although μ-opioid agonist in anterior VP suppressed hedonic 'liking' reactions to sucrose, GABA inhibition or lesion did not appear to alter 'disliking' reactions to sucrose, suggesting that the disruption of decreased 'liking' did not necessarily alter aversive 'disliking', and the 'liking' and the 'disliking' system might be anatomically dissociable.

Why would the posterior part of the VP play a more important role in both 'liking' and 'disliking' of rewards than anterior VP? There are several physiological features of the posterior VP that may be involved. The posterior part of VP appears to have a higher level of enkephalin, higher ratio of non-cholinergic neurons to cholinergic neurons, and less dense presynaptic μ-opioid receptors (Bengtson & Osborne, 2000; Holt & Newman, 2004; Maidment, Brumbaugh, Rudolph, Erdelyi, & Evans, 1989). Furthermore, posterior VP and anterior VP may have different anatomical connections with other brain structures, such as nucleus accumbens, medial prefrontal cortex, insular, and somatosensory cortex (Groenewegen, et al., 1993; Zahm & Heimer, 1988, 1990; Zahm, et al., 1996). However, it is not clear at

this point about the functional roles of these physiological differences between posterior VP and anterior VP.

There is lots of physiological evidence that suggests interactions between opioid and GABA might exist in VP. Anatomically, VP receives both GABA and opioid projections (Zahm, et al., 1985), particularly from nucleus accumbens. Correspondingly, VP neurons express both GABA and μ-opioid receptors (Churchill, Bourdelais, Austin, Zahm, & Kalivas, 1991). Physiologically, GABA and μ-opioid in VP have been suggested to functionally negate each other. For example, μ-opioid agonist in VP attenuated GABA-induced inhibitions of neuronal activity (Chrobak & Napier, 1993; Johnson & Napier, 1997; Napier & Mitrovic, 1999). Furthermore, μ-opioid agonist and GABA antagonist had similar behavioral effects; they both caused increases in food intake and locomotion, suggesting that μ-opioid and GABA are antagonistic of each other. There are also studies showing that μ-opioid and GABA interact at the behavioral level; micoinjections of either of the two drugs can modulate behavioral effects elicited by the other. For example, pretreated GABA (A) agonist in VP attenuated μ-opioid agonist-induced locomotion (Austin & Kalivas, 1990). Thus, μ-opioid and GABA in VP interact not only at the neuronal level but also at the behavioral level. Results from the current study further suggest that GABA and μ-opioid may interact to mediate hedonic 'liking' and aversive 'disliking' aspects of the reward processing.

VP role and reward

VP has been shown to play an important role in positive rewards, such as food, drugs, sex, and social affiliation (Aragona & Wang, 2004; Berridge, 2009a; Berthoud & Morrison, 2008; Anna Rose Childress, et al., 2008; Kelley, 2004; Rauch, et al., 1999; Tzschentke, 1998). The current study further suggests that GABA inhibition and neuronal loss in VP cause elevation of negative aversion to a sucrose sweet taste. Together, the results demonstrate that VP is capable of mediating both positive and negative aspects of reward, which is confirmed by the existence of opioid 'liking' hotspot and GABA/lesion 'disliking' hotspot in caudal VP in rats. Human imaging studies further support this notion by showing that human VP

mediates the processing positive and negative emotions. For example, the caudal VP and rostral VP have been shown to respond to pictures of appetizing food and disgusting food respectively (Calder, et al., 2007), and both 'hot' and 'cold' emotional processes (Schaefer, et al., 2003).

Some evidence from human studies supports the idea that VP may be needed for positive hedonic 'liking' in humans. One recent clinical case describes that a long-term human drug addict with partial lesions to the ventral pallidum (along with ventral globus pallidus) reported to experience the disappearance of all drug cravings, depressed mood (described as anhedonia by the authors), hopeless feelings, social isolation, etc. Most strikingly, the patient could not even feel pleasure from drinking alcohol, suggesting that VP damage disrupts both the pleasure and the craving for reward (Miller, et al., 2006). In a second recent clinical case, a patient with bilateral damage to the globus pallidus (the lesion is described as perhaps extending into the VP by the authors) reported an inability to feel emotions, flat affect, loss of interests, and a profound lack of motivation (Vijayaraghavan, et al., 2008). Together, the results from the two clinical cases suggest that VP may also be necessary for the processes of affective 'liking' in humans. Similar to the case of rats where lesion in posterior VP caused suppression of hedonics, human patients with VP lesions appeared to have impairments in processing positive affect as well.

It is important to note that the impairment of neurotransmission in VP, even when VP is anatomically intact, also contributes to the loss of positive emotions and the elevation of negative emotions. In one PET scan study by Zubieta et al, the deactivation of μ-opioid receptors in human VP was shown to be associated with 'the increases in negative affect ratings and the reductions in positive affect ratings during the sustained sadness state' (Zubieta, et al., 2003), suggesting that the normal functioning of neurochemical systems in VP plays an equally crucial role in modulation of affective 'liking' and 'disliking'.

In regard to negative 'disliking', the current study provides the first evidence that inhibition of most caudal part of the VP suppressed 'liking' to sucrose taste and replaced it with increased aversive 'disliking'. Although the experimental design was

focused on affective reactions to food tastes, the results may have broader implications on emotional disgust and moral disgust. This is because recent findings appear to support Darwin's hypothesis about emotional disgust and moral disgust, which may evolve from the more primitive food aversive rejections (Phillips, et al., 1997; Rozin & Fallon, 1987; Rozin, Haidt, & Fincher, 2009). There are human studies that suggests that the basal ganglia is involved in disgust circuit, and the malfunction of basal ganglia, which includes VP and other structures, is linked to disgust-associated disorders, such as OCD, Huntington's disease, and perhaps Parkinson's disease (Adolphs, 2002; Adolphs, et al., 2003; Chapman, et al., 2009; Olatunji, et al., 2010; Phillips, et al., 1997; Sprengelmeyer, et al., 1996; Sprengelmeyer, et al., 1997; Surguladze, et al., 2010; Suzuki, et al., 2006). For example, patients with Huntington's disease or OCD, which neuronal degeneration or abnormal neural activity occur in basal ganglia, had difficulty recognizing facial expression of disgust (Sprengelmeyer, et al., 1996; Sprengelmeyer, et al., 1997). Although no study has yet examined VP's role in disgust- associated disorders, it is likely the normal functioning of VP is require for the processes of disgust if it stands true that food disgust and emotional disgust share the same neural circuit in the brain. The strongest evidence perhaps come from the study by (Calder, et al., 2007), in which they showed the neural activity in anterior human VP was associated with individual sensitivity to images of disgusting food. It will be of particular importance for future studies to clarify VP's role in emotional disgust and disgust-associated psychopathology.

Finally, VP has been suggested to mediate the evaluation of reward and the transition of the reward value into appropriate reactions (Kelley, Baldo, Pratt, et al., 2005; Pecina, et al., 2006; Pessiglione, et al., 2007; K. S. Smith, et al., 2009; Waraczynski & Demco, 2006; Zahm, 2006). Previous studies have shown that malfunction or loss of function of VP would cause impairments in behavioral tasks that require reward evaluation, such as visual discrimination, reversal learning, or conditioned place preference for food reward (Everitt, et al., 1987; Ferry, Lu, & Price, 2000; McAlonan, Robbins, & Everitt, 1993b; Robbins, et al., 1989). Our results further point out that excessive GABA signal or neuronal loss in VP would turn a

sweet taste that is originally 'liked' into a nasty taste that is 'disliked' although the physical sensation of the sucrose solution is essentially the same. Therefore, the normal functioning of VP might be critical for attributions of both positive and negative values to sensory information and rewards. The imbalance between neurotransmission, for example, GABA and μ-opioid, or lesion in VP may disrupt normal processing of reward evaluation and lead to the inability to inhibit inappropriate reactions. The relationships between 'liking' and 'disliking' circuits in VP and how they transfer reward values into motivation and actions deserve more attention in the future studies.
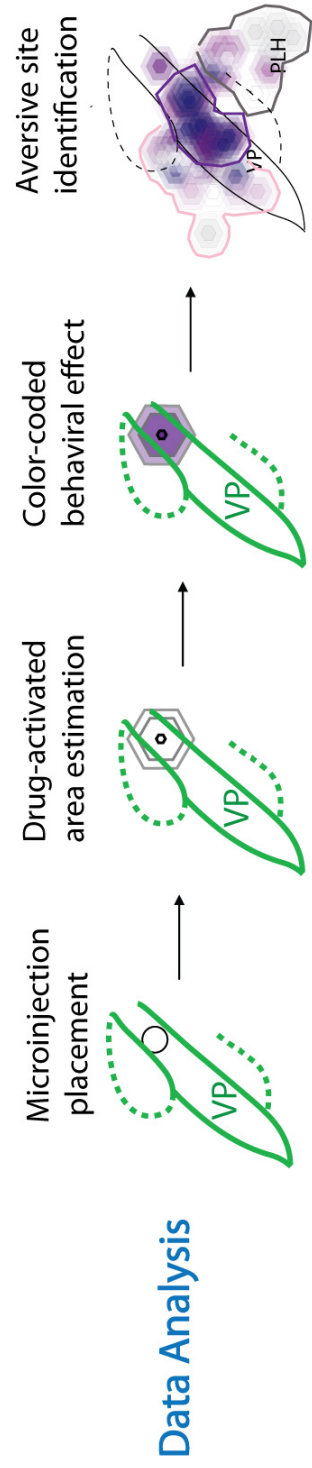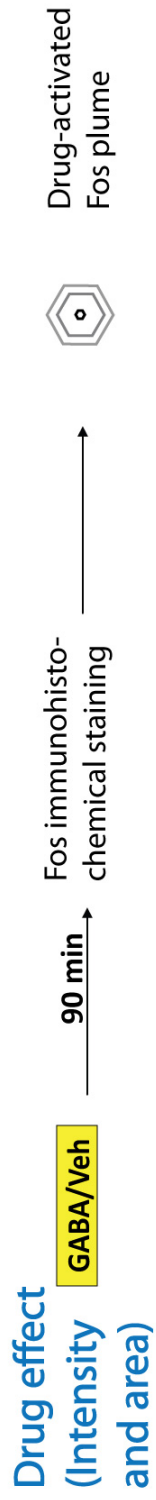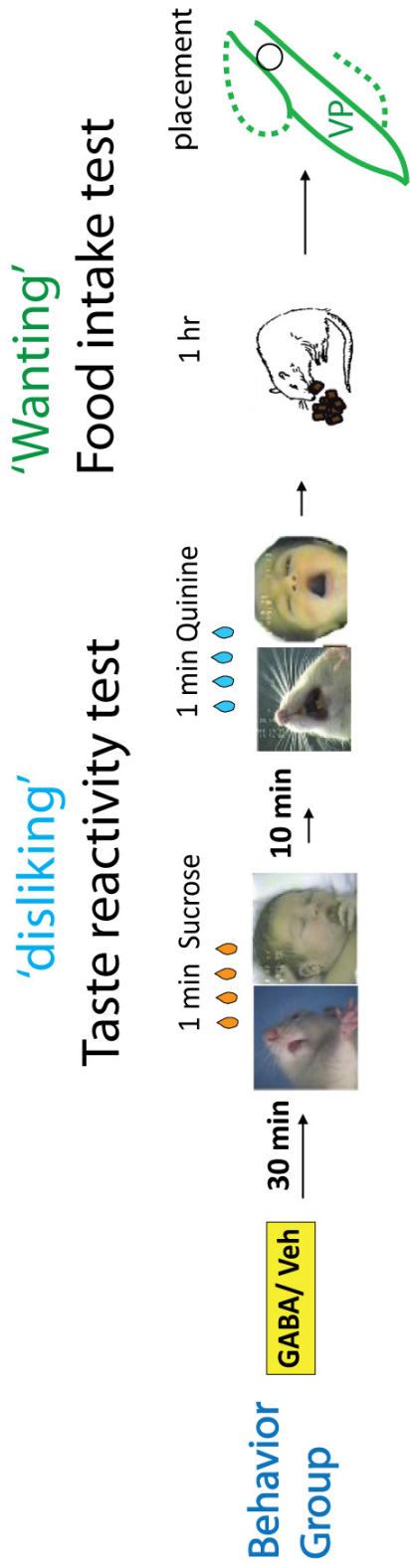
Figure 2-1 General design of the experiment. Two groups of rats are used to construct maps of 'disliking' response to sucrose and maps of 'disliking' response to quinine. In the first group of rats (behavior group), within-subject design is used to test GABA and lesion effects over vehicle. Rats in this group are tested with taste reactivity test to quantify the intensity of 'disliking' response to sucrose or quinine after drug microinjections (ACSF or GABA agonists), and stained with cresyl violet for microinjection center identification and lesion quantification. Rats in the second group (Fos group) are used to construct GABA Fos plume maps, which quantify the intensity and area of neuronal inhibition caused by GABA microinjections. The size of the symbols represents the averaged intensity and area of GABA-induced Fos inhibition in VP. The two groups are combined to construct maps of 'liking' response to sucrose or 'disliking' response to quinine by color coding the behavioral effects (from the first group) onto the Fos plume symbols (from the second group) placed onto the microinjection centers identified by histology (first group). The colors of the symbols represent the increases or decreases of behavioral effects (e.g. 'liking' or 'disliking' reactions). The site where drug caused most intense behavioral changes thus emerge, as indicated by the localized cluster of colored symbols.
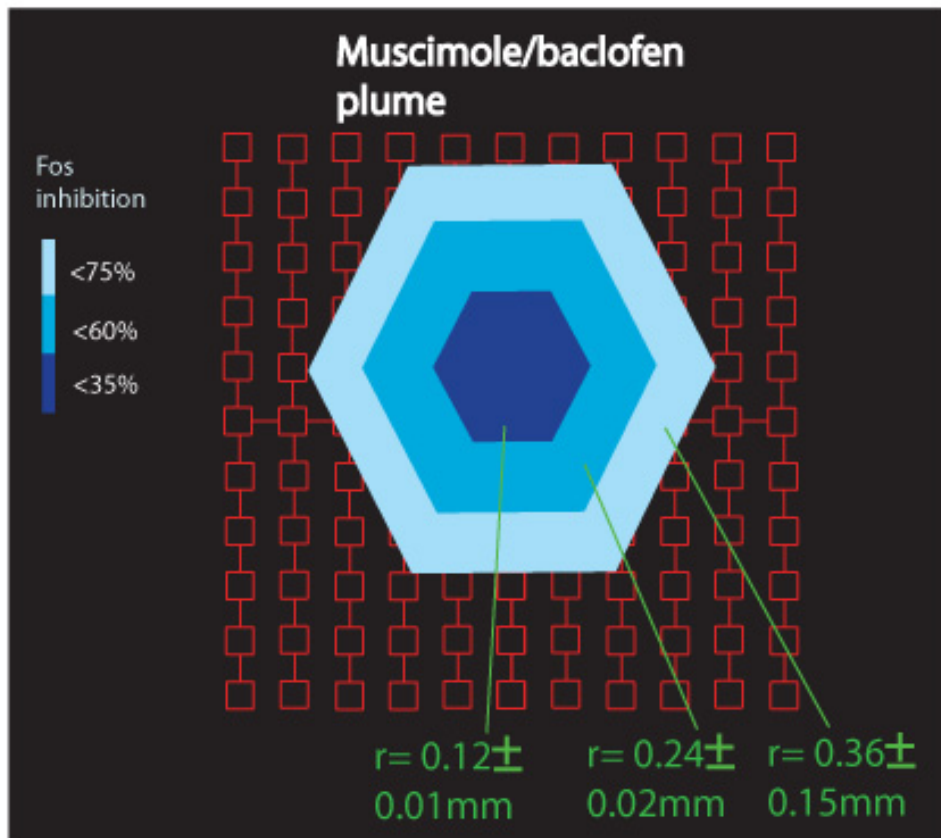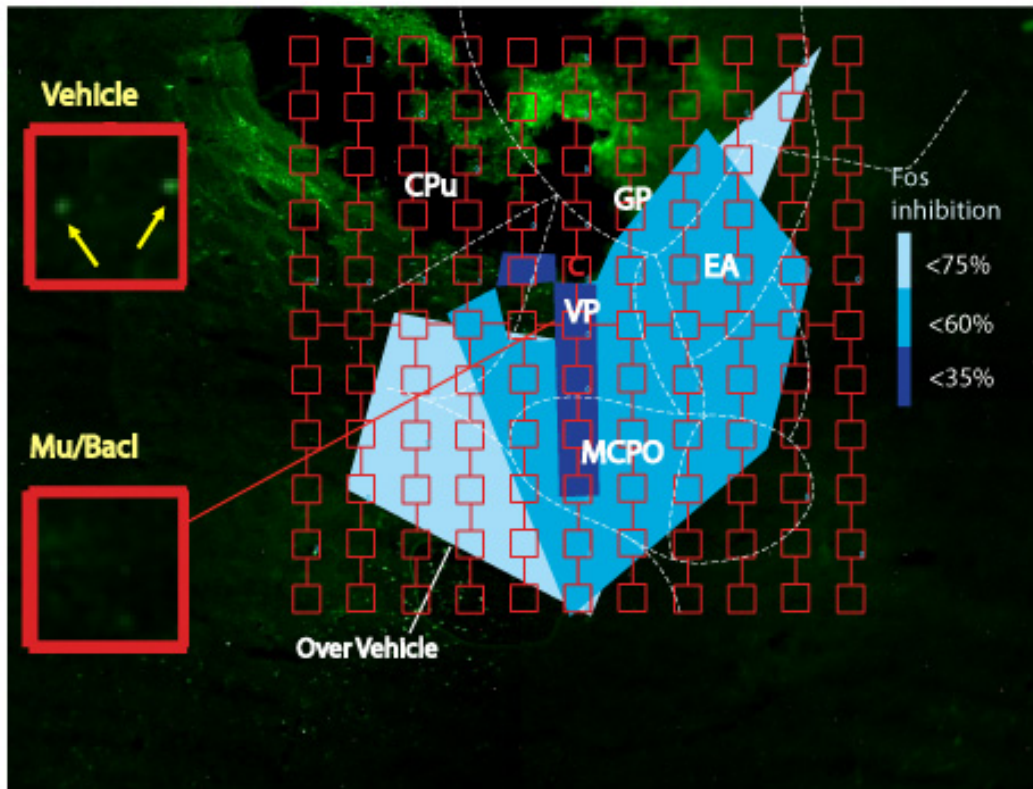
Figure 2-2 GABA Fos plume in VP. *Fos Plume Sampling Method (top)*: The method used to sample Fos expression around injection site is illustrated. Fos-expressing neurons are counted in 50 x 50 µm blocks on 8 sampling arms (0°, 45°, 90°, 135°, 180°, 225°, 270°, 315°) extending from the center of microinjection center at 50 µm intervals. Insets show sample tissue blocks from equivalent sites that were injected with vehicle (ACSF) or with GABA agonists (0.1µg muscimol, 0.1µg baclofen). Fos expression appears as green oval dot under the microscope at 10X magnification (yellow arrow). The color scale demonstrated the colors used for different extents of Fos suppression (over vehicle control levels). Colored areas indicated the neural substrates of 35% (or 65% inhibition; dark-blue), 60% (or 40% inhibition; sky-blue), 75% (or 25% inhibition; light-blue) GABA Fos expression compared to vehicle Fos expression of relevant neural substrate. *Averaged GABA Fos Plume (bottom)*: Mean (SEM) plume radii are shown for GABA microinjections in VP. Inner dark- blue hexagon represents an area of 65% inhibition. Middle sky-blue hexagon represents an area of 40% inhibition. Outer light-blue hexagon represents 25% inhibition.
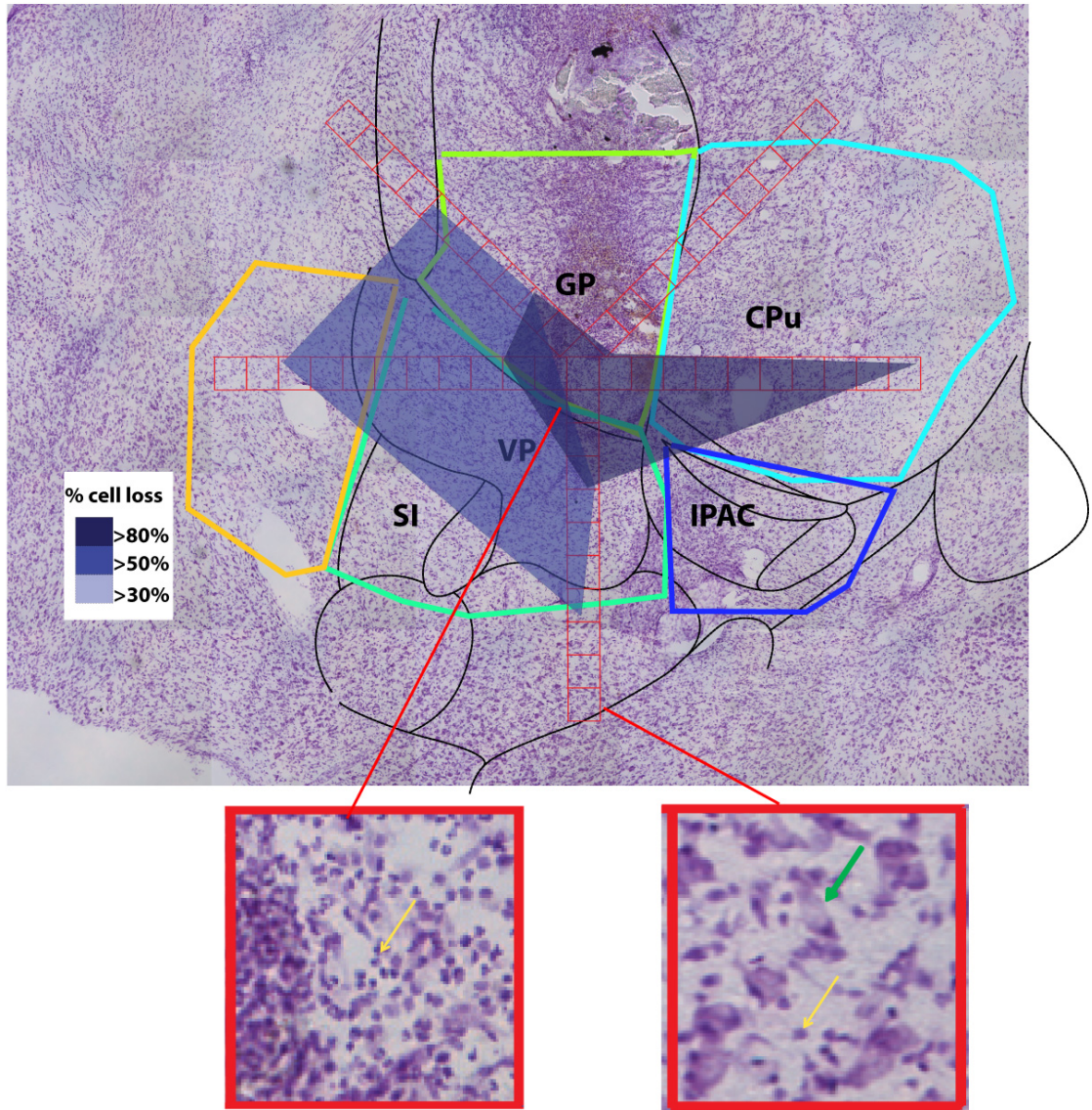
Figure 2-3 Excitotoxin lesion neuronal loss in VP. *Quantification of Neuronal Loss* : The method used to quantify neuronal loss is illustrated. Neurons are counted in 125 x 125 μm blocks on 5 sampling arms (red) extending from the microinjection center. The bottom left sampling box represents neural tissue that has severe neuronal loss and contains only glial cells (yellow arrow), which appear as small dense dark purple dots. The bottom right sampling box represents healthy neural tissue that contains neurons (green arrow), which appear as large heterogeneous oval shapes, and glial cells (40X magnification). Color lines represent the boundaries of different subareas that each brain slice is divided into. Neuronal count of each sampling box is compared with the baseline neuronal number established from healthy tissues of the same subareas to quantify the extent of neuronal loss. The color scale demonstrated the colors used to represent different levels of neuronal loss. Colored areas indicated the neural substrates of 80% (dark-blue), 50% (sky-blue), 30% (light-blue) neuronal loss compared to healthy tissues of relevant neural substrate.
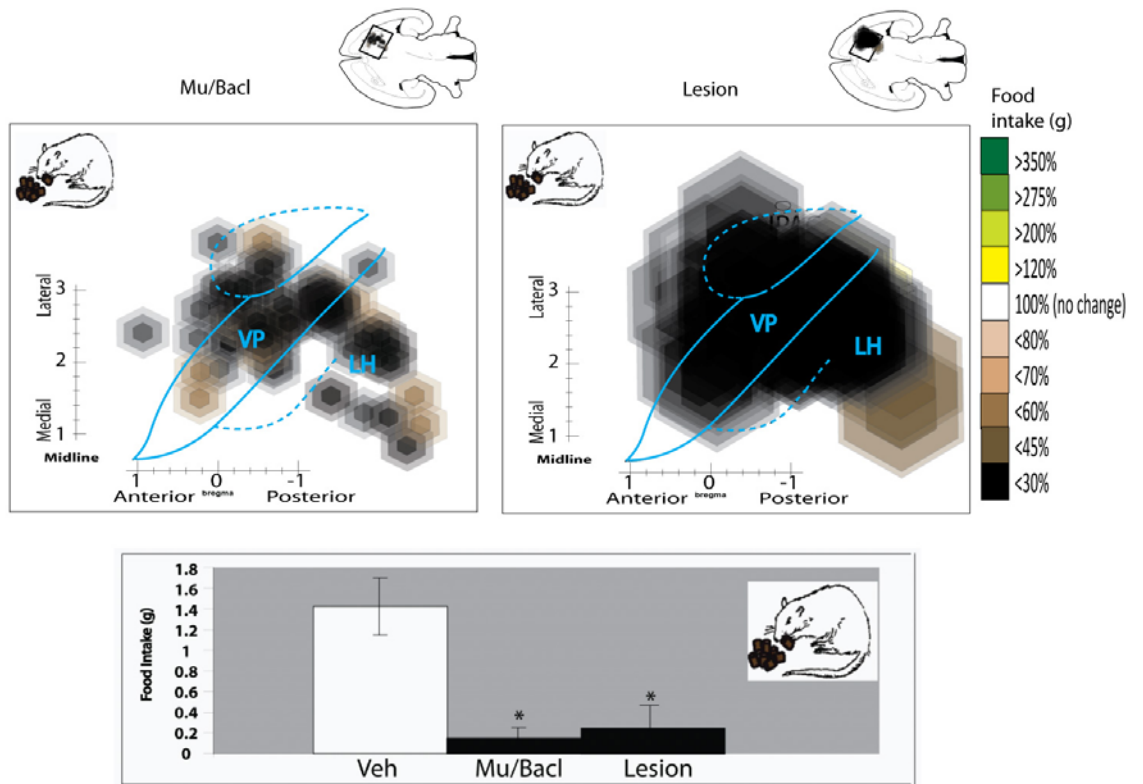
Figures 2-4 GABA and lesion in VP suppress food intake. Food intake suppressions following GABA agonist (0.1 μg muscimol, 0.1μg baclofen)(*top left*) microinjections or excitotoxin lesions (*top right*) are mapped rat-by-rat onto a horizontal view of VP, showing both the within-subject changes in food intake relative to vehicle (color) and the functional spreads of the microinjection based on Fos plume data (size). For GABA hexagon, inner symbols represent 65% Fos inhibition, middle symbols represent 40% Fos inhibition, and outer symbols represent 25% Fos inhibition. For lesion hexagon, inner symbols represent 80% neuronal loss, middle symbols represent 50% neuronal loss, and outer symbols represent 30% neuronal loss. Bar graph (bottom) shows the averaged food intake (g) over the 1-hr food intake session. Error bars represent SEM. * indicates decreases in food intake ($p < 0.05$) compared to vehicle.
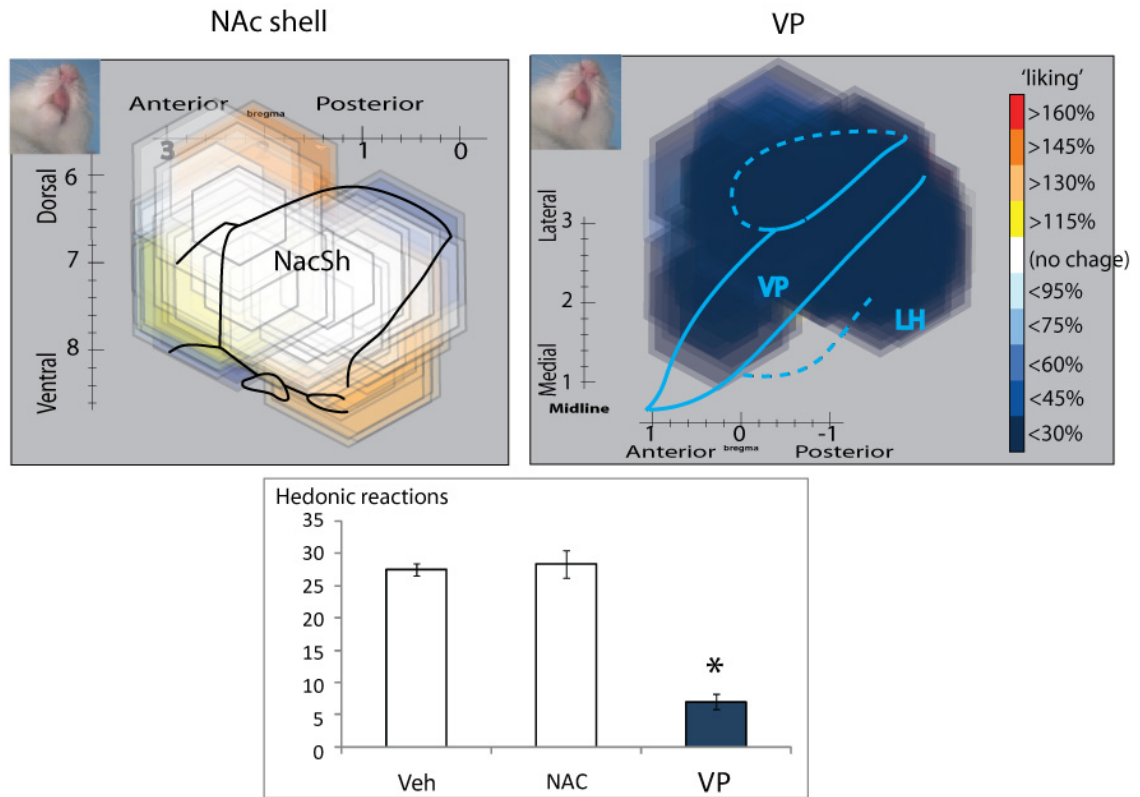
Figure 2-5 Lesions in VP but not NAc decrease 'liking' to sucrose. Map of 'liking' suppression after lesions of VP (*top right*) is shown in a horizontal view of VP. Map of 'liking' suppression after lesions of NAc shell (*top left*) is shown in a sagittal view of Nac shell. The symbol logic is identical to figure 2-3. Bar graph (bottom) shows the averaged hedonic reactions over the 1-min taste reactivity test. Error bars represent SEM. * indicates decreases in hedonic reactions (p< 0.05) compared to vehicle. Lesions of VP decrease 'liking' reactions to sucrose, while lesions of NAc shell have no effect on 'liking' reactions to sucrose.
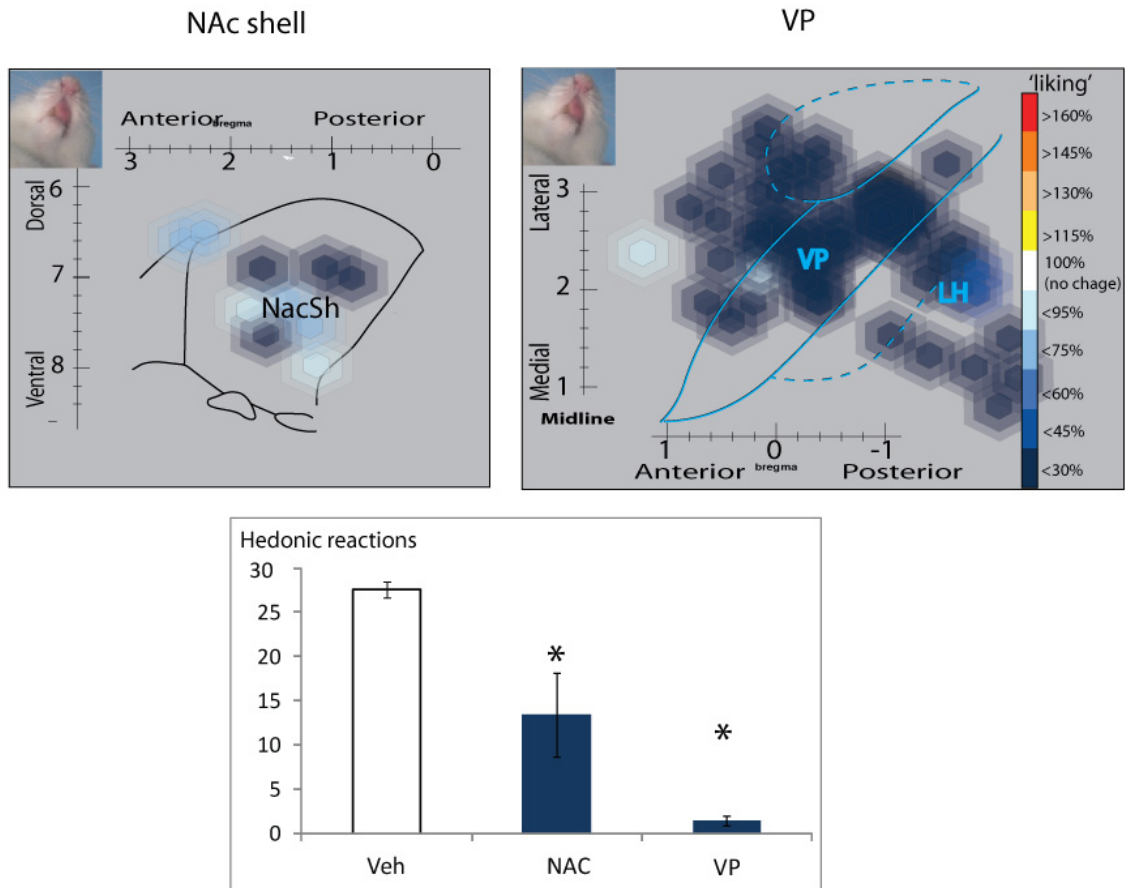
Figure 2-6 GABA in VP and caudal NAc shell decrease 'liking' to sucrose. Map of 'liking' suppression after microinjections of GABA agonists in VP (*top right*) is shown in a horizontal view of VP. Map of 'liking' suppression after microinjections of GABA agonists in NAc shell (*top left*) is shown in a sagittal view of Nac shell. The symbol logic is identical to figure 2-3. Bar graph (*bottom*) shows the averaged hedonic reactions over the 1-min taste reactivity test. Error bars represent SEM. * indicates significant decreases in hedonic reactions ($p < 0.05$) compared to vehicle. GABA agonists in VP decrease 'liking' reactions to sucrose compared to vehicle. GABA agonists in the caudal part of the NAc shell decrease 'liking' reactions to sucrose.
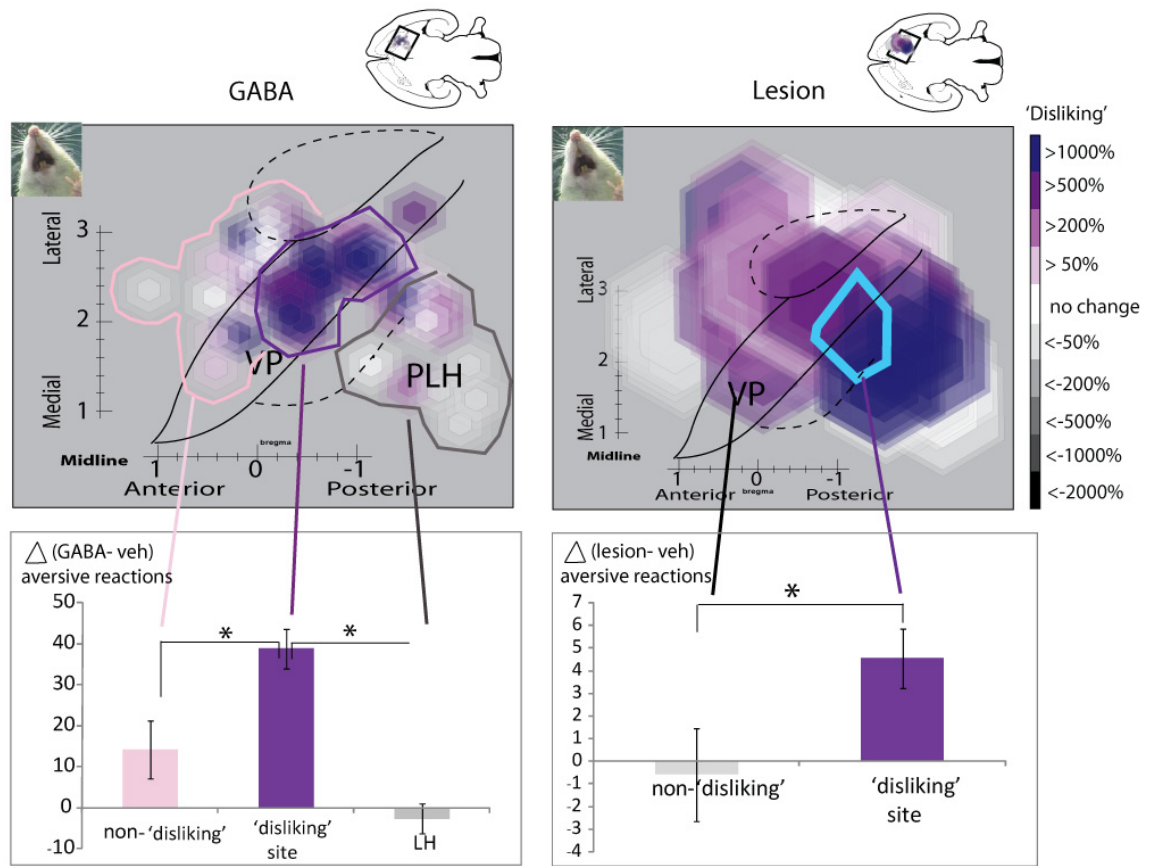
Figure 2-7 GABA and lesion in VP aversive sites. Maps of 'disliking' elevation to sucrose after microinjections of GABA agonists in VP (*top left*) and after lesions of VP (*top right*) are shown in a horizontal view of VP. The unanimous lesion site shared by 6 'aphagic and aversive' animals is circled by thick blue line (*top right*). The symbol logic is identical to figure 2-3. Bar graphs (bottom left and bottom right) show the averaged aversive reactions over vehicle in the corresponding subareas (thin color lines) in the 1-min taste reactivity test. Error bars represent SEM. * indicates decreases in aversive reactions ($p < 0.05$) compared to vehicle. Microinjections of GABA agonists and lesions in the caudal VP increase 'disliking' reactions to sucrose compared to vehicle. The two aversive sites highly overlap but are not identical.
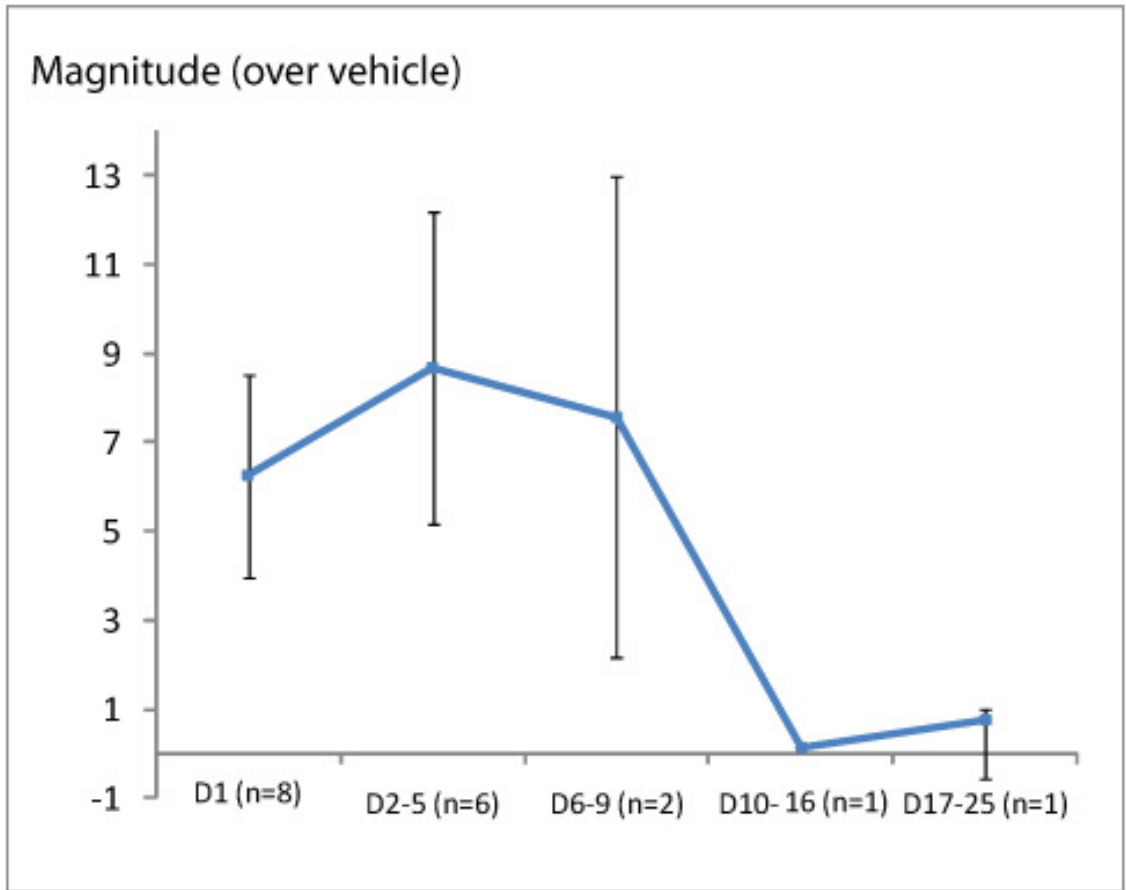
Figure 2-8 Timeline of lesion-caused aphagia and aversion to sucrose. On the second day after lesion, all rats (n=3) are aphagic and show aversion to sucrose. From the second day to the 5th day after lesion, six out of the eight rats remain aphagic and showed aversion. After day 5, only two out of the eight rats are still aphagic and aversive to sucrose. After day 10, only one rat is still aphagic but not aversive. All rats are fully recovered and start to eat normal rat chow by day 15. The recovery of lesion-induced aversion and lesion-induced aphagia proceeds roughly together.
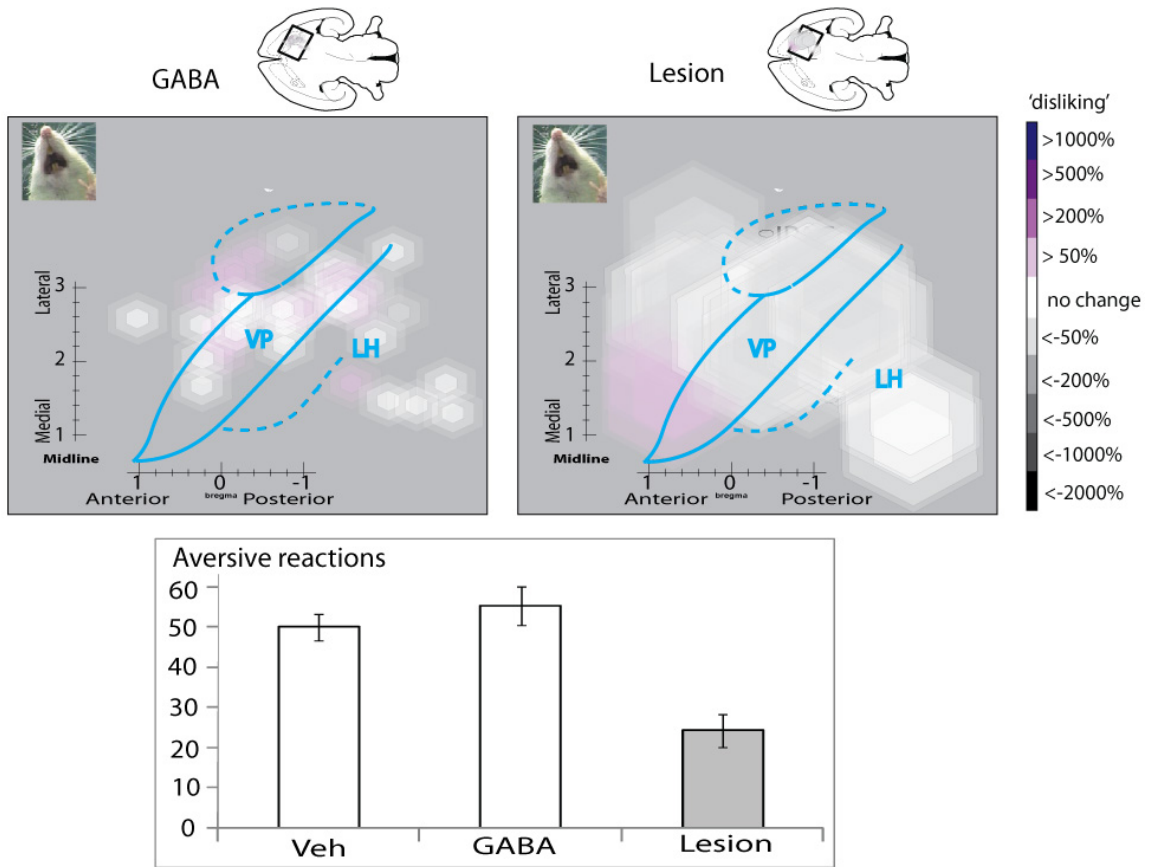
Figure 2-9 GABA and lesion in VP have no effect on 'disliking' to quinine. Maps of changes of aversive reactions to quinine after microinjections of GABA agonists in VP (*top left*) and lesions of VP (*top right*) are shown in a horizontal view of VP. The symbol logic is identical to figure 2-3. Bar graphs (bottom left and bottom right) show the averaged aversive reactions over vehicle over the 1-min taste reactivity session. Error bars represent SEM. * indicates decreases in aversive reactions (p< 0.05) compared to vehicle. Microinjections of GABA agonists or lesions in the VP have no effects on 'disliking' reactions to quinine.
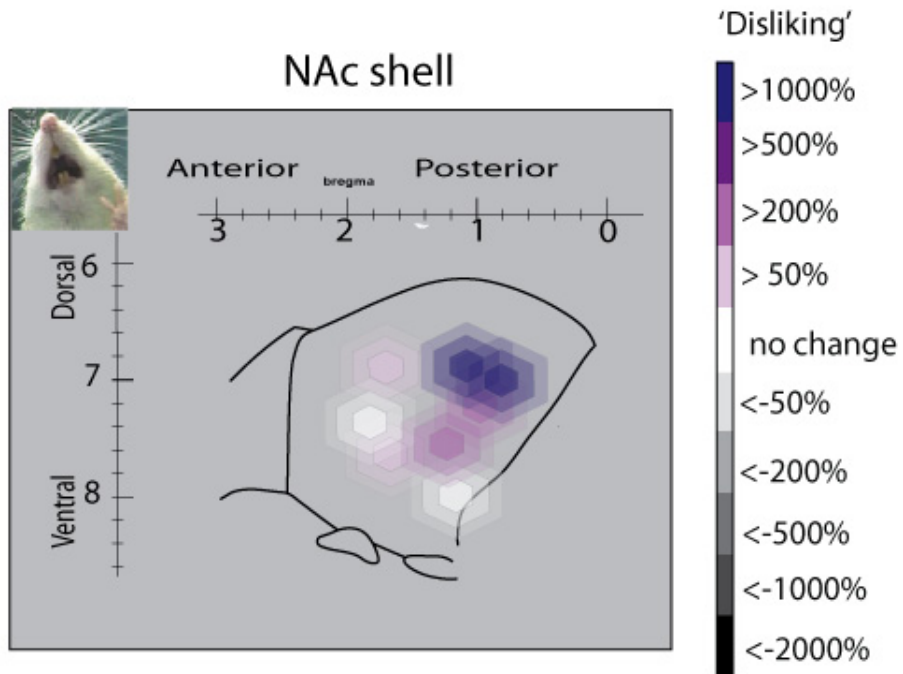
Figure 2-10 GABA in caudal NAc shell increases aversive 'disliking' to sucrose.
Map of 'disliking' changes to sucrose infusion after microinjections of GABA agonists in the NAc shell is shown in a sagittal view of the NAc shell. The symbol logic is identical to figure 2-3. Microinjections of GABA agonists in the caudal part of the NAc shell increase 'disliking' reactions to sucrose.
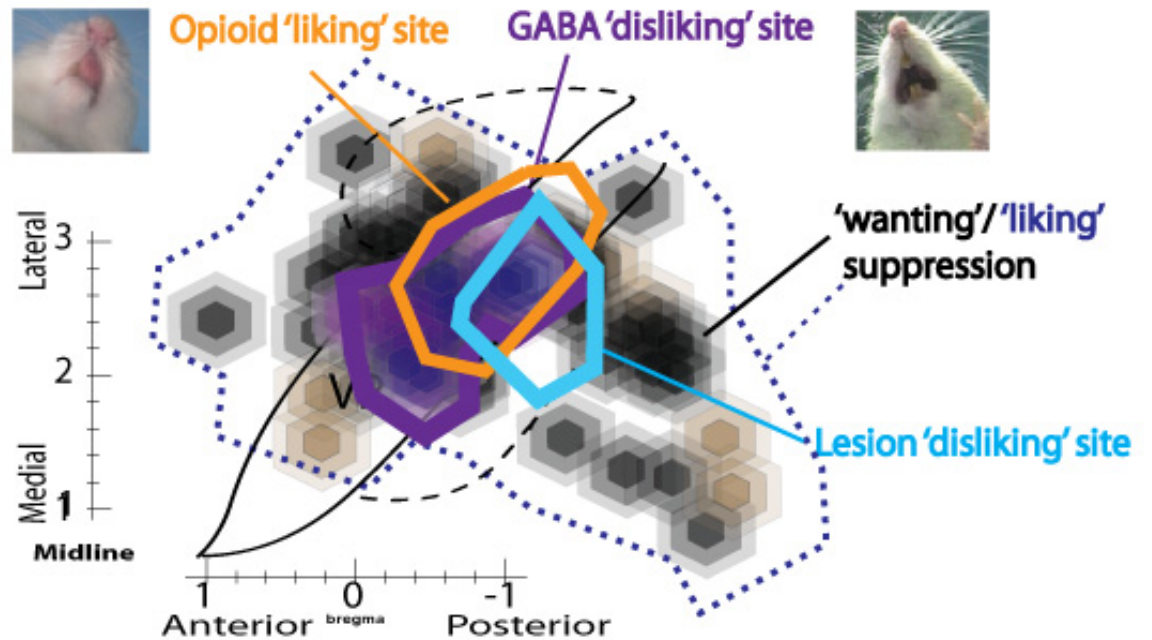
Figure 2-11 Comparison of GABA aversive site/lesion aversive site and opioid hedonic hotspot. Lesion and GABA suppress food 'wanting' and normal 'liking' to sucrose in the VP and surrounding areas (black area and dark blue dashed line). Within this larger loss of 'wanting' and loss of 'liking' area, GABA (purple line) and lesion (blue line) increased 'disliking' to sucrose in a more concentrate site in caudal VP. The GABA aversive site and the lesion aversive site highly overlap with the opioid hedonic hotspot identified previously in our lab (orange line) (Smith et al. 2005).

Chapter 3

Orexin Hedonic Hotspot in The Ventral Pallidum

Introduction

Interactions between the palatability of food and physiological states, such as hunger and satiety, have long intrigued neuroscientists. One situation we have all experienced is that food tastes better when we are hungry. This is the case of hunger state magnifying the palatability of foods and making foods more 'liked'. Another situation is that we usually feel as if we have room for more dissert after a full meal. This can be attributed to how the high palatability of dessert overrides the physiological satiety state and motivates us to eat even more. The misattribution of food palatability when hungry and the overindulgence of highly palatable food when full are likely to be the causes of clinical problems in recent decades, such as obesity and eating disorders.

Orexin neurons in the lateral hypothalamus (LH) serve an important role in bridging homeostasis and reward because of their three key abilities: detecting homeostasis, anatomically projecting to reward-related neural substrates, and modulating behaviors associated with reward. As a mediator in homeostasis, orexin neurons are able to monitor ambient fluctuations in the levels of nutrients and appetite-related hormones and alter their intrinsic electrical activity accordingly (Acuna-Goycolea & van den Pol, 2004; Asakawa, et al., 2002; Burdakov & Alexopoulos, 2005; Cai, et al., 1999; Diano, et al., 2003; Dube, et al., 2000; Ganjavi &

Shapiro, 2007; Griffond, et al., 1999; Lopez, et al., 2000; Niimi, et al., 2001; Perello, et al., 2009; Shiraishi, et al., 2000; Takenoya, et al., 2005; Toshinai, et al., 2003; Yamanaka, et al., 2000). For example, orexin neurons have higher mRNA expression or Fos expression in response to hypoglycemia induced by insulin or by fasting (Cai, et al., 2001; Griffond, et al., 1999). Increases of either extracellular glucose concentration or leptin induces marked hyperpolarization and cessation of action potentials in orexin neurons (Burdakov, Gerasimenko, & Verkhratsky, 2005; Cai, et al., 1999; Shiraishi, et al., 2000), while a decrease of glucose concentration or an increase of ghrelin concentration induces depolarization and increases firing frequency (Burdakov & Gonzalez, 2009; Liu, Morris, Spiller, White, & Williams, 2001; Yamanaka, et al., 2003). Anatomically, orexin neurons project widely throughout the brain, including reward-related structures, such as septal area, amygdala, ventral tegmental area (VTA), nucleus accumbens (NAC), ventral pallidum (VP), and parabrachial nucleus (PBN), suggesting that orexin neurons detect and pass on information about homeostasis to reward circuits and modulate reward processing (Backberg, Hervieu, Wilson, & Meister, 2002; Baldo, et al., 2003; Cutler, et al., 1999; Galas, et al., 2001; Marcus, et al., 2001; Nixon & Smale, 2007; Peyron, et al., 1998; Trivedi, et al., 1998). In the role of regulating reward processes, orexin neurons in the lateral hypothalamaus (apart from orexin neurons in the perifornical area and dorsomedial hypothalamus that are traditionally linked to arousal and stress) have recently been intensively studied because of their involvements in reward for food, drugs, and sex. With food reward, previous research has shown that orexin circuit is critically involved in food intake, food 'wanting' or motivation to work for food, and food reward 'learning' in a conditioned place preference paradigm (Baird, et al., 2009; Borgland, et al., 2009; Choi, Davis, Fitzgerald, & Benoit, 2010; Harris & Aston-Jones, 2006; Kotz, et al., 2002; Rodgers, et al., 2000; Sakurai, et al., 1998; Sharf, Sarhan, Brayton, et al., 2010; Thorpe, et al., 2003). Orexin knockout mice have been shown to have lower anticipatory activity for food and decreased food intake, yet develop late onset obesity, indicating abnormal homeostasis and reward processing (Akiyama, et al., 2004; Hara, Yanagisawa, & Sakurai, 2005). Taken together, the results suggest that orexin

neurons are an important interface between internal homeostatic state and food reward processing. However, it is unclear if and how orexin is involved in 'liking' aspect of the food reward.

Ventral pallidum might be the best candidate to test if orexin is involved in food 'liking' due to its unique role in processing reward 'liking' and its tight anatomical connections with LH orexin neurons. Functionally, the ventral pallidum, besides the NAc shell, is by far the only other known structure that is *sufficient* for processing hedonic 'liking' of food reward (Pecina & Berridge, 2005; Pecina, et al., 2006; K. S. Smith & Berridge, 2005). Microinjections of μ-opioid agonist (DAMGO) into a cubic-millimeter 'hedonic hotspot' in posterior VP enhanced rats' orofacial 'liking' reactions to sucrose along with an increase of food appetite, indicating that an increase of VP activity by opioid can *sufficiently* enhance hedonic 'liking'. Anatomically, the VP has been shown to have a tight link with the LH orexin neurons, which are activated by arcuate neuropeptide Y (NPY) during hunger state (Berridge, et al., 2010; Dube, et al., 2000; Kelley, Baldo, & Pratt, 2005; Niimi, et al., 2001; Yamanaka, et al., 2000) and send projections to reward related areas, including the NAc and the VP (Backberg, et al., 2002; Baldo, et al., 2003; Cutler, et al., 1999; Galas, et al., 2001; Marcus, et al., 2001; Nixon & Smale, 2007; Peyron, et al., 1998; Trivedi, et al., 1998). One recent study by Baldo et al. (2003) further pointed out that the intensive LH orexin projections to the VP, as assessed by fiber tracing, are particular dense in an area in the posterior VP that expands from -0.4mm to -0.92 mm posterior to bregma, which is exactly where the opioid hedonic 'liking' hotspot is located (Baldo, et al., 2003). Corresponding to the dense orexin projections, VP contains a significant level of OrexinA and OrexinB receptors (Marcus, et al., 2001), and sends reciprocal output to the LH neurons (Groenewegen, et al., 1993; Haber, et al., 1993; Zahm, et al., 1996). The anatomical evidence suggests that a functional interaction between LH orexin neurons and VP neurons may exist. The facts that the posterior VP is involved in hedonic 'liking' processes (via μ- opioid signal) and receives intensive orexin input raise one important

question- does orexin signal in the same region in posterior VP also mediate reward 'liking'.

Goals of the study

In this study, we investigated if orexin neurotransmission in posterior VP mediated the hedonic impact of a natural sweet taste, sucrose. To quantify orexin-induced changes in palatability of sucrose, we used taste reactivity paradigm (a measurement based on homologous orofacial expressions of affective 'liking' and 'disliking' reactions elicited by tastes in human infants, apes, monkeys, and rats) to quantify changes in hedonic 'liking' reactions to sucrose. Orexin-induced changes in aversive 'disliking' reactions to a bitter quinine taste were also quantified as comparison and control. To quantify the intensity and area of drug-induced neuronal activation, we used Fos plume technique (a measurement based on drug-induced Fos activation in tissues surrounding microinjection tips) to label and measure Fos protein expression caused by orexin microinjections. To precisely locate the most intense orexin-induced 'liking' effect in the ventral pallidum, we combine results from taste reactivity and Fos plume measurement, and present the combined results with color symbols. The size of the symbols represents the averaged intensity and area of orexin-induced Fos activation in VP, while the colors of the symbols represent the increases or decreases of 'liking' when microinjecting orexin at the exact same sites. Our results indicated that orexin microinjections in posterior VP significantly amplified hedonic 'liking' reactions to sucrose, while leaving aversive 'disliking' reactions to quinine unchanged. Also, orexin signal in surrounding areas, particularly LH, which is medial and posterior to VP, had no effect on hedonic 'liking' to sucrose.

Materials and Methods

General design

Split-and-recombine design was used to construct maps of 'liking' response to sucrose or maps of 'disliking' response to quinine. Rats are split into two separate groups, the behavioral taste reactivity group (n=26) and the Fos plume measurement group (n=10). Rats in the behavioral taste reactivity group (group 1)

were implanted with both cranial cannulae and oral cannulae, tested with taste reactivity test to quantify the intensity of 'liking' and 'disliking' responses to sucrose or quinine after drug microinjections, and euthanized for microinjection center identification. Rats in the Fos group (group 2) were implanted with cranial cannulae, handled and microinjected with drugs, and euthanized for Fos activation quantification. Fos protein activation around the microinjection center was compared to that in the non-surgical tissues in order to estimate the area and intensity of neuronal activation caused by orexin microinjections.

Results from both groups are combined to construct maps of 'liking' responses to sucrose or 'disliking' responses to quinine by color coding the behavioral effects (group 1 result) onto the Fos plume symbols (group 2 result) placed onto the microinjection centers identified by histology (group 1 result). The reason to split was to ensure that Fos activation and behavior effects were measured under the same conditions in which drugs had effects and to avoid the diminishment of drug efficacy on Fos activation after repeated microinjections. The reason to recombine was to map the 'liking' or 'disliking' behavioral effects onto the precise areas around the microinjection centers where orexin microinjections were likely to have effects based on Fos plume estimations.

Experiment 1 assesses orexin effect on hedonic 'liking' reactions to sucrose in the VP. Experiment 2 identifies the specific area in the VP that orexin has strongest effects on.

Surgery

Under anesthesia (80mg/kg ketamine HCL, 10 mg/kg xylazine, and 0.2 ml atropine sulfate), male Sprague-Dawley rats (n=26, 250-400g) were bilaterally implanted with guide cannulae (23 gauge) positioned 2 mm above the ventral pallidum (VP), or surrounding structures, for microinjection of drugs. Coordinates for implantation were: anteroposterior (AP), 0 to -0.8 mm; mediolateral (ML), ± 3 mm; dorsoventral (DV), -5.5 mm. The cannulae were anchored to the skull with stainless steel screws and dental cement. Obturators were inserted in guide cannulae to prevent clogging.

In the same surgery, all rats were bilaterally implanted with intraoral cannulae (PE-100 tubing) for delivery of the taste solutions. Oral cannulae enter the mouth lateral to the first maxillary molar, travel beneath the zygomatic arch, and exit the dorsal head near the skull screws. Oral cannulae did not disrupt normal eating. All rats were given at least 7 days to recover prior to the first test day.

Drugs and microinjections

OrexinA (10nM in 0.2µl ACSF) was dissolved in ACSF. Within-subject comparison design was applied. To ensure that order effects did not distort our data, microinjection order was counterbalanced between orexin and vehicle. Tests were spaced at least 48 h apart. On a particular test day, each rat received bilateral microinjections of 0.2 µl per side of either orexin or vehicle. Microinjections were made through a stainless-steel injector cannula (29 gauge) connected to PE-20 tubing and a syringe pump at a rate of 0.2 µl /min. Microinjector tips were held in place for an additional 1 minute after infusion to allow drug to diffuse away from the cannula tip and to avoid backflow. After microinjections, obturators were inserted back to the cranial cannulae to prevent clotting or infection, and rats were placed into the transparent taste reactivity chamber with intraoral fluid delivery tubing attached (described in behavioral taste reactivity testing).

Behavioral taste reactivity testing

Rats were handled and habituated in the taste reactivity chamber for 4 consecutive days for 30 minutes each before behavioral testing, and received a mock injection of vehicle on the final habituation day. On each test day, hollow fluid delivery tubing (PE-50 connected to a PE-10 delivery nozzle) was attached to the intraoral cannula to deliver solutions. The other end of the tubing was attached to a syringe pump, which infuses solutions at a stable rate of 1ml/min. All rats received sucrose solution (1%, 1 minute duration) 30 minutes after orexin/vehicle microinjections. Oral facial 'liking' and 'disliking' responses were videorecorded via an angled mirror placed underneath the transparent floor during the one- minute test. A small group of rats (n=9) received additional quinine infusions ($3x10^{-4}$M, 1 minute duration) 10 minutes after sucrose infusions. Quinine was always infused and tested after sucrose because hedonic 'liking' responses to sucrose are generally

more vulnerable to contamination from the prior taste compared to aversive 'disliking' responses to quinine.

Taste reactivity video scoring

Hedonic, aversive, and neutral response patterns were scored offline using frame-by-frame slow-motion videotape analysis developed to assess hedonic *vs.* aversive taste valuations (Berridge, 2000). Hedonic responses included rhythmic tongue protrusions, lateral tongue protrusions, and paw licks. Aversive responses included gapes, headshakes, face washes, forelimb flails, and chin rubs. Neutral responses included passive dripping of solution out of the mouth, ordinary grooming, and mouth movements. Neutral responses, which are less consistently linked to hedonic/aversive taste valuation, included grooming, passive dripping of solution out of the mouth, rhythmic mouth movement, and doing nothing. All video analyses were conducted blind to the microinjection contents and cannula placements using Observer software (Noldus, Netherlands).

A time-bin scoring procedure was used to ensure that taste reactivity components of different relative frequency still contributed equally to the final affective hedonic/aversive totals (Berridge 2000). For example, rhythmic mouth movements, passive dripping of solution, paw licking, and grooming typically occur in long bouts and were thus scored in 5 s time bins (5 s equals one bout occurrence). Tongue protrusions, which occur in shorter bouts, were scored in 2 s time bins (2 s equals one bout occurrence). The other behavioral components (lateral tongue protrusions, gapes, forelimb flails, head shakes, and chin rubs) typically occur in discrete events and were thus scored as single occurrences each time they occurred (e.g., one gape equals one occurrence). Finally, Individual totals were calculated for hedonic versus aversive categories by adding all response scores within an affective category for that rat. A hedonic 'liking' reaction total was the sum of occurrences for lateral tongue protrusions, rhythmic tongue protrusions, and paw licks. Similarly, an aversive 'disliking' reaction total was the sum occurrences of gapes, headshakes, face washes, forelimb flails, and chin rubs.

Histology

After completion of behavioral experiments, rats received an overdose of sodium pentobarbital (0.2g/kg). Rats' brains were extracted and post-fixed with 10% formalin in 0.1 M phosphate buffer (PB) for a few days and then transferred to 30% sucrose in 0.1M PB. Brains were sectioned with a freezing microtome into 60 μm, stained with cresyl violet, and mapped for the centers of the microinjections according to Paxinos and Watson atlas (2008).

General procedures for Fos plume measurement

Fos activation was assessed by c-Fos immunohistochemistry and visualized under the microscope (Figure 3-1). To quantify the area and the intensity of orexin-induced neuronal activation, Fos plumes caused by orexin microinjections were compared to Fos plumes caused by vehicle microinjections and non-surgical tissues (Figure 3-1, top). Orexin-induced Fos plumes were thus constructed and further divided into different intensity zones. The radii for each intensity zone were calculated and averaged. Assuming a spherical shape of functional drug diffusion, the averaged radii were applied to calculate the volumes of different Fos intensity zones and to construct the averaged Fos plume symbol of different intensities on the 2-D plane (Figure 3-1, bottom).

Fos-like protein immunohistochemistry

Rats in the Fos plume group (n=11) were handled gently for 3 days for 10 min each, similar to the behavioral groups, and then microinjected bilaterally in the VP with either orexin (10 nM, 0.2μl) or vehicle (ACSF). A separate group of non-surgical rats went through the same procedures but were held by the experimenter for 5 minutes instead of having microinjections. Ninety minutes later, rats were overdosed with sodium pentobarbital before transcardial perfusion. After perfusion, brains were removed and placed in 4% paraformaldehyde for 2 hours, transferred to 30% sucrose overnight, sectioned at 40 μm, and stored in 0.1M NaPb, pH 7.4. Alternate series of sectioned slices were processed for immunofluorescence.

For immunofluorescence processing, we used the avidin-biotin procedure (Hsu et al, 1981). In brief, brain sections were rinsed (0.1MNaPB+ 0.2% Triton), pre-blocked (5% Norrmal Donkey Serum (NDS) in 0.1M NaPB and 0.2% Triton), and

incubated in primary antibody (goat anti-Fos 1:500) and 5%NDS overnight at 4° C.
After primary incubation, the slices were once again rinsed, pre-blocked, and
incubated in secondary antibody (4% donkey-anti-goat Alexa 488) and 5% NDS for
2 hours followed by final rinses. The slices were mounted in 0.01M NaPB, let dried,
and cover slipped with anti-fade.

Fos plume maps of orexin-induced neuronal activation spread

The procedure for measuring drug-induced Fos plumes immediately
surrounding a local microinjection site was adopted from previous studies from our
lab (Smith & Berridge, 2005; Mahler et al., 2007).

To visualize and quantify drug-induced Fos activities, Fos-labeled cells on
tissue surface were visualized with 5x–40x magnification under the microscope
followed by image processing. (Figure 3-1) Fos count and Fos plume construction
were done post-hoc by an observer that was blind to the drug conditions. On the
Fos images, sampling blocks (50 x 50 μm) were placed along seven radial arms
emanating from the center of the microinjection site (45, 90, 135, 180, 225, 270,
315°) at 50 μm intervals. The 360° or 0° arm was not sampled because it was
occupied by the microinjection cannulae track. Fos- labeled cells in each individual
sampling box was counted individually (Figure 3-1, insets). In order to establish
baseline of Fos activation, two control conditions were measured: (1) in normal VP
tissue of intact brains without damage from surgical intrusion or gliosis (2) around
the site of vehicle microinjections in the VP to assess Fos activation caused by
microinjector tips and by the pressure from vehicle microinjections. Orexin-induced
Fos activation was also measured and compared to normal and vehicle-induced Fos
at the same site.

Orexin microinjections significantly increased Fos activation compared to
normal tissues. Orexin Fos plumes were mapped as zones of 'intense' or 'moderate'
Fos elevation identified by absolute Fos increases 3X or 2X over normal (Figure 3-
1). In this study, Orexin-induced Fos activation was not significantly stronger
compared to vehicle-induced Fos activation. To determine the averaged radii of
intense or moderate Fos activation zones around the microinjection center, the
distances from the microinjection center to the outer most sampling box of 3X or 2X

Fos elevation were measured and averaged across all seven radial arms in all animals. The averaged radii were applied to determine the size of the hexagon map symbol, which represents the averaged sizes of 3X and 2X Fos activation zones from the microinjection center (Figure 3-1, bottom).

Functional mapping for localization of behavioral effects

The averaged Fos plume and the behavioral data were recombined to map the localization of function (e.g. 'liking'). Microinjection sites from rats in the behavioral group were identified and plotted onto the atlas to represent the center of microinjections. The plume-derived hexagon symbols were superimposed onto the microinjection centers to represent estimated zones of intense or moderate activation caused by orexin. The magnitudes of the behavioral effects of each microinjection were color coded onto the hexagons to represent the functional zones in which the behavioral effects were induced by drug. Therefore, each hexagon symbol on the map illustrates the location of the microinjection in a particular rat tested for behavior, the sizes of zones of intense or moderate neuronal activation caused by the microinjection (based on averaged Fos-plume radii), and the magnitude of behavioral effect (e.g. 'liking' responses) when drug is injected at this specific site (based on taste reactivity test). The bilateral cannulae for each rat were collapsed into one unilateral map and plotted separately (two sites per rat).

Statistical analyses

All behavioral analyses were two-tailed and α was always set at $p<0.05$. Paired samples t-tests or one-way ANOVAs were used to test orexin effects on different behaviors. Between-subjects ANOVAs were used to determine anatomical location effects of microinjection sites (e.g. hotspot vs. non-hotspot).

Results

Fos plume mapping: identifying zones of local neuronal activation

Fos plume maps help to identify the spheres and the intensity of neuronal activation caused by microinjection of drugs. In traditional microinjection studies, only the microinjection centers are identified and plotted to represent the location where drug could cause behavioral effects. However, it is necessary to know how far

the drug spreads to stimulate tissue extending away from the microinjection site in order to map the substrates responsible for drug-induced effects on behavior.

Fos plume mapping, a methodology that applies IEG activation, has been shown to be a useful tool to identify specific areas responsible for the drug-induced behavioral effects, as described in previous studies (Mahler, et al., 2007; Reynolds & Berridge, 2008; K. S. Smith & Berridge, 2005). In this study, orexin A microinjections may induce Fos expression either directly through acting on the same neurons that contain orexin receptors and express Fos or indirectly through modulating nearby neurons which contain orexin receptors and in turn activate adjacent neurons that express Fos. In either case, a Fos plume reflects spheres of local functional modulation induced by the drug and provides quantitative information on sphere intensity and size. Most importantly, the boundaries of the plume reveal the functional zones likely to be responsible for orexin-induced behavioral effects, even if drug molecules spread further beyond the plume and are insufficient to trigger gene transcription or have functional effects.

Orexin microinjections produced roughly spherical and concentric zones of Fos enhancement (Figure 3-1). Different zones of activation are defined based on the intensity of Fos activation. The inner intense zone was defined as the zone in which Fos expression was increased by 3X above control 'normal' ventral pallidum tissue from uninjected rats. The outer moderate zone, which was further away from the drug microinjection center, was defined as the zone in which Fos expression was increased by 2X above control 'normal' ventral pallidum tissue from uninjected rats. The averaged radius of each zone, gathered from 6 to 10 Fos plumes, was applied to calculate the volume of drug-induced functional spheres and to determine the size of hexagon symbol we used to represent functional drug effect on the 2-D plane.

The size of the Fos plume depends on the dosage and the speed of microinjection. Microinjection of 10 nM 0.2μl Orexin A over one minute followed by maintaining the microinjectors in place for another minute produced an averaged Fos plume with a mean radius of 0.065 ± 0.006 mm for the inner intense zone and a mean radius of 0.16 ± 0.01 mm for the outer moderate zone. We chose to use normal

non-surgical tissues as control because vehicle Fos expression might cast a ceiling effect on anandamide-induced Fos effect. In order to avoid the underestimation of drug functional effect, we chose to compare orexin Fos expression with normal tissues.

Fos plume maps were later generated by combining the results from the Fos group(averaged Fos plume) and the results from the behavioral group (changes of 'liking' or 'disliking' caused by drug microinjections at a specific site).

Orexin enhances sucrose hedonic impact in posterior VP

Averaged hexagon-shaped Fos symbols with an inner zone (radius:0.065 ± 0.006 mm) and an outer zone (radius: 0.16 ± 0.01 mm) were placed onto each microinjection site identified from rats in the behavioral group to represent the intense and moderate functional drug activation spread from the center. The Fos symbols were later color-coded based on the magnitude of the behavioral effects, defined by drug-induced changes of 'liking' or 'disliking reactions over vehicle, produced by orexin A microinjections at the corresponding site.

Orexin A microinjections on average (n=26) caused an overall 15.8% increase of hedonic 'liking' reactions (sum of rhythmic tongue protrusion, lateral tongue protrusion, and paw lick scores) elicited by sucrose infusion compared to control levels after vehicle microinjections ($F_{1,76}$=8.025, p<0.01). Sucrose infusion elicited mainly hedonic 'liking' reactions and rarely aversive 'disliking' reactions. Orexin A selectively enhanced the number of hedonic 'liking' reactions elicited by sucrose and did not affect the number of aversive 'disliking' reactions. Although further study is needed to identify the effect of orexin A on hedonic 'liking' reactions to sucrose in anterior ventral pallidum, the decreased 'liking' gradient spreading from posterior VP to anterior VP suggests that the most intense 'liking' enhancement caused by orexin A is most likely in posterior VP.

Orexin hedonic hotspot in posterior VP

A hedonic hotspot is defined as a specific brain site where drug microinjections elicit the most intense hedonic 'liking' enhancement. In the case of orexin-amplified hedonic 'liking' reactions to sucrose, a hedonic hotspot is where

orexin could elicit >60% hedonic enhancement to a sweet taste. Orexin caused an average of $0.34 \pm 0.12\%$ increase (11.57 ± 3.87 more hedonic reactions compared to vehicle) of hedonics in the hedonic hotspot as shown in figure 3-3. In comparison, orexin caused only $0.1 \pm 0.05\%$ increases of hedonics in the non-hotspot zone and $0.024 \pm 0,047$ % increases of hedonics in the lateral hypothalamus (Figure 3-3). Orexin in the hotspot enhanced hedonic 'liking' to sucrose significantly more compared to the non-hotspot area (p=0.048) and LH (p=0.015). This orexin hedonic 'liking' hotspot was found to be concentrated in posterior VP (roughly AP -0.2~ -0.9mm, DV -7.3~ -8 mm, ML ±2.2~ 3mm), which was posterior to interventricular foramen and between the rostral tip of the lateral hypothalamus and the caudal tip of the olfactory tubercle. The center of the orexin 'liking' hot spot was located at 0.7 mm posterior to bregma, 2.4 mm medial lateral to bregma, and 8 mm ventral to the skull. The area of intense orexin 'liking' enhancement extended from this center all the way forward to middle VP (AP -0.2 mm), where VP is ventral to anterior commissure, lateral to substantia innominata and lateral preoptic area, and medial to the interstitial nucleus of the posterior limb of the anterior commissure, and downward caudal VP (AP -0.9mm), where VP is lateral to the most anterior tip of the lateral hypothalamus and joints with the most anterior part of the sublenticular extended amygdala (SLEA).

Orexin does not affect hedonic 'liking' in the anterior VP or the LH

Orexin exclusively produced intense hedonic enhancement in the posterior VP, but not in globus pallidus, sublenticular extended amygdala, or lateral hypothalamus. While orexin A in the hedonic hotspot produced > 30% intense enhancement of hedonic 'liking' reactions to sucrose, it produced 11% changes of hedonics in the immediate surrounding areas (SLEA, ventral globus pallidus), no change (0%) in the anterior VP or in the lateral hypothalamus (Figure 3-3).

It is of particular interest that at the dosage of 10nM, 0.2μl orexin failed to enhance hedonic 'liking' in the LH. Despite the fact that orexin-producing neurons in lateral hypothalamus (LH) have been suggested to mediate rewards for food and

drug, results from the current study suggest that LH orexin neurons are not involved in the hedonic 'liking' aspect of reward.

Although the current study showed that orexin intensely enhanced hedonic 'liking' in the posterior VP, no microinjection sites were located in the anterior VP. It requires future studies to elucidate the specific role of orexin A on hedonic 'liking' in the anterior VP, or whether or not orexin A decreases hedonic 'liking' in a pattern similar to μ- opioid signal (K. S. Smith & Berridge, 2005). As for now, it is safe to conclude from the current study that a concentrated and highly effective hedonic 'liking' hotspot induced by orexin A focuses in the posterior VP.

Orexin does not affect aversive 'disliking' reactions to quinine

In contrast to the enhancement of hedonic 'liking' reactions to sucrose, microinjections of Orexin A in posterior VP had no significant effect on aversive 'disliking' reactions to quinine (Figure 3-4). Oral infusions of bitter quinine elicited mainly aversive 'disliking' reactions, such as gapes, head- shakes, etc, and hardly any hedonic 'liking' reactions (vehicle: 51.8 ± 11.8 aversive reactions; orexin: 44.14 ± 8.4 aversive reactions, $F_{1, 12}$= 2.72, p= 0.61). Orexin changed neither the low level of 'liking' reactions nor the dominant 'disliking' reactions to quinine.

Discussion

Orexin enhanced 'liking' reactions to sucrose in the posterior VP

Our results demonstrated that orexin enhanced hedonic 'liking' reactions to sucrose in posterior VP. The orexin hedonic hotspot, where orexin has the most intense enhancement of 'liking' reactions to sucrose, is limited to the posterior portion (1/3) of the VP that is anterior to the sublenticular extended amygdala (SLEA), and anterior an lateral to the lateral hypothalamus (LH), and caudal to the olfactory tubercle (Figure 3-3).

Orexin did not change aversive 'disliking' reactions to a bitter quinine taste, even when microinjections of orexin at the same site enhanced 'liking' reactions to a sweet sucrose taste. This selective enhancement pattern suggests that orexin only enhances the hedonic 'liking' value of a tasty taste without affecting the aversive 'disliking' value of a nasty taste. In other words, orexin appeared to make a sweet

taste even tastier and more 'liked', but did not make a bitter taste nastier or more 'disliked'. This result is consistent with previous findings about how orexin selectively promotes motivation for positive high-fat food/drug reinforcers and modulates synaptic plasticity only under conditions of positive salience but not arousal or aversion (Aston-Jones, et al., 2009; Borgland, et al., 2009; Choi, et al., 2010; Harris & Aston-Jones, 2006; Harris, et al., 2005; Sharf, Sarhan, Brayton, et al., 2010). However, we cannot draw conclusion from the current study about whether orexin-induced selective enhancement of hedonic 'liking' in the posterior VP also drives motivation, or 'wanting', for positive rewards. Future studies are required to establish the role of VP orexin in 'wanting' aspect of the reward processes.

Orexin in VP does not enhance motor activity or arousal

The orexin-induced enhancement of hedonic 'liking' reactions to sucrose is not due to motor effects, arousal, or reflex. Orexin microinjections in posterior VP only amplified hedonic 'liking' to sucrose, but not aversive 'disliking' to sucrose, or 'liking'/'disliking' to quinine. If orexin enhanced 'motor activity' in general, at least orofacial aversive 'disliking' reactions to quinine would be enhanced by orexin microinjections at the same site in which orexin enhanced hedonic 'liking' reactions to sucrose infusion. However, orexin enhanced only hedonic 'liking' aspects of reward without affecting aversive 'disliking' reactions to quinine, even in the orexin hotspot where the strongest enhancement of 'liking' was produced. If orexin enhanced arousal to infusion of sweet/bitter solutions, we would expect to see enhancements of 'disliking' reactions to sucrose or 'liking'/'disliking' reactions to quinine. However, none of the above aspects were altered by orexin microinjections in VP. Although orexin has long been linked to stress and arousal (Boutrel, et al., 2005; Carr & Kalivas, 2006; Ford, et al., 2005; Harris & Aston-Jones, 2006; Sharf, Sarhan, & Dileone, 2010; Yamanaka, et al., 2003; Zhu, Onaka, Sakurai, & Yada, 2002), the fact that only the hedonic 'liking' reactions to sucrose were enhanced ruled out the possibility of general arousal or solution infusion-elicited stress. Further support of the notion that orexin in VP enhanced 'liking' but not arousal or stress came from the observation that rats appeared to be calm and did not show higher locomotion or rearing behavior in the taste reactivity chambers during testing

(personal observation). Similar to the reasons listed above, we do not think orexin-induced enhancement of 'liking' reactions to sucrose was due to reflex because of its specificity to hedonic aspect of positive reward.

Orexin hedonic hotspot highly overlaps with opioid hedonic hotspot

The orexin hedonic hotspot in the posterior VP, where orexin elicits the most intense enhancement of 'liking' reactions to sucrose, has a high level of overlap with the opioid hedonic hotspot previously discovered in our lab (K. S. Smith & Berridge, 2005). This anatomical overlap implies a potential interaction between opioid and orexin- together they regulate reward processing and promote motivation for reward.

Previous research has found evidence that supports orexin- opioid interaction and suggests neither of the two systems can function normally without the other in processing food reward (Clegg, et al., 2002; Georgescu, et al., 2003; Sweet, et al., 2004; Zheng, et al., 2007). Systemic inhibition of opioid pathways or local blockade of opioid in NAc (but not LH) disrupted orexin-induced feeding effects (Clegg, et al., 2002; Sweet, et al., 2004). Similarly, pharmacological inhibition of orexin signal in the ventral tegmental area blocked high fat appetite induced by opioid microinjections in NAc shell (in an area similar to the opioid hedonic hotspot found in our lab) (Zheng, et al., 2007). These results demonstrated the importance of functional crosstalk between orexin and opioid in food reward, and further suggested that the communication between opioid in NAc and orexin neurons in LH might be one link between reward and homeostasis.

Several reasons made us believe that the connections between VP and LH could be another link between reward and homeostasis, and the posterior VP may serve as an important converging point between orexin and opioid circuits for reward. Anatomically, VP has reciprocal connections with both LH and NAc and is located between NAc and LH (Backberg, et al., 2002; Baldo, et al., 2003; Cutler, et al., 1999; Galas, et al., 2001; Groenewegen, et al., 1993; Groenewegen, Wright, Beijer, & Voorn, 1999; Haber & Knutson, 2010; Haber, et al., 1993; Marcus, et al., 2001; Nixon & Smale, 2007; Peyron, et al., 1998; Trivedi, et al., 1998; Zahm, et al., 1996). Functionally, results from the current study showed that VP contains hedonic

hotspots for both orexin and opioid in a similar site in posterior VP. Taken together, these results suggest that VP converges information from opioid circuit via NAc inputs and orexin circuit via LH inputs, and both signals can sufficiently enhance 'liking' of reward. It would be of particular importance for future studies to identify whether orexin and opioid in hedonic hotspots in posterior VP are both required for enhancement of reward 'liking'.

Orexin in lateral hypothalamus does not mediate reward 'liking'

Results from the current study showed that orexin in the LH is not involved in 'liking' of reward despite its importance in other aspects of reward shown by previous studies. It has been hypothesized that orexin neurons in LH are critical in combining information about internal states and external cues, and promote motivated behaviors via synaptic output to dopamine neurons in VTA (Alberto, et al., 2006; Aston-Jones, et al., 2010; Borgland, et al., 2009; Borgland, Taha, Sarti, Fields, & Bonci, 2006; Kelley, Baldo, & Pratt, 2005). As a detector of internal states and homeostasis, LH orexin neurons can alter their electrical activity in response to concentration changes of nutrients and appetite-hormones (Burdakov & Alexopoulos, 2005; Burdakov, et al., 2005; Cai, et al., 1999; Liu, et al., 2001; Shiraishi, et al., 2000; Yamanaka, et al., 2003). As a receiver of external cues, LH orexin neurons respond to both drug- and food- associated cues, and the level of neuronal activation is correlated with the extent of preferences for food and drug rewards (Aston-Jones, et al., 2009; Aston-Jones, et al., 2010; Harris, et al., 2005; Harris, et al., 2007). Through projections to reward-related brain areas, an increase of orexin signal can sufficiently promote motivation to work for food and drug rewards (Aston-Jones, et al., 2009; Borgland, et al., 2009; Choi, et al., 2010; Harris & Aston-Jones, 2006; Harris, et al., 2005; Sharf, Sarhan, Brayton, et al., 2010), and mediate anticipatory activity for food rewards when being food deprived (Akiyama, et al., 2004). Although results from the current study showed that orexin in LH is not sufficient for hedonic 'liking', it does not rule out the possibility that LH orexin can regulate behaviors to respond to 'liking' of reward or even actively regulate 'liking' of reward. One mechanism that would allow LH orexin neurons to communicate with 'liking' circuit might exist between the two-way connections between VP

hedonic hotspot and LH. Future studies are needed to identify whether functional connectivity exists between neurons in the orexin hedonic hotspot in posterior VP and orexin neurons in LH.

Orexin's role in reward and homeostasis- hunger-induced misattribution of food palatability vs. high palatability-driven override of satiety

How does hunger modulates food palatability? How does highly- palatable food hijack homeostasis and motivates us to eat even more? The reciprocal connections between orexin 'liking' hot spot in posterior VP and LH orexin neurons might be one critical link that explains the interaction between 'food palatability' and 'physiological state and homeostasis'.

The projection from LH orexin neurons to orexin hedonic hotspot in posterior VP may mediate the mechanism underlying alliesthesia, a phenomenon that describes physiological state -induced pleasantness of a stimulus (or hunger-induced food 'liking'). One potential mechanism was proposed by Kelly et al (2005): LH orexin neurons are activated during hunger state by NPY, send projections to activate glutamate neurons in the thalamic paraventricular nucleus, which in turn activate large acetylcholine-containing interneurons in the NAc shell and indirectly activate nearby enkephalin neurons. Supposedly, these enkephalin neurons include those in the opioid hedonic hotspot in the NAc shell, whose activation leads to an enhancement of hedonic 'liking' of rewards. Therefore, through this indirect LH-thalamic paraventricular- NAc shell route, hunger state could indirectly recruit opioid hedonic hotspot and enhance palatability of foods. Here we propose a second mechanism based on the finding of a VP orexin hedonic hotspot in posterior VP: LH orexin neurons directly recruit the orexin hedonic 'liking' hotspot in the caudal VP. Instead of indirectly recruiting the NAc opioid hotspot via glutamatergic neurons in the thalamic paraventricular nucleus and cholinergic neurons in NAc shell, we hypothesize that LH orexin neurons recruit 'liking' circuit via direct anatomical projections to the orexin hedonic hotspot in posterior VP. Thus, during hunger state, LH orexin neurons are activated by NPY neurons and directly enhance palatability of foods through the activation of VP orexin hotspot. The overpowering

of this circuit may lead to the misattribution of palatability to food reward and increase the motivation to eat even when satiated.

The projection from VP orexin 'liking' hot spot to LH orexin neurons may mediate the mechanism of 'liking'- induce override of satiety state or 'liking'- induced motivation to eat. Orexin in posterior ventral pallidum enhanced 'liking' reactions to a palatable sweet taste. VP neurons in a similar region as the orexin 'liking' hot spot project to LH orexin neurons, which are involved in homeostasis and motivation to eat. It is possible that the hedonic factors of food activate VP hot spot, which sends output to LH orexin neurons and hijacks homeostasis. In other words, the tastes of highly palatable foods may trigger the activation of VP 'liking' hot spots, and neurons in this region send the 'liking' signal to LH orexin neurons to either suppress hunger state or promote motivation to eat or both.   Orexin role in drug reward

In the past decade, a series of studies, particularly those from the Aston-Jones group, provide new insights on orexin's prominent role in drug rewards. Orexin neurons have been shown to respond to reward (food, morphine, cocaine)-associated cues as measured by Fos activation, and the level of activation varies in proportion to animals' preference of the reward in a conditioned place preference task (Aston-Jones, et al., 2009; Aston-Jones, et al., 2010; Choi, et al., 2010; Harris, et al., 2005; Harris, et al., 2007). The blockade of orexin neurotransmission disrupts addiction- associated behaviors, including the acquisition of morphine conditioned place preference, alcohol consumption, cue-induced reinstatement of cocaine-seeking behavior, or nicotine- induced reinstatement (Aston-Jones, et al., 2009; Harris, et al., 2007; Moorman & Aston-Jones, 2009; R. J. Smith, et al., 2009). One study particularly relevant to the current study showed that orexin neurons may be able to track hedonic processing during protracted withdrawal; Fos expression of LH orexin neurons were higher when morphine withdrawn animals were tested with drug preference (compared to normal animals) and lower when these same animals were tested with food or novelty preference, which corresponded with these animals' behavior (Aston-Jones, et al., 2009). This particular finding indicated that orexin might mediate the hedonic value of drug reward as well. While orexin

hedonic hotspot in VP was shown to mediate the hedonic value of food rewards, it is still unclear whether VP orexin is involved in the hedonic aspect of the drug rewards.
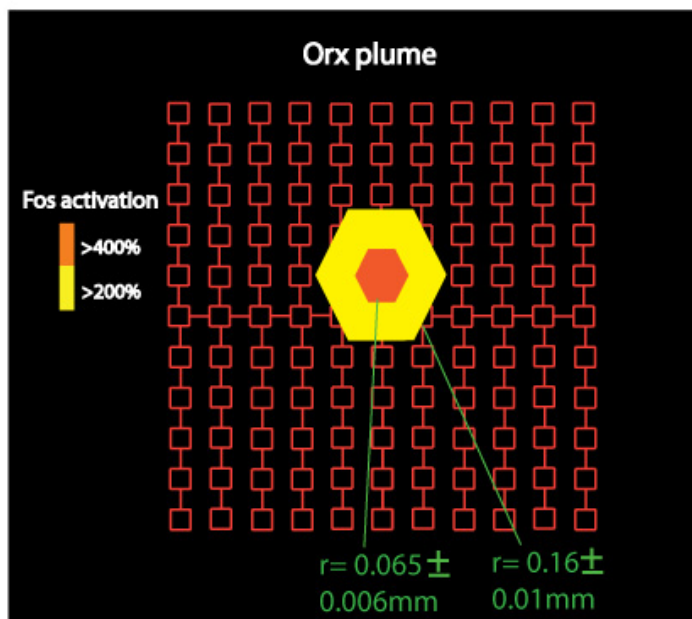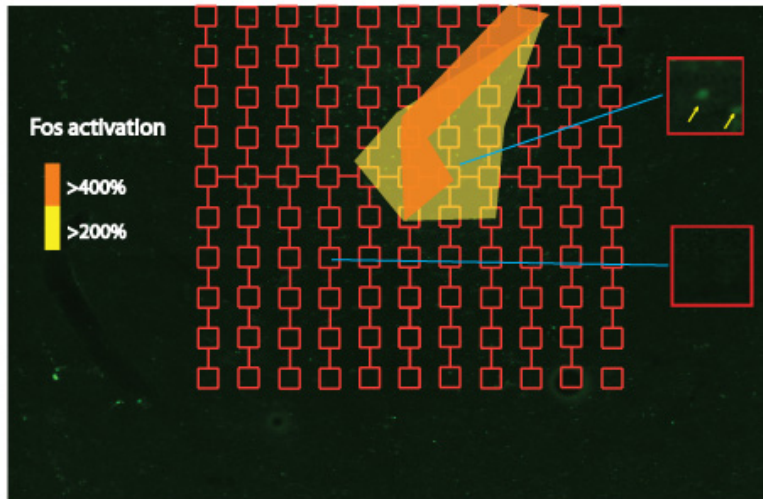
Figure 3-1 Orexin Fos plume in VP. Fos Plume Sampling Method (top): The method used to sample Fos expression around injection site is illustrated. Fos-expressing neurons are counted in 50 x 50 μm blocks on 8 sampling arms (0o, 45o, 90o, 135o, 180o, 225o, 270o, 315o) extending from the center of microinjection center at 50 μm intervals. Insets show sample tissue blocks from equivalent sites that were injected with vehicle (ACSF) or with orexin A (10nM in 0.2μl). Fos expression appears as green oval dot under the microscope at 10X magnification (yellow arrow). The color scale demonstrated the colors used for different extents of Fos elevation (over vehicle control levels). Colored areas indicated the neural elevation of 200% (yellow) and 400% (orange) orexin Fos expression compared to healthy normal tissues. Averaged orexin Fos Plume (bottom): Mean (SEM) plume radii are shown for orexin microinjections in VP. Inner orange hexagon represents an area of 200% activation. Outer yellow hexagon represents an area of 400% activation.
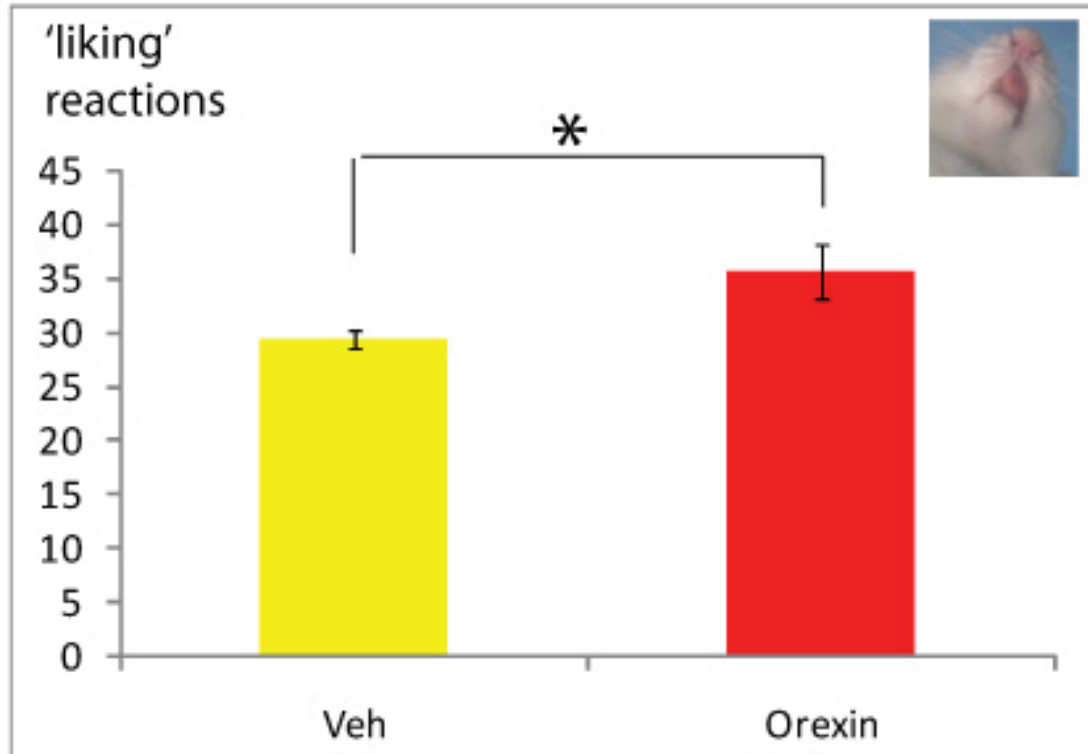
Figure 3-2 Orexin in VP increases 'liking' to sucrose. Averaged total hedonic 'liking' reactions (n= 26) to sucrose are shown after orexin microinjections (red) and vehicle microinjections (yellow) in the 1-min taste reactivity test. * indicates significant increases of hedonic reactions (p<0.05) compared to vehicle.
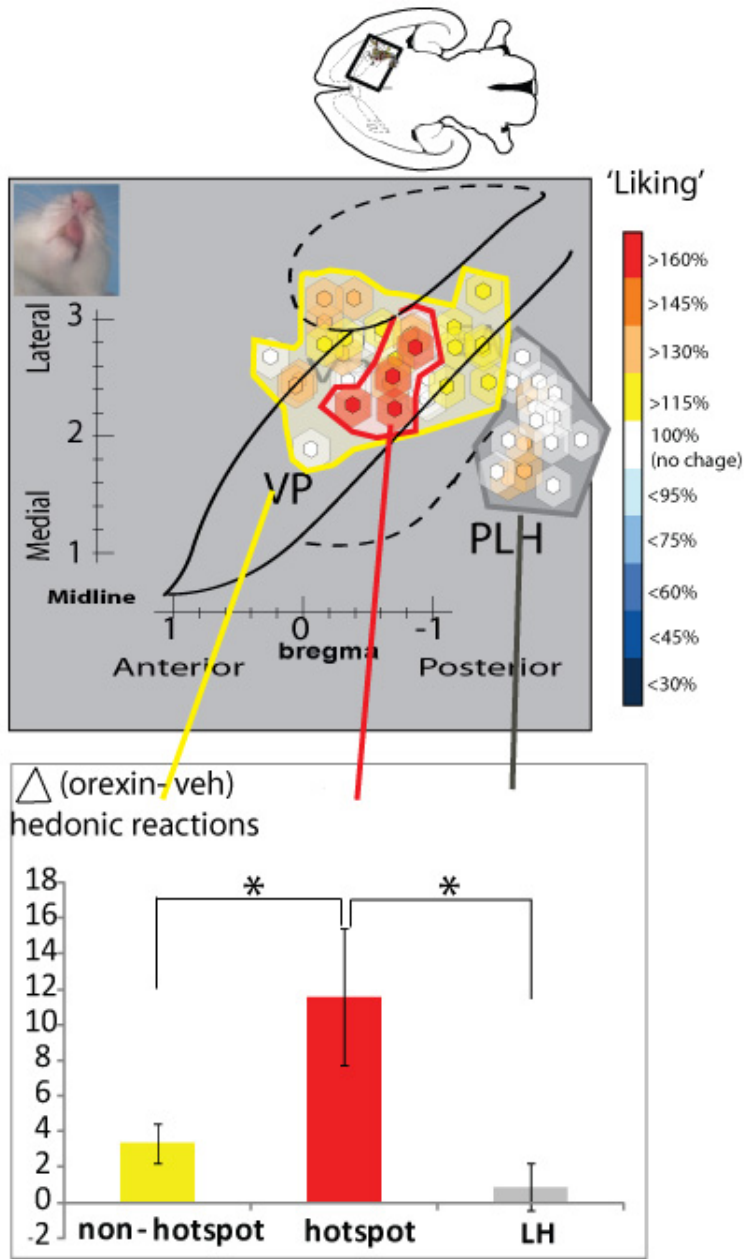
Figure 3-3 Orexin in VP hedonic sites increase 'liking' to sucrose. 'Liking' enhancements following oreixn microinjections are mapped rat-by-rat onto a horizontal view of VP, showing both the with-subject change in hedonic 'liking' reactions relative to vehicle (color) and the functional spread of the microinjection based on Fos plume data (size). For orexin hexagon, and inner symbols represent 400% Fos activation, outer symbols represent 200% Fos activation. Bar graphs (bottom left and bottom right) show the averaged hedonic reactions over vehicle in the corresponding subareas (thin color lines) in the 1-min taste reactivity test. Error bars represent SEM. * indicates significant increases in hedonic reactions (p< 0.05) compared to vehicle. Microinjections of orexin in the caudal VP increase 'liking' reactions to sucrose compared to vehicle.
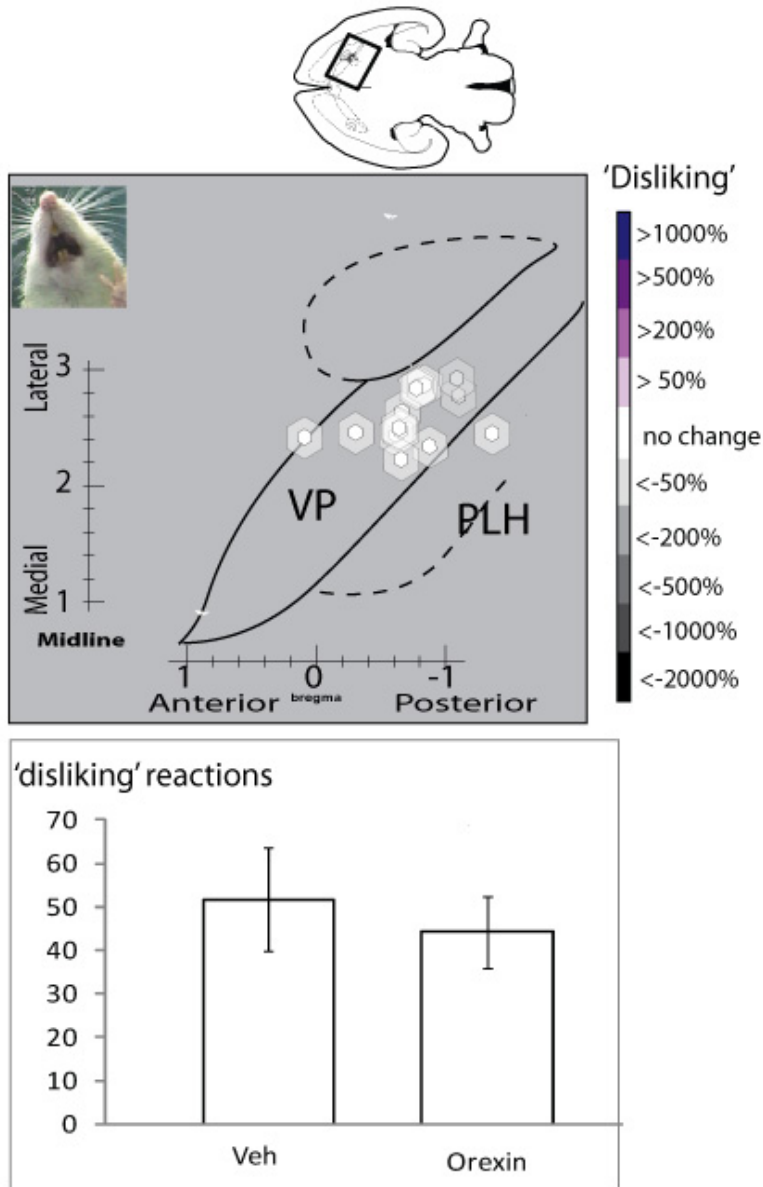
Figure 3-4 Orexin in VP has no effect on 'disliking' to quinine. Map of 'disliking' changes to quinine after microinjections of orexin in VP (top) is shown in a horizontal view of VP. The symbol logic is identical to figure 3-2. Bar graph (bottom) shows the averaged aversive reactions over vehicle over the 1-min taste reactivity session. Error bars represent SEM. Microinjections of orexin in the VP have no effects on 'disliking' reactions to quinine.
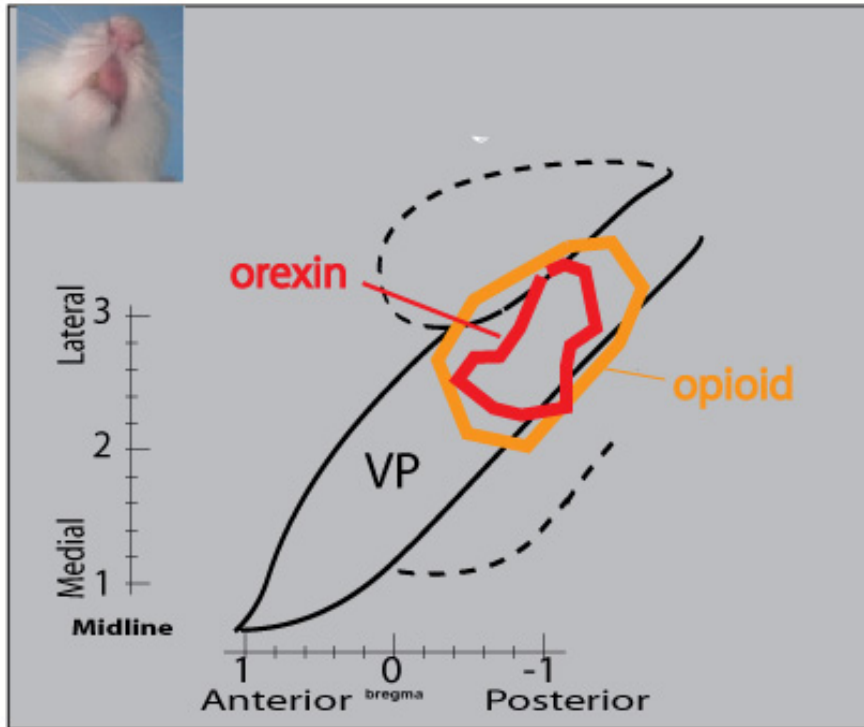
Figure 3-5 Comparison of orexin hedonic hotspot and opioid hedonic hotspot.
Orexin increases 'liking' to sucrose in the caudal VP (red line). The orexin hedonic
hotspot highly overlaps with the opioid hedonic hotspot identified previously in our
lab (orange line) (Smith et al.2005).

Chapter 4

Endocannabinoid Hedonic Hotspot in VP

Introduction

Cannabinoid drugs, such as THC, have been shown to increase appetite (e.g. marijuana 'munchies'), especially for sweet food, and are associated with pleasure in both humans and animals. In animals, systemic microinjections of cannabinoid drugs or endogenous cannabinoid neurotransmitters stimulate eating behavior and food intake (DiPatrizio & Simansky, 2008; Koch & Matthews, 2001; Mahler, et al., 2007; Shinohara, et al., 2009; Solinas, et al., 2008; Williams & Kirkham, 2002; Williams, et al., 1998). In humans, cannabinoids have long been known as medical treatments for a number of conditions associated with food appetite and reward, such as chemotherapy-induced nausea and loss of appetite, AIDS-associated depression, weight loss, etc (Solinas, et al., 2008). The identification and characterization of mechanisms underlying cannabinoids' functional roles will further clarify the neuronal control of food appetite and better improve medical use of cannabinoids.

The discovery of two subtypes of G protein-coupled cannabinoid receptors, the CB1-R and CB2-R, as well as the isolation of different endogenous ligands, such as anandamide and 2-arachidonoylglycerol (2-AG), have opened new directions in research of endocannabinoid system and food appetite. Between the two types of

receptors, CB1 receptors have been suggested to play a more important role in brain reward processes (Cooper, 2004; Osei-Hyiaman, et al., 2005; Pagotto, Marsicano, Cota, Lutz, & Pasquali, 2006; Solinas & Goldberg, 2005; Williams & Kirkham, 1999). Anatomically, CB1-Rs are highly expressed in brain areas associated with reward, such as nucleus accumbens (NAc), ventral pallidum (VP), and ventral tegmental area (VTA), whereas CB2-Rs are present mainly in the periphery (Glass, et al., 1997; Mailleux, et al., 1992). Functionally, CB1 receptors are thought to mediate most of the rewarding effects of the endocannabinoid system in the brain, such as increased eating. As for the two endogenous ligands for cannabinoid receptors, anandamide, a partial agonist for CB1 receptor, and 2-AG have both been shown to be involved in food reward processing. Microinjections of anandamide and 2-AG not only enhance food appetite in normal animals but also induce over-eating in sated animals (Cooper, 2004; Kirkham, Williams, Fezza, & Di Marzo, 2002; Mahler, et al., 2007; Solinas, et al., 2008; Williams & Kirkham, 1999, 2002). Together, the results suggest that the activation of endocannabinoid system facilitates food reward processing via endogenous receptors, predominantly CB1Rs, and endocannabinoids.

Cannabinoid-mediated increases of food palatability have been hypothesized to mediate the enhancement of food appetite. Previous studies have demonstrated that systemic microinjections of CB1 agonist increase intake of sweet, palatable food rewards more than regular rat chows (Koch & Matthews, 2001), while CB1 antagonist reduce intake of palatable food and solutions more than water or laboratory chows (Simiand, Keane, Keane, & Soubrie, 1998). These results suggest that the hedonic impact of reward is more sensitive to cannabinoid manipulations in the brain. In experiments designed to specifically target the hedonic impact of reward, systemic microinjections of THC and/or anandamide in rats increase affective orofacial 'liking' reactions to sucrose solutions or the number of voluntary licking bouts at a sucrose spout in rats (Higgs, et al., 2003; Jarrett, et al., 2005; Mahler, et al., 2007), suggesting that cannabinoids can sufficiently enhance the palatability of food rewards. Conversely, food-related manipulations, such as deprivation and satiety, produce changes in dialysis levels of endogenous anandamide and 2-AG in NAc and other brain areas (Kirkham, Williams, Fezza, & Di

Marzo, 2002), suggesting that endocannabinoid system responds to changes of homeostatic states and is involved in appetitive phase of the food reward processing. Taken together, these results indicate that the endocannabinoid system is able to monitor the homeostatic hunger/satiety state and accordingly modulate feeding behavior at least partly through altering the palatability of food.

The neuronal circuitry of cannabinoid hedonic effects is so far unclear. Although several studies suggest that cannabinoid manipulations in neural substrates in the ventral limbic reward circuit affect intake of palatable food or solutions, only one study so far has particularly examined the brain substrate where cannabinoid manipulations sufficiently alter the hedonic impact of reward. In this study, Mahler et al. (2007) reported a hedonic hotspot in NAc shell where anandamide can increase the hedonic impact of sweetness pleasure. In this NAc anandamide hedonic hotspot, local microinjections of anandamide in dorsal medial shell of the NAc produced >150% increases in hedonic 'liking' reactions to sucrose along with an increase of food 'wanting' measured by food intake. However, it is unclear if there are other endocannabinoid hotspots in the brain. If other anandamide hedonic hotspots exist, anandamide microinjections in those sites would similarly increase 'liking' reactions to sucrose taste.

Ventral pallidum (VP) is an especially likely candidate of cannabinoid-mediated hedonic impact of reward due to its unique role in processing reward 'liking' and its tight anatomical and functional connections with neurons in NAcSh. Anatomically, NAcSh sends compressed output to VP, and VP sends reciprocal output back to NAcSh, suggesting that functional interactions may exist between NAcSh and VP (Churchill & Kalivas, 1994; Heimer & Wilson, 1975; Mogenson & Yang, 1991; Zahm, 2000). Indeed, NAcSh and VP interact and function together in one circuit to mediate opioid-facilitated hedonic 'liking' of food reward. Both posterior VP and dorsal medial NAcSh contain a cubic- millimeter opioid 'liking' hotspot where microinjections of μ-opioid agonist (DAMGO) amplify rats' orofacial 'liking' reactions to sucrose along with an increase of food appetite (Pecina & Berridge, 2005; Pecina, et al., 2006; K. S. Smith & Berridge, 2005). The activation of

hedonic hotspot either in posterior VP or dorsal medial NAcSh recruits the other hedonic hotspot, and the interaction and normal functioning of both hotspots are required for opioid-mediated hedonic 'liking' (K. S. Smith & Berridge, 2007). The tight anatomical and functional connection between NAcSh and VP suggests that if endocannabinoid neurotransmission in dorsal medial NAcSh mediates hedonic impact of reward, it is likely that posterior VP also plays a sufficient role in amplification of hedonic 'liking' of sweet sucrose taste. The physiological evidence that VP contains significant level of endogenous endocannabinoid receptors in rats and humans further supports the hypothesis of an endocannabinoid 'liking' hotspot in posterior VP.

Goals of the study

      In this study, we investigated if anandamide/ endocannabinoid neurotransmission in posterior VP mediates the hedonic impact of a natural sweet taste. To quantify anandamide-induced changes of palatability of sucrose, we used the taste reactivity paradigm (a measurement based on homologous orofacial expressions of affective 'liking' and 'disliking' reactions elicited by tastes in human infants, apes, monkeys, and rats) to quantify changes in hedonic 'liking' reactions to sucrose. To quantify the intensity and area of drug-induced neuronal activation, we used Fos plume technique (a measurement based on drug- induced Fos activation in tissues surrounding microinjection tips) to label and measure Fos protein expression caused by anandamide microinjections. To precisely locate the most intense anandamide-induced 'liking' effect in the ventral pallidum, we combine results from the taste reactivity test and the Fos plume measurement, and present the combined results with color symbols. The size of the symbols represents the averaged intensity and area of anandamide-induced Fos activation in the VP, while the colors of the symbols represent the increases or decreases of 'liking' when microinjecting anandamide at the exact same sites. Our results indicated that anandamide microinjections in posterior VP significantly amplified hedonic 'liking' reactions to sucrose. Also, anandamide signal in surrounding areas had no effect on hedonic 'liking' to sucrose.

Materials and Methods

General design

Split-and-recombine design (fig.1) was used to construct maps of 'liking' responding to sucrose or maps of 'disliking' response to quinine. Rats are split into two separate groups, the behavioral taste reactivity group (n=13) and the Fos plume measurement group (n=6). Rats in the behavioral taste reactivity group (group 1) were implanted with both cranial cannulae and oral cannulae, tested with taste reactivity test to quantify the intensity of 'liking' and 'disliking' responses to sucrose after drug microinjections, and euthanized for microinjection center identification. Rats in the Fos group (group 2) were implanted with cranial cannulae, handled and microinjected with drugs, and euthanized for Fos activation quantification. Fos protein activation around the microinjection center was compared to that in the non-surgical tissues in order to estimate the area and intensity of neuronal activation caused by anandamide microinjections.

Results from both groups are combined to construct maps of 'liking' responses to sucrose by color coding the behavioral effects (group 1 result) onto the Fos plume symbols (group 2 result) placed onto the microinjection centers identified by histology (group 1 result). The reason to split was to ensure that Fos activation and behavior effects were measured under the same conditions in which drugs had effects and to avoid the diminishment of drug efficacy on Fos activation after repeated microinjections. The reason to recombine was to map the 'liking' or 'disliking' behavioral effects onto the precise areas around the microinjection centers where anandamide microinjections were likely to have effects based on Fos plume estimations.

Experiment 1 assesses anandamide effect on hedonic 'liking' reactions to sucrose in the VP. Experiment 2 identifies the specific area in the VP that anandamide has strongest effects on.

Surgery

Under anesthesia (80mg/kg ketamine HCL, 10 mg/kg xylazine, and 0.2 ml atropine sulfate), male Sprague-Dawley rats (n=13, 250-400g) were bilaterally

implanted with guide cannulae (23 gauge) positioned 2 mm above the ventral pallidum (VP), or surrounding structures, for microinjection of drugs. Coordinates for implantation were: anteroposterior (AP), 0 to -0.8 mm; mediolateral (ML), ± 3 mm; dorsoventral (DV), -5.5 mm. The cannulae were anchored to the skull with stainless steel screws and dental cement. Obturators were inserted in guide cannulae to prevent clogging.

In the same surgery, all rats were bilaterally implanted with intraoral cannulae (PE-100 tubing) for delivery of the taste solutions. Oral cannulae enter the mouth lateral to the first maxillary molar, travel beneath the zygomatic arch, and exit the dorsal head near the skull screws. Oral cannulae did not disrupt normal eating. All rats were given at least 7 days to recover prior to the first test day.

Drugs and microinjections

Anandamide was dissolved in tocrisolve and diluted to dose (25ng) with ACSF. Similarly, tocrisolve was diluted with ACSF as vehicle control. Within-subject comparison design was applied. To ensure that order effects did not distort our data, microinjection order was counterbalanced between anandamide and vehicle/tocrisolve. Tests were spaced at least 48 h apart. On a particular test day, each rat received bilateral microinjections of 0.2 µl per side of either anandamide or vehicle. Microinjections were made through a stainless-steel injector cannula (29 gauge) connected to PE-20 tubing and a syringe pump at a rate of 0.2 µl /min. Microinjector tips were held in place for an additional 1 minute after infusion to allow drug to diffuse away from the cannula tip and to avoid backflow. After microinjections, obturators were inserted back to the cranial cannulae to prevent clotting or infection, and rats were placed into the transparent taste reactivity chamber with intraoral fluid delivery tubing attached (described in behavioral taste reactivity testing).

Behavioral taste reactivity testing

Rats were handled and habituated in the taste reactivity chamber for 4 consecutive days for 30 minutes each before behavioral testing, and received a mock injection of vehicle on the final habituation day. On each test day, hollow fluid delivery tubing (PE-50 connected to a PE-10 delivery nozzle) was attached to the

intraoral cannula to deliver solutions. The other end of the tubing was attached to a syringe pump, which infuses solutions at a stable rate of 1ml/min. All rats received sucrose solution (1%, 1 minute duration) 30 minutes after anandamide/vehicle microinjections. Oral facial 'liking' and 'disliking' responses were video-recorded via an angled mirror placed underneath the transparent floor during the one- minute test.

Taste reactivity video scoring

Hedonic, aversive, and neutral response patterns were scored offline using frame-by-frame slow-motion videotape analysis developed to assess hedonic *vs* aversive taste valuations (Berridge, 2000). Hedonic responses included rhythmic tongue protrusions, lateral tongue protrusions, and paw licks. Aversive responses included gapes, head shakes, face washes, forelimb flails, and chin rubs. Neutral responses included passive dripping of solution out of the mouth, ordinary grooming, and mouth movements. Neutral responses, which are less consistently linked to hedonic/aversive taste valuation, included grooming, passive dripping of solution out of the mouth, rhythmic mouth movement, and doing nothing. All video analyses were conducted blind to the microinjection contents and cannula placements using Observer software (Noldus, Netherlands).

A time-bin scoring procedure was used to ensure that taste reactivity components of different relative frequency still contributed equally to the final affective hedonic/aversive totals (Berridge, 2000). For example, rhythmic mouth movements, passive dripping of solution, paw licking, and grooming typically occur in long bouts and were thus scored in 5 s time bins (5 s equals one bout occurrence). Tongue protrusions, which occur in shorter bouts, were scored in 2 s time bins (2 s equals one bout occurrence). The other behavioral components (lateral tongue protrusions, gapes, forelimb flails, head shakes, and chin rubs) typically occur in discrete events and were thus scored as single occurrences each time they occurred (e.g., one gape equals one occurrence). Finally, Individual totals were calculated for hedonic versus aversive categories by adding all response scores within an affective category for that rat. A hedonic 'liking' reaction total was the sum of occurrences for lateral tongue protrusions, rhythmic tongue protrusions, and paw licks. Similarly, an

aversive 'disliking' reaction total was the sum occurrences of gapes, headshakes, face washes, forelimb flails, and chin rubs.

Histology

After completion of behavioral experiments, rats received an overdose of sodium pentobarbital (0.2g/kg). Rats' brains were extracted and post-fixed with 10% formalin in 0.1M phosphate buffer (PB) for a few days and then transferred to 30% sucrose in 0.1M PB. Brains were sectioned with a freezing microtome into 60 µm, stained with cresyl violet, and mapped for the centers of the microinjections according to Paxinos and Watson atlas (2008).

General Procedures for Fos plume measurement

Fos activation was assessed by c-Fos immunohistochemistry and visualized under the microscope. To quantify the area and the intensity of anandamide-induced neuronal activation, Fos plumes caused by anandamide microinjections were compared to Fos plumes caused by vehicle microinjections and non-surgical tissues (Figure 4-1, top). Anandamide-induced Fos plumes were thus constructed and further divided into different intensity zones. The radii for each intensity zone were calculated and averaged. Assuming a spherical shape of functional drug diffusion, the averaged radii were applied to calculate the volumes of different Fos intensity zones and to construct the averaged Fos plume symbol of different intensities on the 2-D plane (Figure 4-1, bottom)

Fos-like protein immunohistochemistry

Rats in the Fos plume group (n=6) were handled gently for 3 days for 10 min each, similar to the behavioral groups, and then microinjected bilaterally in the VP with either or vehicle (tocrisolve). A separate group of non-surgical rats went through the same procedures but were held by the experimenter for 5 minutes instead of having microinjections. Ninety minutes later, rats were overdosed with sodium pentobarbital before transcardial perfusion. After perfusion, brains were removed and placed in 4% paraformaldehyde for 2 hours, transferred to 30% sucrose overnight, sectioned at 40 µm, and stored in 0.1M NaPB, pH 7.4. Alternate series of sectioned slices were processed for immunofluorescence.

For immunofluorescence processing, we used the avidin-biotin procedure (Hsu et al, 1981). In brief, brain sections were rinsed (0.1MNaPB+ 0.2% Triton), pre-blocked (5% Norrmal Donkey Serum (NDS) in 0.1M NaPB and 0.2% Triton), and incubated in primary antibody (goat anti-Fos 1:500) and 5%NDS overnight at 4ºC. After primary incubation, the slices were once again rinsed, pre-blocked, and incubated in secondary antibody (4% donkey-anti-goat Alexa 488) and 5% NDS for 2 hours followed by final rinses. The slices were mounted in 0.01M NaPB, let dried, and cover slipped with anti-fade.

Fos plume maps of anandamide-induced neuronal activation spread

The procedure for measuring drug-induced Fos plumes immediately surrounding a local microinjection site was adopted from previous studies from our lab (Smith & Berridge, 2005; Mahler et al., 2007).

To visualize and quantify drug-induced Fos activities, Fos-labeled cells on tissue surface were visualized with 5x–40x magnification under the microscope followed by image processing. Fos count and Fos plume construction were done post-hoc by an observer that was blind to the drug conditions. On the Fos images, sampling blocks (50 x 50 μm) were placed along seven radial arms emanating from the center of the microinjection site (45, 90, 135, 180, 225, 270, 315°) at 50 μm intervals (Figure 4-1, top). The 360° or 0° arm was not sampled because it was occupied by the microinjection cannulae track. Fos- labeled cells in each individual sampling box was counted individually (Figure 4-1, boxes). In order to establish baseline of Fos activation, two control conditions were measured: (1) in normal VP tissue of intact brains without damage from surgical intrusion or gliosis (2) around the site of vehicle microinjections in the VP to assess Fos activation caused by microinjector tips and by the pressure from vehicle microinjections. Anandamide-induced Fos activation was also measured and compared to normal and vehicle-induced Fos at the same site.

Anandamide microinjections significantly increased Fos activation compared to normal tissues. Anandamide Fos plumes were mapped as zones of 'intense' or 'moderate' Fos elevation identified by absolute Fos increases 3X or 2X over normal. In this study, anandamide-induced Fos activation was not significantly stronger

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compared to vehicle-induced Fos activation. To determine the averaged radii of intense or moderate Fos activation zones around the microinjection center, the distances from the microinjection center to the outer most sampling box of 3X or 2X Fos elevation were measured and averaged across all seven radial arms in all animals. The averaged radii were applied to determine the size of the hexagon map symbol, which represents the averaged sizes of 3X and 2X Fos activation zones from the microinjection center (Figure 4-1, bottom).

Functional mapping for localization of behavioral effects

The averaged Fos plume and the behavioral data were recombined to map the localization of function (e.g. 'liking'). Microinjection sites from rats in the behavioral group were identified and plotted onto the atlas to represent the center of microinjections. The plume-derived hexagon symbols were superimposed onto the microinjection centers to represent estimated zones of intense or moderate activation caused by anandamide. The magnitudes of the behavioral effects of each microinjection were color coded onto the hexagons to represent the functional zones in which the behavioral effects were induced by drug. Therefore, each hexagon symbol on the map illustrates the location of the microinjection in a particular rat tested for behavior, the sizes of zones of intense or moderate neuronal activation caused by the microinjection (based on averaged Fos-plume radii), and the magnitude of behavioral effect (e.g. 'liking' responses) when drug is injected at this specific site (based on taste reactivity test). The bilateral cannulae for each rat were collapsed into one unilateral map and plotted separately (two sites per rat).

Statistical analyses

All behavioral analyses were two-tailed and α was always set at $p<0.05$. Paired samples t-tests were used to test anandamide effects on different behaviors. Between-subjects ANOVAs were used to determine anatomical location effects of microinjection sites (e.g. hotspot vs. non-hotspot).

Results

Fos plume mapping: identifying zones of local neuronal activation

Fos plume maps help to identify the spheres and the intensity of neuronal activation caused by microinjection of drugs. In traditional microinjection studies, only the microinjection centers are identified and plotted to represent the location where drug could cause behavioral effects. However, it is necessary to know how far the drug spreads to stimulate tissue extending away from the microinjection site in order to map the substrates responsible for drug-induced effects on behavior.

Fos plume mapping, a methodology that applies IEG activation, has been shown to be a useful tool to identify specific areas responsible for the drug-induced behavioral effects, as described in previous studies. In this study, anandamide microinjections may have induced Fos expression either directly through acting on the same neurons that contain anandamide receptors and express Fos or indirectly through modulating nearby neurons, which contain anandamide receptors and in turn activate adjacent neurons that express Fos. In either case, a Fos plume reflects spheres of local functional modulation induced by the drug and provides quantitative information on sphere intensity and size. Most importantly, the boundaries of the plume reveal the functional zones likely to be responsible for anandamide-induced behavioral effects, even if drug molecules spread further beyond the plume and are insufficient to trigger gene transcription or have functional effects.

Anandamide microinjections produced roughly spherical and concentric zones of Fos enhancement. Different zones of activation are defined based on the intensity of Fos activation (Figure 4-1). The inner intense zone was defined as the zone in which Fos expression was increased by 3X above control 'normal' ventral pallidum tissue from uninjected rats. The outer moderate zone, which was further away from the drug microinjection center, was defined as the zone in which Fos expression was increased by 2X above control 'normal' ventral pallidum tissue from uninjected rats. The averaged radius of each zone, gathered from 6 to 10 Fos plumes, was applied to calculate the volume of drug-induced functional spheres and

to determine the size of hexagon symbol we used to represent functional drug effect on the 2-D plane (Figure 4-1, bottom).

The size of the Fos plume depends on the dosage and the speed of microinjection. Microinjection of 25 ng/0.2μl anandamide over one minute followed by maintaining the microinjectors in place for another minute produced an averaged Fos plume with a mean radius of 0.063 ± 0.021 mm for the inner intense zone and a mean radius of 0.175 ± 0.035 mm for the outer moderate zone compared to normal tissues. We chose to use normal non-surgical tissues as control because vehicle Fos expression might cast a ceiling effect on anandamide-induced Fos effect. In order to avoid the underestimation of drug functional effect, we chose to compare Anandamide Fos expression with normal tissues.

Fos plume maps were later generated by combining results from the Fos group (averaged Fos plume) and results from the behavioral group (changes of 'liking' or 'disliking' caused by drug microinjections at a specific site).

Anandamide in VP enhances sucrose 'liking'

Anandamide overall enhanced 'liking' to sucrose (t_{10}=-3.06, p<0.05). Among eleven VP animals, anandamide microinjections in the posterior half (but not anterior half) of the VP on average caused 26% (31.09 ± 1.43 hedonic reactions) increases of hedonic 'liking' reactions (sum of rhythmic tongue protrusion, lateral tongue protrusion, and paw lick scores) elicited by sucrose sweet taste compared to control levels (22.36 ± 2.35 hedonic reaction) after vehicle/tocrisolve microinjections (Figure 4-2). Sucrose infusion elicited mainly hedonic 'liking' reactions and reached the average of 31.09 ± 1.43 hedonic reactions over one minute in the control condition. At the same time, anandamide suppressed aversive disliking reactions (72%, $t_{10} = -3.06$, $p = 0.012$) to sucrose. Anandamide selectively enhanced the number of hedonic 'liking' reactions elicited by sucrose without affecting the number of aversive 'disliking' reactions elicited by sucrose, suggesting that the effect of hedonic enhancement caused by anandamide was not due to general arousal to sucrose infusion.

Anandamide hedonic 'liking' hotspot in posterior VP

Averaged hexagon-shaped Fos symbols with an inner zone (radius:0.06 ± 0.02 mm; 3X Fos increase over the normal condition) and an outer zone (radius: 0.18 ± 0.04 mm; 2X Fos increase over the normal condition) were placed onto each microinjection site identified from rats in the behavioral group to represent the intense and moderate functional drug activation spread from the center. The Fos symbols were later color-coded based on the magnitude of behavioral effects, defined by drug-induced changes of 'liking' or 'disliking' reactions over vehicle, produced by anandamide microinjections at the corresponding site. Different colored clusters, which represent the overall behavioral effect from microinjection sites located in this specific subregion of the VP, would emerge and help us identify the most critical site for anandamide 'liking' enhancement. From the anandamide 'liking' Fos plume map, it appeared that anandamide increased hedonic 'liking' reactions to sucrose in posterior 1/2 of the VP. Although further study is needed to identify the effect of anandamide on hedonic 'liking' reactions to sucrose in anterior ventral pallidum, the decreased 'liking' gradient spreading from posterior VP to anterior VP suggests that the most intense 'liking' enhancement caused by anandamide was in posterior VP.

The most critical 'liking' site where anandamide could caused >60% increases of hedonic reactions was found to be concentrated in posterior VP (roughly AP -0.5~ -0.9mm, DV -7.3~ -8 mm, ML ±2.2~ 3mm), which was posterior to interventricular foramen and between the rostral tip of the lateral hypothalamus and the caudal tip of the olfactory tubercle. The center of the anandamide 'liking' hot spot was located at 0.9 mm posterior to bregma, 2.5 mm medial lateral to bregma, and 7.8 mm ventral to the skull. The area of intense anandamide 'liking' enhancement extended from this center all the way forward to middle VP (AP +0.1 mm), where VP is ventral to anterior commissure, lateral to substantia innominata and lateral preoptic area, and medial to the interstitial nucleus of the posterior limb of the anterior commissure. Anandamide in the hedonic 'liking' hotspot produced a stronger enhancement of hedonic 'liking' reactions to sucrose than in sites outside

of the hedonic hotspot ($F_{1, 11}$=51.20, p<0.05). On average, anandamide caused 29% hedonic enhancement to sucrose compared to vehicle in the hedonic 'liking' hotspot and only 15% hedonic enhancement compared to vehicle in the areas around the anandamide 'liking' hotspot (Figure 4-3).

Other control sites outside of the posterior VP

At other anatomical control sites in brain structures outside of the VP, anandamide failed to increase hedonic 'liking' reactions to sucrose. Anandamide microinjections in caudate putamen (n=4), which is dorsal and lateral to VP, did not increase or decrease hedonic reactions to sucrose. Similarly, anandamide microinjections in sublenticular extended amygdala and lateral hypothalamus (n=2), which are dorsal and medial to VP, failed to alter hedonic reactions to sucrose as well. While future studies on anandamide's ability to enhance hedonic 'liking' reactions in the most posterior end of VP, where it connects with sublenticular extended amygdala, and ventral part of globus pallidus, where it connects with dorsal VP, are needed to precisely map the posterior and dorsal boundaries of the anandamide hedonic hotspot in VP, the overall pattern of anandamide 'liking' Fos plume map from the current study indicates that anandamide acted primarily in posterior VP to cause amplification of positive hedonic 'liking' reactions to a sweet sucrose taste.

Discussion

Anandamide enhanced 'liking' reactions to sucrose in posterior VP

Our results demonstrated that anandamide enhanced hedonic 'liking' reactions to sucrose in the posterior VP. In particular, the anandamide hedonic hotspot, where anandamide has the most intense enhancement of 'liking' reactions to sucrose, is concentrated in the posterior portion of the VP that is anterior and lateral to the lateral hypothalamus and caudal to the olfactory tubercle.

Anandamide suppressed aversive 'disliking' reactions to sucrose, even when microinjections of anandamide at the same site enhanced 'liking' reactions to a sweet sucrose taste. This selective enhancement pattern suggests that anandamide only enhances the hedonic 'liking' value, rather than enhancing general arousal.

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However, the low baseline value of aversive reactions to sucrose may cause overestimation of the actual behavioral effect. Thus, it is of particular importance for future studies to examine anandamide-induced aversive 'disliking' reactions to quinine, which provides more precise evaluation of the functional specificity of anandamide. This result is consistent with previous findings, which demonstrate how systemic THC (CB1 agonist) and NAc shell anandamide microinjections selectively enhanced 'liking' reactions to sucrose(Higgs, et al., 2003; Jarrett, et al., 2005). It is also consistent with reports that anandamide-induced enhancements of food intake interact strongly with palatability. Some previous studies even further suggest that cannabinoid manipulations only alter intake of palatable rewards that contain sweet or fat, rather than regular food chow pellets or water that does not contain sweet or fat, by showing that systemic THC microinjections, local anandamide microinjections in NAcSh, or local 2-AG microinjections in parabrachial nucleus selectively enhanced intake of palatable foods/solutions without affecting intake of normal rat chows/ water/quinine (DiPatrizio & Simansky, 2008; Koch & Matthews, 2001; Simiand, et al., 1998). Further studies are required to determine whether anandamide-induced selective enhancement of hedonic 'liking' in posterior VP also drives 'wanting' for regular foods, or/and highly palatable (high sweet high fat) food rewards.

Anandamide hedonic hotspot is concentrated in the caudal VP

With Fos plume technique, we were able to quantify the functional effect, or the extent of neuronal activation, of anandamide spread, and more precisely locate the most critical site where anandamide caused increased 'liking' to sucrose. In this study, we operationally defined a hedonic hotspot where anandamide produced >60% increases in 'liking' reactions to sucrose. Anandamide microinjections in a hedonic hotspot in the posterior VP were significantly more effective than sites that surround this area for enhancing 'liking' reactions to sucrose. For example, within the hedonic hotspot, anandamide caused an average of 29% hedonic enhancement, while in areas surrounding the hedonic hotspot, anandamide caused 0- 15% hedonic enhancement. The crucial anatomical substrate for anandamide-induced 'liking' (center at AP -0.9 mm, ML ±2.5mm, DL -7.8 mm) was located ventral to

anterior commissure and globus pallidus, caudal to olfactory tubercle and interventricular foramen, and anterior to the rostral tip of the sublenticular extended amygdala. The results suggest that VP is heterogeneously involved in processing hedonic 'disliking' of rewards depending on where anandamide activation occurs. Also, the results further point out the importance that different subareas within the same structure, in this case VP, can be differentially involved in the same functional processes.

Anandamide hedonic hotspot highly overlaps with opioid hedonic hotspot

The anandamide hedonic hotspot in posterior VP highly overlaps with an opioid hedonic hotspot previously mapped in the posterior VP by Smith and Berridge in our lab (where the µ opioid agonist DAMGO amplified 'liking' reactions) (K. S. Smith & Berridge, 2005). This anatomical overlap implies a potential interaction between opioid and anandamide- they may together regulate reward processing and promote motivation for reward.

Previous research has found evidence that suggests cannabinoid and opioid may interact to mediate food and drug reward processes. For example, in the case of food reward, the inactivation of opioid pathway by opioid antagonist naloxone attenuated or blocked cannabinoid-induced feeding and feeding-reinforced behavior (Solinas & Goldberg, 2005; Williams & Kirkham, 2002b), while inactivation of endocannabinoid pathway by microinjections of CB1 antagonist SR 141716 in NAc shell blocked NAc shell µ- opioid elicited hyperphagia (Skelly, Guy, Howlett, & Pratt, 2010). In the case of drug reward, opioid antagonist naloxone attenuated or blocked cannabinoid agonist self-administration (Navarro, et al., 2001). In reverse, cannabinoid antagonists blocked heroin self-administration in rats, morphine-induced conditioned place preference, and morphine self-administration in mice (Alvarez-Jaimes, Polis, & Parsons, 2008; Azizi, Haghparast, & Hassanpour-Ezatti, 2009; Ellgren, et al., 2008; Navarro, et al., 2001). A recent finding further suggests that opioid antagonist/cannabinoid antagonist can modulate cannabinoid/opioid-induced c-Fos expression in similar reward-related brain areas, such as VTA, NAc, thalamic paraventricular nucleus, etc (Allen, McGregor, Hunt, Singh, & Mallet, 2003;

Singh, Verty, Price, McGregor, & Mallet, 2004) Finally, one study examined the functional interaction between opioid system and cannabinoid system and showed that the combination of cannabinoid antagonist SR 141716 and opioid antagonist naloxone generated a supra-additive anorectic effect (Kirkham & Williams, 2001; Solinas & Goldberg, 2005) .Together, these results indicate that the simultaneous activation of both opioid and cannabinoid systems is required for the normal functioning of food and drug reward processes. It would be of particular importance for future studies to identify the interaction between cannabinoid system and opioid system in hedonic 'liking' aspect of the reward, and whether or not anandamide and opioid hedonic hotspots in the medial NAc shell or the posterior VP are both required for enhancement of reward 'liking'.

Anandamide hedonic hotspot also overlaps with the orexin hedonic hotspot

The anandamide hedonic hotspot identified in this project highly overlaps with the orexin hedonic hotspot identified in Project 2 (Chapter 3), suggesting that the two neurotransmitter systems may interact to mediate reward processes. Recent studies have indeed highlighted the existence of a functional interaction between the endocannabinoid system and the orexin system. One *in vitro* study by Hilairet et al. (Hilairet, Bouaboula, Carriere, Le Fur, & Casellas, 2003) first demonstrated that when CB1 and Orexin 1 receptors are co-expressed, a major CB-A dependent enhancement of the orexin A potency was found. Verty et al. (2009) further confirmed that the interaction between endocannabinoid system and orexin system *in vivo* by showing that CB 1 antagonist rimonabant (SR 141716) activated neurons in hypothalamic regions, as identified by Fos activation, colocalized with orexin peptides that are immunohistochemically labeled (Verty, Boon, Mallet, McGregor, & Oldfield, 2009). Functionally, pretreated rats with subeffective doses of Rimonabant (CB1 antagonist) attenuated orexin- A induced feeding (Crespo, Gomez de Heras, Rodriguez de Fonseca, & Navarro, 2008), supporting the idea that cannabinoid and orexin A systems share a common mechanism in food reward. However, it is still unclear how the endocannabinoid system and opioid system interact to mediate reward 'liking'.

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Anandamide's role in reward

Why does food taste better when we are hungry, or how does hunger modulate food palatability? The anandamide 'liking' hot spot in the dorsal medial NAc shell and in the posterior VP might play critical roles in alliesthesia, which is a phenomenon that describes physiological state -induced pleasantness of a stimulus (or hunger-induced food 'liking'). Previous studies have shown that the level of endocannabinoids in the brain can be modulated by hunger state of the body (Kirkham, et al., 2002; Solinas, et al., 2008). For example, Kirkham and colleagues reported that 24-hr fasting increased the levels of endocannabinoids, anandamide and/or 2-AG, in forebrain limbic structures, including nucleus accumbens. Also, microinjections of 2-AG in the same area reliably caused hyperphagia(Kirkham, et al., 2002). Together, the results suggest a potential role of endocannabinoids in alliesthesia: hunger induced high level of endocannabinoids, which may recruit the anandamide 'liking' hotspots in NAcSh and VP (Berridge, et al., 2010; Glass, et al., 1997; Mailleux, et al., 1992), and result in enhanced palatability of food along with over-eating. It is noteworthy that endocannabinoids also modulates mesolimbic dopamine activity in VTA (Cheer, Kendall, Mason, & Marsden, 2003; Melis, et al., 2004; Riegel & Lupica, 2004; Szabo, Siemes, & Wallmichrath, 2002), which may enhance motivation, or 'wanting' of foods without affecting 'liking' (Zangen, Solinas, Ikemoto, Goldberg, & Wise, 2006). Also, hunger state raises the level of endocannabinoid (2-AG) in hypothalamus, which has long been shown to be involved in the regulation of homeostasis and feeding (Kirkham, et al., 2002). Although the functional role of endocannabinoids in hypothalamus is still unclear, it is possible that endocannabinoid system can bypass limbic system and directly regulate eating via hypothalamus under certain conditions.

In clinical cases, the dysregulation of the endocannabinoid system has been linked to eating disorders and obesity. For example, in women with anorexia nervosa or in subjects with binge-eating disorders, their plasma levels of anandamide are significantly higher than normal subjects (Monteleone, et al., 2005).Also, the polymorphism of the CB1 gene has been shown to correlate with the susceptibility to different types of anorexia nervosa (Siegfried, et al., 2004). And the

mutation of gene encoding the primary enzyme that inactivates endocannabinoids has been reported to be associated with overweight and obesity (Sipe, Waalen, Gerber, & Beutler, 2005). Additional experiments will be required to identify whether the functioning of NAcSh and VP is affected and whether 'liking' of food reward is changed under these clinical conditions.
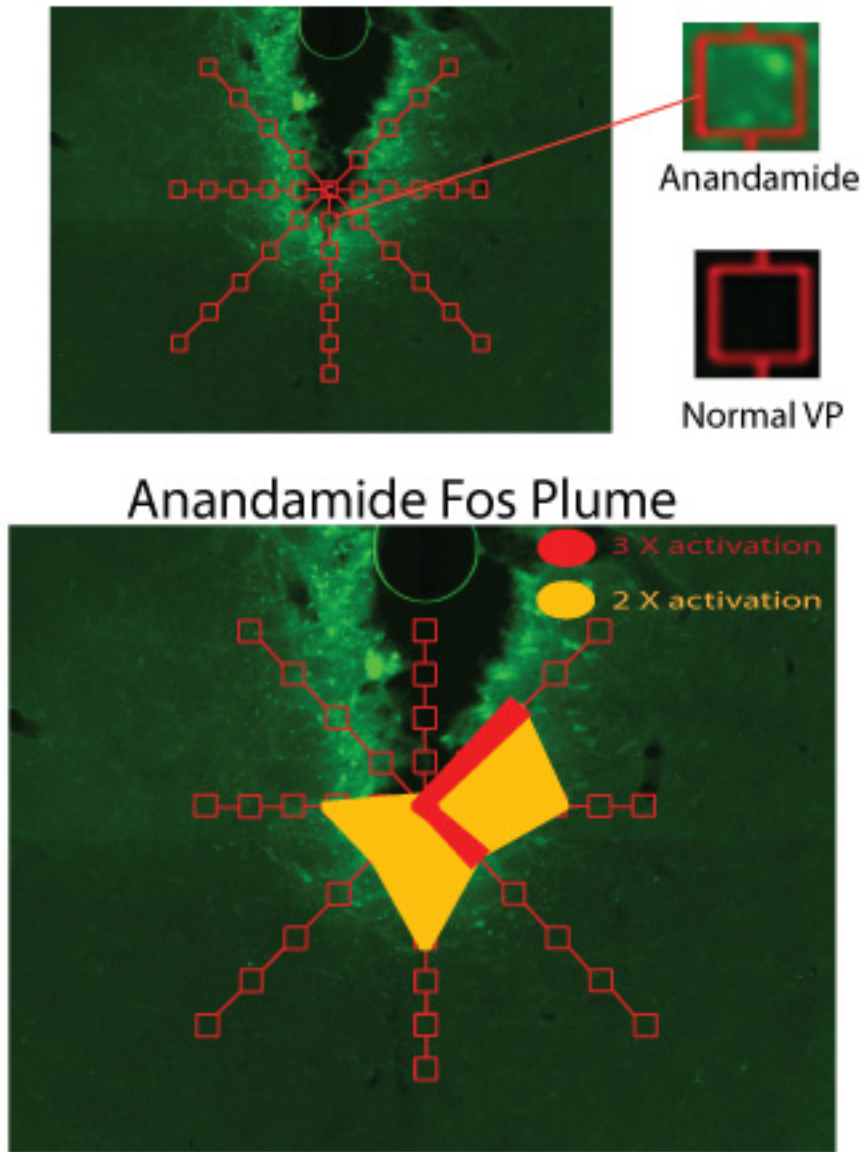
Figure 4-1 Anandamide Fos plume in VP. *Fos Plume Sampling Method (top)*: The method used to sample Fos expression around injection site is illustrated. Fos-expressing neurons are counted in 50 x 50 μm blocks on 8 sampling arms (0°, 45°, 90°, 135°, 180°, 225°, 270°, 315°) extending from the center of microinjection center at 50 μm intervals. Insets show sample tissue blocks from equivalent sites that were injected with vehicle (tocrisolve) or with anandamide (25ng). Fos expression appears as green oval dot under the microscope at 10X magnification. The color scale demonstrated the colors used for different extents of Fos elevation (over vehicle control levels). Colored areas indicated the neural elevation of 200% (yellow) and 400% (orange) orexin Fos expression compared to healthy normal tissues.
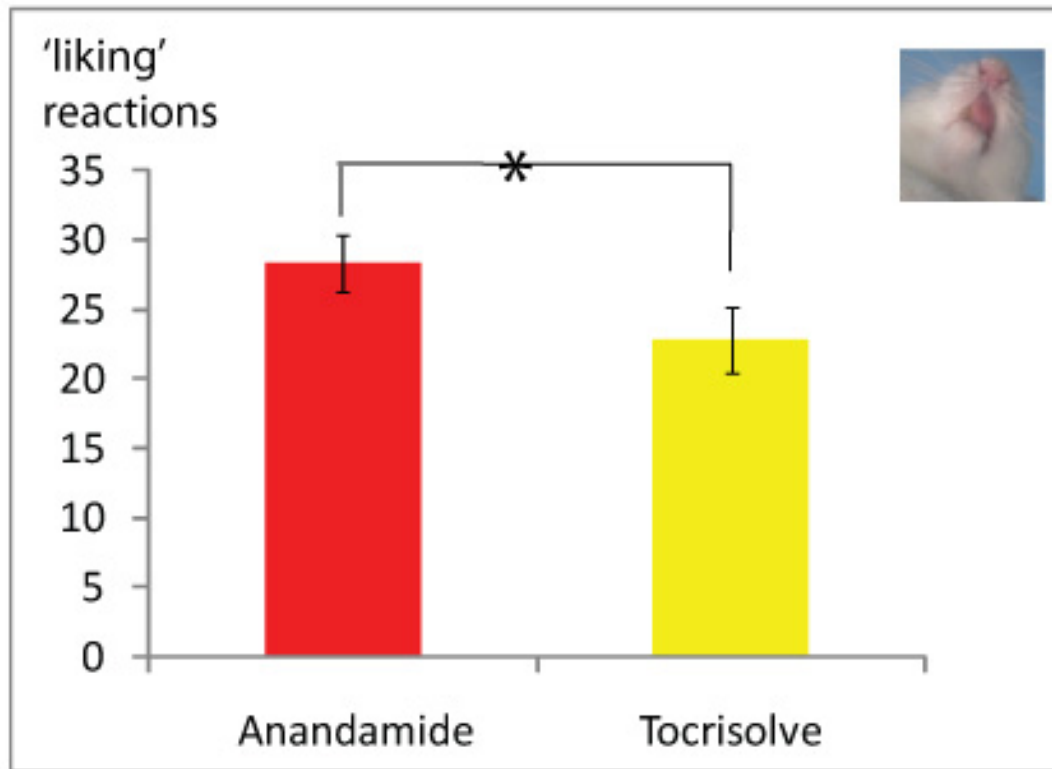
Figure 4-2 Anandamide in VP increases 'liking' to sucrose. Averaged total hedonic 'liking' reactions to sucrose are shown after anandamide microinjections (red) and vehicle microinjections (yellow) in the 1-min taste reactivity test (n=6). * indicates significant increases of hedonic reactions ($p<0.05$) compared to vehicle.
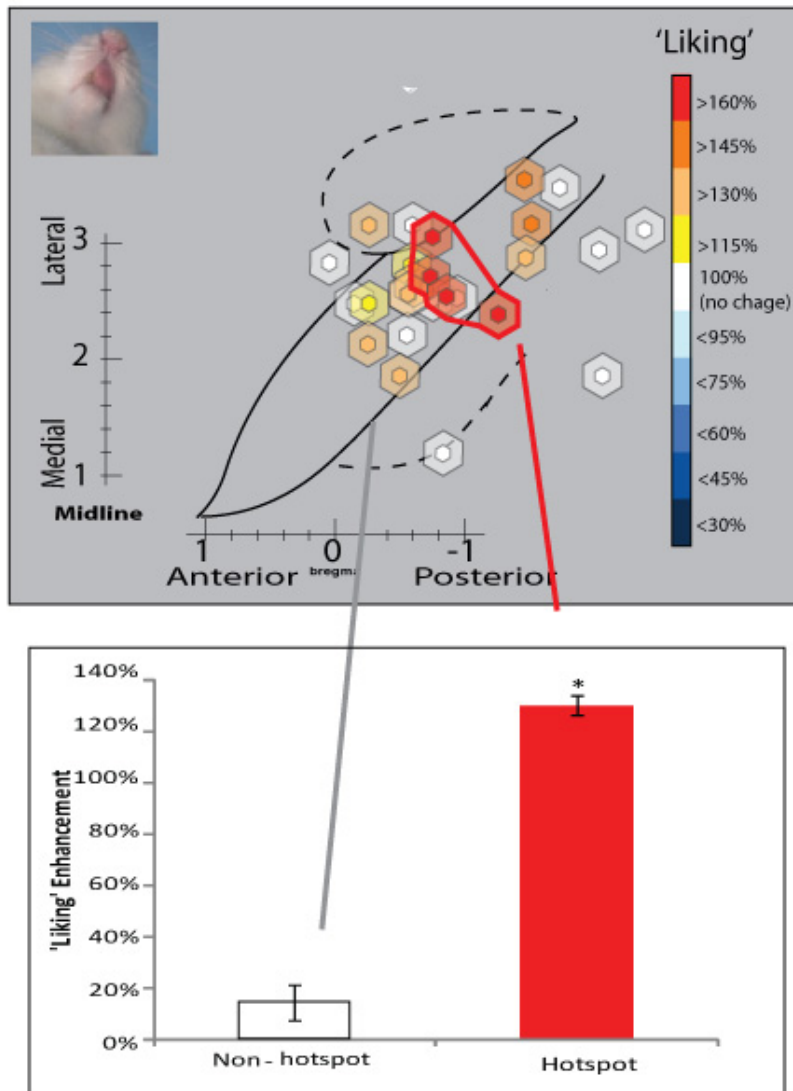
Figure 4-3 Anandamide in VP hedonic sites increase 'liking' to sucrose. 'Liking' enhancements following anandamide microinjections are mapped rat-by-rat onto a horizontal view of VP, showing both the within-subject changes in hedonic 'liking' reactions relative to vehicle (color) and the functional spreads of the microinjections based on Fos plume data (size). For anandamide hexagon, and inner symbols represent 400% Fos activation, outer symbols represent 200% Fos activation. Bar graphs (bottom left and bottom right) show the averaged hedonic enhancement over vehicle in the corresponding subareas (thin color lines) in the 1-min taste reactivity test. Error bars represent SEM. * indicates significant increases in hedonic reactions (p< 0.05) compared to vehicle. Microinjections of anandamide in the caudal VP increase 'liking' reactions to sucrose compared to vehicle.
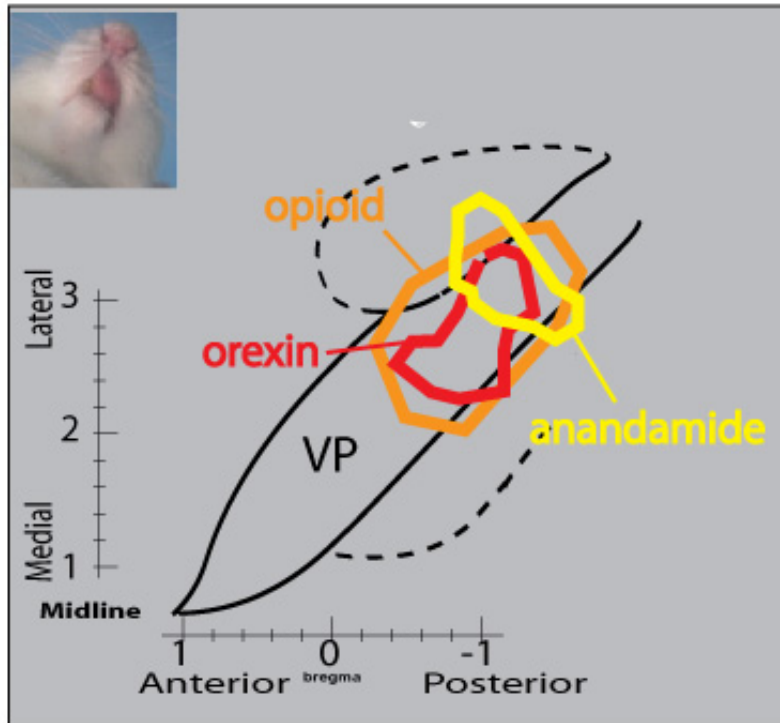
Figure 4-4 Comparison of anandamide, orexin, and opioid hedonic hotspots.
Anandamide (yellow line), orexin (red line), and μ-opioid (orange line) increase 'liking' to sucrose in the caudal VP. The anandamide hedonic hotspot highly overlaps with the opioid hedonic hotspot and the orexin hotspot identified previously in our lab (Smith et al.2005).

Chapter 5

Conclusion

Summary of Findings

The projects described in this dissertation were designed to study causational mechanisms for hedonic 'liking' and aversive 'disliking' processes, focusing specifically on the ventral pallidum (VP) and comparing it with the nucleus accumbens (NAc). Although both VP and NAc have been shown to contain an opioid hedonic 'liking' hotspot, where μ- opioid agonist DAMGO *sufficiently* enhances 'liking', it was unclear whether both hotspots are *necessary* for normal functioning of 'liking'. It was also not previously known if the DAMGO hotspot was anatomically isomorphic with other neurochemical hotspots in VP, as demonstrated here for anandamide and for orexin. Finally, it was not known if the hotspots for positive 'liking' of sensory pleasure were involved in aversive 'disliking' processes as well, especially after damage or dysfunction of the localized hotspots.

In Project 1 (Chapter 2), I identified a posterior VP site, nearly isomorphic with the previously identified μ-opioid hedonic hotspot, as the only neural substrate *necessary* for hedonic 'liking'. By contrast, the NAc shell hotspot is not *necessary* for positive hedonic enhancements, in which loss of function does not disrupt the processing of normal 'liking'. I also functionally mapped an aversive 'disliking' site in posterior VP, which highly overlaps with the μ- opioid hedonic hotspot. In Project 2

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(Chapter 3), I discovered an orexin hedonic 'liking' hotspot in the posterior VP. Orexin intensely enhanced 'liking' reactions to sucrose in this 'liking' hotspot. In Project 3 (Chapter 4), I identified yet another neurochemical hedonic signal for the 'liking' hotspot in posterior VP via endocannabinoid (anandamide) transmission. Both the orexin hedonic 'liking' hotspot and the anandamide hedonic 'liking' hotspot highly overlap with the μ-opioid hedonic hotspot previously discovered in our lab in the posterior VP (K. S. Smith & Berridge, 2005), indicating that all three neurochemical signals may act on the same anatomical hotspot.

Over all, these findings shed light on the neural mechanisms through which sensory signals are painted with hedonic gloss and become 'liked', and how the inhibition, or malfunction, of this mechanism can turn 'liking' of a sweet taste into 'disliking' of the same taste.

Results from these projects have implications relating to three broad issues in brain research: (1) the functional role VP plays in rewards (2) how different neurotransmitter systems interact to mediate 'liking', and (3) the interactions between limbic affective system and hypothalamic homeostasis regulation. These are each discussed below followed by a brief consideration of how the findings may help advance human research in disorders related to reward, such as obesity, depression, drug addictions, etc.

The Ventral Pallidum: a Critical Structure for Reward

Traditional notions of VP function: motor output and relay station

The ventral pallidum was first identified as a distinct structure by Heimer and Wilson in 1975 (Heimer & Wilson, 1975), and was suggested to position as a major output for the ventral striatum (nucleus accumbens). This notion of VP as a striatal output contributed to the functional hypotheses of VP as a motor expression site. For example, Mogenson et al. proposed that between the NAc- VP projections, limbic motivational signals are translated into motor signal. VP passes the signals onto brainstem motor nuclei (e.g. pedunculopontine nucleus) and transmits motor signals to spinal motor nuclei (Mogenson & Yang, 1991).The motor-output view of VP function is supported by the evidence that the NAc and the VP interact to

mediate locomotion stimulation (Austin & Kalivas, 1989; Jones & Mogenson, 1980; Mogenson & Yang, 1991) and by evidence that neurochemical modulation in VP alters locomotion (Austin & Kalivas, 1991; Fletcher, Korth, Sabijan, & DeSousa, 1998; Gong, Justice, & Neill, 1997; Kalivas, Klitenick, Hagler, & Austin, 1991; Kretschmer, 2000).

However, transferring input from the NAc to brainstem motor-related targets is only one feature of the VP. The VP also receives convergent inputs from orbitofrontal, prefrontal, and infralimbic cortex, the amygdala, lateral hypothalamus, ventral tegmental area, parabrachial nucleus, subthalamic nucleus, and other structures related to reward (Carnes, et al., 1990; Fuller, et al., 1987; Groenewegen & Berendse, 1990; Grove, 1988; Klitenick, et al., 1992; Maurice, et al., 1997; Napier, et al., 1991; Turner, et al., 2001; Usuda, et al., 1998a; Zaborszky, et al., 1997; Zahm, et al., 1985). Conversely, VP projects reciprocally to most of its input sources, including NAc, and re-enters cortocolimbic loops via mediodorsal nucleus of thalamus (Churchill & Kalivas, 1994; Groenewegen & Berendse, 1990; Groenewegen, et al., 1993; Haber, et al., 1985a; Haber, et al., 1993; Heimer, et al., 1987; Kincaid, et al., 1991; Young, et al., 1984; Zahm, et al., 1987). Such anatomical connectivity sets the stage for VP to actively mediate and modulate reward processing, rather than passively passing on motor signals to the brainstem.

VP Function in Reward

VP as necessary cause, sufficient cause, and neural encoder for reward

Several studies have demonstrated that posterior VP might be a site necessary for normal 'liking' and incentive 'wanting' of reward. VP cell loss, or temporary VP inhibition, causes loss of incentive 'wanting' for food reward and replaces 'liking' to a sweet sucrose taste with 'disliking' (Cromwell & Berridge, 1993; Schallert, Whishaw, & Flannigan, 1977; Shimura, et al., 2006; Stellar & Corbett, 1989). In addition, VP has been shown to be sufficient for reward enhancement, particularly in a hedonic hotspot in posterior VP where opioid agonist causes > 100% increases of 'liking' reactions to sucrose (Pecina, et al., 2006; K. S. Smith & Berridge, 2005). Lastly, recent electrophysiological studies from the Aldridge lab in

the university of Michigan have revealed that VP neurons integrate physiological state information in order to dynamically encode hedonic value of taste rewards and incentive salience of the reward-predictive cues (Tindell, et al., 2004; Tindell, et al., 2005; Tindell, et al., 2009; Tindell, et al., 2006).

The nucleus accumbens shell has also been shown to be a *sufficient* cause for reward 'liking' and food 'wanting'. For example, similar to the µ-opioid hedonic hotspot in posterior VP, dorsal medial NAc shell also contains a µ-opioid hedonic hotspot (Pecina & Berridge, 2005). Increased eating and other reward measures have also been observed following GABA agonist or glutamate antagonist manipulations in the NAc. Also, NAc neurons have been demonstrated to respond with a firing rate inhibition to an intraoral sucrose taste and during the presentation of Pavlovian cues for sucrose (Nicola, 2007; Roitman, Wheeler, & Carelli, 2005; Taha & Fields, 2005), and a firing rate excitation to an intraoral quinine taste and during aversive state predictive cue before cocaine (Calu & Schoenbaum, 2008; Roitman, et al., 2005; Wheeler, et al., 2008). However, nucleus accumbens does not seem to play a *necessary* role for food 'wanting'- NAc cell loss does not cause impairments in food intake, instrumental conditioning for food rewards, or responding for food rewards (Albertin, et al., 2000; Dworkin, et al., 1988; Meredith, Ypma, & Zahm, 1995; Parkinson, et al., 1999).

The Inhibition of Posterior VP Suppressed Hedonic 'Liking' and Caused Aversive 'Disliking' (Project 1)

In a mapping study using Fos immunohistochemistry, intracranial drug microinjections, and behavioral microanalyses, I found that GABA inhibition in caudal VP suppressed 'wanting' for food or water, and replaced hedonic 'liking' reactions to a sucrose sweet taste with 'disliking' reactions, consistent with one previous report (Shimura, et al., 2006). In addition, I functionally mapped the subareas in that VP that are involved in GABA-induced loss of 'wanting', decreased 'liking', and increased 'disliking'. I found that the neural substrates for the above three functions are arranged in concentric areas, with loss of 'wanting' area and 'liking' suppression area occupying a larger area in VP, and the 'disliking' site being

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more concentrated. This aversive 'disliking' site occupied a 0.8mm^3 zone in caudal VP and perhaps most rostral part of the sublenticular extended amygdala. By using neuronal count, excitotoxin lesions, and behavioral microanalyses, I found that lesions in a similar area in the caudal VP and sublenticular extended amygdala caused aphagia (loss of food appetite) and the replacement of 'liking' to sucrose with 'disliking'. This lesion 'aversive' site was larger, and more medial and posterior to the GABA 'aversive' site, but the two sites highly overlapped in posterior VP.

Between the GABA aversive site and the lesion aversive site, we believe that the GABA aversive site reveals the truest boundaries of the neural substrate where loss of function causes aversion to a sweet taste. As discussed in Chapter 2, transient GABA inhibition has the advantage (over lesion) to provide more intense anatomical and functional challenges, but does not have the shortages of traditional lesion techniques, like functional compensation, incomplete neuronal damage, etc (Lomber, et al., 1996; Waraczynski & Demco, 2006). In addition, the extent of neuronal damage has been proved to be important for the generation of aversion, as shown in one previous study (Cromwell & Berridge, 1993) and the current study. For example, for animals with lesion centers located at similar sites, the major difference that causes some of them to show aversion to sucrose and some do not is the extent of neuronal loss. Animals with over 74% neuronal loss in the caudal VP showed intense 'disliking' reactions to sucrose infusion, while animals with only 53% neuronal loss in a similar site do not. Because GABA causes more localized and more intense inhibition in neuronal substrates, as assessed by GABA Fos plumes, the aversive site mapped by GABA inhibition may reveal a more precise area that is crucial for aversive 'disliking' to sucrose.

VP GABA and reward

These results support a significant role for the VP GABAergic signal in taste reward processing, consistent with other studies of VP. Most relevant study comes from the Shimura et al. (2006), in which they showed that VP muscimol microinjections dramatically decreased 'liking' reactions and replaced them with increased 'disliking' reactions in response to saccharin infusion. Further evidence demonstrated that the sucrose-licking pattern of rats with GABA inhibition in VP

resembled quinine- licking pattern, along with a decrease in food intake (Taha, Katsuura, Noorvash, Seroussi, & Fields, 2009). Together with results from the current study, it appears that VP inhibition mediates loss of food appetite along with changes in hedonic value of reward. However, it is important to note that the loss of food appetite does not always come with changes in hedonic value of reward. For example, results from the current study showed that GABA inhibition in lateral hypothalamus, which is medial and posterior to VP, caused loss of food appetite without altering the hedonic value of reward. Also, while GABA inhibition caused suppression of food consumption, GABA blockade by microinjections of GABA antagonists actually caused enhancement of food consumption (K. S. Smith & Berridge, 2005; Stratford, et al., 1999). However, this enhancement of food consumption was not due to an increase of reward palatability, as measured by taste reactivity test. Another important line of support came from electrophysiological studies by Tindell et al, where they showed that the hedonic value of a taste solution (sucrose or salt) was encoded by VP neurons, and these VP neurons tracked the change of hedonic value of a nasty salty taste during salt depletion. Importantly, VP neurons fired with a lower rate when the highly concentrated salty solution elicited aversive reactions in the normal state, suggesting that aversive 'disliking' coding is associated with stronger GABA inhibition. Together, theses studies established a precise relationship between GABAergic signaling in the VP and reward- increasing inhibition blocks feeding along with a decrease of hedonic value, while decreasing inhibition increases food intake without changes of hedonic value.

GABA neurotransmission antagonizes opioid signal in VP

It is noteworthy that the GABA aversive 'disliking' site identified in the current study highly overlaps with the opioid hedonic 'liking' hotspot identified previously in our lab. This finding is consistent with the notion that GABAergic signal and opioid signal in VP play antagonistic roles. Anatomically, GABA and met-enkephalin receptors are co-localized on axon terminals in VP (Groenewegen, et al., 1993; Zahm & Heimer, 1988, 1990; Zahm, et al., 1996; Zahm, et al., 1985). Physiologically, electrophysiological recording studies have demonstrated that GABA agonist and μ- opioid negate each other (Chrobak & Napier, 1993; Johnson &

Napier, 1997; Napier & Mitrovic, 1999). For example, morphine shortened the duration of the NAc-evoked short latency excitations and attenuated the magnitude of the long-latency inhibition, which was similar to the effect of GABA antagonist bicuculline but opposite of GABA agonist, suggesting that opioid and GABA play opposite roles in VP (Chrobak & Napier, 1993). Behaviorally, microinjections of VP GABA agonists in the VP blocked μ-opioid-induced hyperactivity, suggesting that GABA signal is capable of modulating opioid effects (Austin & Kalivas, 1990). CTAP, an opioid antagonist, in VP blocks cocaine-induced decreases of GABA in VP, as well as cocaine-reinstatement, suggesting that opioid is capable of modulating GABA transmission presynaptically (X. Tang, K. McFarland, S. Cagle, & P. Kalivas, 2005). Together, these results suggest that an antagonistic relationship between GABA and μ-opioid in VP physiologically and functionally. However, future studies are required to clarify how GABA and opioid interact in posterior VP to mediate positive 'liking' and negative 'disliking' of reward.

GABAergic reward signal in other structures: nucleus accumbens and parabrachial nucleus

　　While GABA neurotransmission mediates only aversive 'disliking' in VP, GABA is capable of mediating hedonic 'liking' aspect of reward in other brain structures, such as NAc and parabrachial nucleus (PBN). Benzodiazepines (which amplifies the effects of GABA transmission) have long been suggested to amplify food palatability (Cooper, 1980; Cooper & Estall, 1985). Microinjections of GABA agonist muscimol in the medial NAc shell elicit a hedonic gradient that goes from positive to negative along the anterior to posterior axis; in most rostral part of the NAc shell, GABA amplifies hedonic 'liking' reactions to sucrose, while in most caudal part of the NAc shell, GABA increases aversive 'disliking' reactions to sucrose (Faure, et al., 2010; Reynolds & Berridge, 2001, 2002). The results from the current study are consistent with the previous findings. Another site where GABA enhances 'liking' is the parabrachial nucleus in the brainstem. Microinjections of a benzodiazepine drug into the parabrachial nucleus or the forth ventricle increases 'liking' reactions to sucrose infusion as well as food intake (Pecina & Berridge, 1996;

Soderpalm & Berridge, 2000). Overall, GABA mediates 'liking' in anterior part of the medial NAc shell and the PBN, and 'disliking' in caudal part of the medial NAc shell and posterior VP. It is still unknown whether the two 'disliking' sites interact with each other to mediate 'disliking', or the precise GABA 'liking' hotspots in PBN.

GABA in VP is critical for controlling 'liking' and 'disliking' of reward

Future studies are needed to clarify if there is a 'disliking' circuit mediated by GABA neurotransmission, how GABA and opioid function together to mediate 'liking', or how 'liking' and 'disliking' signals may interact with each other to modulate reward processing. The results from the current study (together with the previous finding) suggest that caudal VP may serve as a critical 'checkpoint' for the hedonic value of reward. The elevation of 'liking' caused by opioid requires the functioning of opioid signals in both NAc and VP hotspots. Similarly, GABA-elicited 'liking' requires the normal functioning of opioid signal as well. One potential mechanism is that PBN receives opioid signal via anatomical connections with VP and/or NAc (Groenewegen, et al., 1993; Usuda, et al., 1998a)(Norgren, 1976; Saper and Loewy, 1980; Groenewegen et al. 1993; Usuda et al., 1998), and the opioid signal plays a permissive role for GABA to amplify 'liking' in VP. As discussed previously, GABA signal in posterior VP is antagonistic of opioid signal, suggesting that GABA could be a critical factor at this checkpoint in terms of whether or not opioid signal could be passed on to the PBN. In reverse, opioid signal negates GABA signal (as well as dopamine signal, but not glutamate) in posterior VP, implicating that opioid in the checkpoint in caudal VP could disrupt the recruitment of GABAergic network across subcortical areas (e.g. PBN).

Sublenticular extended amygdala and reward

One other structure in which loss of function causes decreased 'liking' and increased 'disliking' may be the sublenticular extended amygdala (SLEA). Results from the current study suggest that both of the GABA 'liking' suppression site and the lesion 'liking' suppression site cover the anterior part of the SLEA, suggesting that SLEA loss of function may impair normal 'liking' of reward. In addition, SLEA loss of function may also cause aversive 'disliking' to a sweet taste (but we can not draw definite conclusion about this notion from the current study). The caudal

boundary of the GABA aversive site ends around the most anterior tip of the SLEA, where it joints with the most caudal part of the VP. However, it is unclear if the SLEA is crucially involved in aversive processes, since we do not have animals with microinjection centers located in the SLEA. While the caudal part of the GABA aversive site seems to be touching the anterior part of the SLEA, the real neural substrate that causes aversion elevation may come from the posterior VP, which is where the microinjection centers are located. In the case of the lesion aversive site, although the site encompasses a large portion of the SLEA, it is unclear whether or not the SLEA is truly involved in aversive processes due to the limitations of lesion techniques discussed earlier. Future studies are required to design experiments that specifically target the SLEA in order to clarify whether SLEA plays a role in aversion. However, it is noteworthy to point out that the SLEA has been suggested to play an important role in reward as discussed below.

The sublenticular extended amygdala (sometimes referred to as caudal substantia innominata) together with central nucleus of amygdala (CeA), medial nucleus of amygdala (MeA), the interstitial nucleus of the posterior limb of the anterior commissure (IPAC) and bed nucleus of stria terminalis (BNST) are neuroanatomically grouped together under the macrosystem of extended amygdala. Since the work of neuroanatomists George Alheid, Lennart Heimer, and Jose de Olmos (Alheid, 2003; de Olmos & Heimer, 1999; Heimer, Harlan, Alheid, Garcia, & de Olmos, 1997) in the 1980s, the concept of extended amygdala became widely accepted and was considered to be different from the ventral-striato-pallidal system based on histological, architectural, and developmental evidence.

The SLEA has been shown to mediate both rewarding and aversive stimuli. In rodents, SLEA neurons are activated by brain stimulations (Arvanitogiannis, Flores, Pfaus, & Shizgal, 1996; Arvanitogiannis, Tzschentke, Riscaldino, Wise, & Shizgal, 2000; Flores, Arvanitogiannis, & Shizgal, 1997). Lesions or inactivation of the SLEA reduced rewarding effect of medial forebrain bundle stimulation (Arvanitogiannis, et al., 1996; Waraczynski, 2003, 2008), reduced self-administration of cocaine (Robledo & Koob, 1993), and disrupted operant performance for sucrose pellets in rats (Brown, Latimer, & Winn, 1996). In humans, SLEA appeared to be involved in

reward and responded to both the expectancy and experience of monetary reward (Breiter, Aharon, Kahneman, Dale, & Shizgal, 2001; Breiter & Rosen, 1999). Recent studies suggest that SLEA may also respond to aversive stimuli. For example, transient activation of SLEA was reported following noxious thermal stimulation (46^oC) in humans (Becerra, Breiter, Wise, Gonzalez, & Borsook, 2001), suggesting that SLEA may be involved in both positive and negative domains of stimuli. Indeed, recent studies support this notion by showing that SLEA responded to both positive and negative emotional content (Liberzon, Phan, Decker, & Taylor, 2003). The malfunction of SLEA has been linked to affective disorders such as post-traumatic stress disorder (PTSD) (Liberzon & Martis, 2006; Liberzon & Sripada, 2008) and unipolar depression (Abler, Erk, Herwig, & Walter, 2007)

The current study suggests that inactivation of the SLEA suppressed hedonic 'liking' and may cause aversive 'disliking' to a sweet taste. Future studies are required to further identify the causal role of SLEA in reward.

Other Neurotransmitters that Amplified Hedonic 'Liking' in VP: Orexin and Anandamide (Project 2 & Project 3)

GABA and μ- opioid are not the only neurotransmitters that mediate 'liking' aspect of reward. Project 2 and 3 (Chapter 3 and 4) identified orexin and cannabinoid as two other neurotransmitters that enhance 'liking' in VP.

In Project 2 (Chapter3), by intracranial microinjection, Fos plume mapping, and taste reactivity test, I found an orexin hedonic 'liking' hotspot located in a 0.8 mm^3 zone in caudal VP. This orexin hotspot is about 80% of the size of the opioid hedonic hotspot in caudal VP. In Project 3 (Chapter4), by similar methodology, an endocannabinoid (anandamide) hedonic hotspot was found to be located in a similar area in caudal VP. The opioid, orexin, and cannabinoid hotspots highly overlap in caudal VP, suggesting that the three neurotransmitters may interact to mediate hedonic 'liking' processing (Figure 5-1).

Reward vs. Homeostasis

Relatively little is known about the interaction between corticolimbic reward circuit and hypothalamic homeostasis control in the integrated regulation of feeding

behavior. As discussed in Chapter 3 and Chapter 4, the link between reward and homeostasis may provide answers to why food tastes better when we are hungry or why we eat more tasty food even when we are full.

Orexin mediates hunger- enhanced food palatability

It has been hypothesize that orexin neurons in the lateral hypothalamus may serve as one important link between hypothalamic homeostasis control and corticolimbic reward circuit. LH orexin neurons are activated by arcuate neuropeptide Y (NPY) during hunger state (Kelley, Schiltz, et al., 2005; Niimi, et al., 2001; Yamanaka, et al., 2000), and send projections to reward related areas, including NAc shell and VP (Backberg, Hervieu, Wilson, & Meister, 2002; Baldo, et al., 2003; Cutler, et al., 1999; Galas, et al., 2001; Marcus, et al., 2001; Nixon & Smale, 2007; Peyron, et al., 1998; Trivedi, et al., 1998). One possible orexin- mediated circuit that recruits nucleus accumbens shell during hunger state was suggested by Kelly et al. (2005). Kelley et al. proposed that during hunger state, orexin neurons in the lateral hypothalamus project to activate glutamate neurons in the thalamic paraventricular nucleus. The glutamatergic thalamic paraventricular neurons in turn project to the nucleus accumbens shell to activate large acetylcholine-containing interneurons. Finally, cholinergic interneurons in medial NAc shell activate nearby enkephalin neurons. These enkephalin-releasing neurons should possibly include those in the cubic- millimeter opioid hedonic hotspot, which together with the hedonic hotspot in VP, mediate hedonic 'liking' to sucrose. Thus, this indirect lateral hypothalamic-thalamic paraventricular-nucleus accumbens circuit may mediate hunger-enhanced palatability of foods.

The discovery of the orexin hedonic hotspot suggests the possible existence of a second mechanism by which orexin neurons in the lateral hypothalamus directly recruit 'liking' circuit via LH- VP link. As discussed in Chapter 3, the projection from LH to VP is particularly dense in posterior VP, which is where the orexin hedonic hotspot is located (Baldo, et al., 2003). We speculate that orexin activation during hunger might directly enhance the hedonic impact of food reward by stimulating neurons in the orexin hedonic hotspot in posterior VP. Thus, orexin might effectively activate the same hedonic hotspot as μ- opioid signal does in VP. In

addition, orexin might stimulate 'wanting' both through these hedonic hotspots and/or through projections to the mesolimbic dopamine neurons in the ventral tegmental area.

Endocannabinoid mediates hunger-enhanced food palatability

Evidence suggests that endocannabinoids may be another potential candidate that links the corticolimbic reward circuit and the hypothalamic homeostasis control. Endocannabinoids are similarly activated by hunger. For example, 24- hr fasting in rats has been shown to raise the levels of endocannabinoids, anandamide and 2-AG in the limbic forebrain, such as NAc shell (Kirkham, et al., 2002). An increase of endocannabinoids during hunger could enhance the incentive salience or hedonic 'liking' for food (Kirkham, 2005, 2009) possibly via recruitments of the hedonic hotspots in medial NAc shell and the hedonic hotspot in posterior VP. Thus, the discovery of an endocannabinoid hedonic hotspot in posterior VP provides a potential mechanism to hunger-enhanced food palatability.

Interactions between μ-opioid, orexin, and endocannabinoids in reward

The opioid, orexin, and cannabinoid hotspots highly overlap in caudal VP, suggesting that the three neurotransmitters may interact to mediate reward processing.

Orexin vs. μ-opioid

As discussed in Chapter 3, results from previous studies suggest that orexin signal and opioid signal interact to process food and drug reward. For example, system inhibition of opioid pathways or local blockade of opioid in NAc (but not LH) disrupted orexin-induced feeding effects (Clegg, et al., 2002; Sweet, et al., 2004). Similarly, pharmacological inhibition of orexin signal the in ventral tegmental area blocked high fat appetite induced by opioid microinjections in NAc shell (in an area similar to the opioid hedonic hotspot found in our lab) (Zheng, et al., 2007). As for drug reward, previous studies showed that LH orexin neurons contain opioid receptors and responded to morphine administration and opioid antagonist-precipitated morphine withdrawal, suggesting that orexin system is involved in

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opioid drug reward (Georgescu et al., 2003). In addition, blockade or lesion of orexin in LH and VTA blocks morphine conditioned place preference, while microinjections of orexin in VTA reinstate morphine preference (Aston-Jones, et al., 2009; Aston-Jones, et al., 2010; Harris, et al., 2005; Harris, et al., 2007; Sharf, Guarnieri, Taylor, & DiLeone, 2010). Overall, it is clear that orexin and opioid signals interact to mediate rewards for foods and drugs. However, future studies are required to identify whether the systems interact closely for hedonic 'liking' processing, such as by mutual recruitment and mutual co-dependence, or instead act independently.

Endocannabinoid vs. μ- opioid

As discussed in Chapter 4, previous research has found evidence that supports cannabinoid-opioid interaction and suggest that neither of the two systems can function normally without the other in processing drug and food rewards. The inactivation of opioid pathways by naloxone attenuated or blocked the reinforcing effect of cannabinoid drugs, as well as cannabinoid-induced feeding and feeding-reinforced behaviors (Navarro, et al., 2001; Solinas & Goldberg, 2005; Williams & Kirkham, 2002b). Similarly, the inactivation of cannabinoid neurotransmission by microinjections of CB1 antagonist disrupted the reinforcing effect of opioid drugs, as well as opioid-induced feeding, and feeding-reinforced behavior (Azizi, et al., 2009; Ellgren, et al., 2008; Navarro, et al., 2001). In addition, recent findings suggest that opioid antagonist/cannabinoid antagonist can modulate cannabinoid/opioid-induced c-Fos expression in reward-related brain areas, such as VTA, NAc, thalamic paraventricular nucleus, etc (Allen et al., 2003; Singh et al., 2004). Finally, previous research examined the functional interaction between opioid system and cannabinoid system and showed that the combination of cannabinoid antagonist SR 141716 and opioid antagonist naloxone generated a supra-additive anorectic effect(Kirkham & Williams, 2001). Taken together, these results clearly indicate that the simultaneous activations of both opioid and cannabinoid systems are required for the normal functioning of food and drug rewards. Future studies are needed to examine whether opioid and cannabinoid systems interact to mediate reward 'liking'.

Orexin vs. endocannabinoid

Recent studies have highlighted the existence of a functional interaction between the endocannabinoid system and the orexin system. One *in vitro* study by Hilairet et al. (2003) first demonstrated that when CB1 and Orexin 1 receptors are co-expressed, a major CB-A dependent enhancement of the orexinA potency was found. Verty et al. (2009) further confirmed the interaction between the endocannabinoid system and the orexin system *in vivo* by showing that CB 1 antagonist rimonabant (SR 141716) activated neurons in the hypothalamic regions, as identified by Fos activation, colocalized with orexin peptides that are immunohistochemically labeled. Functionally, pretreated rats with subeffective doses of Rimonabant (CB1 antagonist) attenuated orexin- A induced feeding (Crespo, et al., 2008), supporting the idea that cannabinoid and orexin A systems share a common mechanism in food reward. However, it is still unclear how the endocannabinoid system and opioid system interact to mediate reward 'liking'.

VP and Reward Circuits

VP role in 'disliking' circuit

Although the VP 'disliking' site identified in this dissertation is the only known brain substrate where neuronal loss and GABA inhibition can flip hedonic 'liking' (to sucrose) to aversive 'disliking', it might not function as the only site that sensory aversion arises. Instead, sensory aversion may result from VP and an interconnected larger 'disliking' circuit that operate as a whole. This aversive 'disliking' circuit stretches from forebrain to brainstem, forming a system that paints 'aversive gloss' onto pure sensory stimuli to form sensory aversion.

Within an interconnected neural hierarchy of affective function, the brainstem functions as an essential first level. For example, in the 1970s, Grill and Norgren further showed that decerebrate rats, with only a hindbrain and midbrain intact, still emitted positive 'liking' reactions to sucrose taste and negative 'disliking' reactions to quinine taste as measured by the taste reactivity test (Grill & Norgren, 1978b). In humans, Steiner (1973) showed that anencephalic infants (born without the forebrain but with a normal brainstem) similarly showed normal tongue

protrusions to sucrose taste, but aversive gapes and head- shakes to quinine solutions (Steiner, 1973). Thus, the brainstem alone is capable of generating balanced affective 'liking' and 'disliking' reactions.

However, adding one more layer of brain substrate, diencephalon (thalamus, hypothalamus, and pineal) actually unbalances the affect value completely to negative 'disliking'. For example, 'thalamic animals', which underwent a surgical preparation that removed brain substrates above the thalamus, expressed only aversive 'disliking' reactions to a sweet taste and lacked normal 'liking' reactions (Grill and Nogren, 1978b). These thalamic animals not only lacked neocortex, but also subcortical areas, including the amygdala, hippocampus, NAc, VP, etc. The fact that thalamic animals predominantly expressed negative affective reactions suggests that the diencephalon contains a circuitry, which pushes brainstem reactions into 'disliking' unless opposed by signals from forebrain structures.

Importantly, adding one layer of subcortical substrate, without the neocortex, above the diencephalon is enough to restore the balance between 'liking' and 'disliking' reactions. For example, 'decorticate rats', which had their neocortex removed but had their subcortical substrate intact, showed completely normal 'liking' reactions to sweet taste and 'disliking' reactions to bitter taste (Grill and Nogren, 1978b). This result suggests that some area in the subcortical substrate negates the imbalanced 'disliking' reactions in 'thalamic animals' and restore normal affect.

So, what structure in the subcortical area is required for the balance of normal affect? Or in the extreme case, what structure is capable of flipping 'liking' into complete 'disliking' when loss of function occurs? Results from the current dissertation suggest that this crucial area is situated in the posterior VP. Excitotoxin lesions and GABA inhibition in similar sites in the posterior VP not only abolish 'liking' reactions to sucrose but also replace them with 'disliking' reactions. This result suggests that the normal functioning of VP is required for the balance of normal pleasure, and the removal of VP flips 'liking' into 'disliking'. One potential mechanism may lie between the anatomical connection between the VP and the thalamus. In the normal condition, GABAergic projection neurons in the VP send

inhibitory output to suppress the thalamic 'disliking' circuit and to ensure the balance between 'liking' and 'disliking'. However, in the condition where VP inhibition or loss of function occurs, thalamus is dis-inhibited from the GABAergic projections from the VP. The affective value thus shifts to complete negativity.

VP role in 'liking' circuit

As we discussed above, VP may be embedded in a larger interconnected 'liking' circuit that paints hedonic gloss onto pure sensations and generates sensory pleasure. Anatomically, VP maintains reciprocal connections with structures related to taste rewards, including the parabrachial nucleus, insular cortex, orbitofrontal cortex, and the NAc (Groenewegen, et al., 1993; Groenewegen, Wright, Beijer, & Voorn, 1999; Haber & Knutson, 2010; Usuda, Tanaka, & Chiba, 1998b). Microinjection studies have further pointed out a particular role of NAc- VP link in reward 'liking' (Pecina, et al., 2006; K. S. Smith & Berridge, 2007). For example, DAMGO in a cubic-millimeter hedonic hotspot in both the NAcSh and the caudal VP enhanced hedonic 'liking' of a sweet taste (Pecina & Berridge, 2005; K. S. Smith & Berridge, 2005). In addition, the normal functioning of opioid signal in both hedonic hotspots is required for 'liking' elevation- opioid blockade in either of the opioid hedonic hotspot disrupts 'liking' enhancement (K.S. Smith & Berridge, 2007). This tight GABAergic connection between NAc and VP in reward 'liking' circuit is further supported by electrophysiological data. NAc neurons have been shown to differentially respond to appetite and aversive rewards. For example, NAc neurons respond with a firing rate inhibition to an intraoral sucrose taste and during the presentation of Pavlovian cues for sucrose (Nicola, 2007; Roitman et al., 2005; Taha & Fields, 2005), but a firing rate excitation to an intraoral quinine taste and during aversive state predictive cue before cocaine (Calue & Schoenbaum, 2008; Roitman et al., 2005; Wheeler et al., 2008). In contrast, VP neurons respond to a sweet sucrose taste and a palatable salty taste in the salt-depleted state with increased firing rate, but to a nasty highly- concentrated salty taste in a normal state with a decrease in firing rate (Tindel et al., 2006). The reversed pattern of VP firing (e.g. increased firing to palatable taste) in correspondence to NAc firing (e.g. decreased firing to palatable taste) raises the possibility that NAc neurons play a disinhibitory role in

hedonic encoding via NAc- VP GABAergic connection. It is likely that rewarding stimuli cause an inhibition of NAc firing, which leads to a weaker GABAergic output to the VP and thus dis-inhibit VP neurons. VP neurons correspondingly respond to the rewarding stimuli with an increase of firing rate.

However, it is important to note that different subregions of the NAc-VP system may be differentially involved in 'liking' processes. For example, GABA microinjections in the anterior NAcSh enhanced 'liking' to sucrose, while GABA in the posterior NAcSh actually caused 'disliking' to sucrose (Faure, et al., 2010; Reynolds & Berridge, 2002). Similarly, GABA in the caudal VP flips 'liking' to 'disliking' to sucrose taste, but GABA in the anterior VP does not (Tindell et al, 2006). The recording sites of the abovementioned studies, which show attenuated NAc firing and elevated VP firing in response to appetitive stimuli, are more focused at the rostral NAcSh and at the caudal VP. This suggests that the notion of excessive GABA in the NAcSh dis-inhibits 'liking' circuit via VP may only exist between the rostral NAcSh and the caudal VP. Future investigations that compare the physiological characteristics of rostral NAcSh/VP neurons and the caudal NAcSh/VP neurons are required to clarify the functional compartmentalization within VP and NAcSh. The findings may provide insights into 'liking' circuit.

Another important structure in the 'liking' circuit is the parabrachial nucleus (PBN) in the brainstem, which receives GABAergic input from the VP (Groenewegen et al., 1993). Interestingly, PNB neurons predominantly respond to sucrose taste with attenuated firing rate (Lei, Yan, Shi, Yang, & Chen, 2007), suggesting that NAc-VP- PBN may form a hierarchy of 'liking' circuitry. Upon the infusion of sucrose taste, NAc neurons decreased firing, dis-inhibit VP neurons (increased firing), which in turn suppress PBN neurons.

VP Function in Human Studies: Implication for Clinical Neuropsychology

VP role in 'liking'

As a critical structure for hedonics and incentive motivation, VP dysfunction may contribute to maladaptive behaviors or clinical disorders of affect and reward.

As discussed in Chapter 2, some human clinical evidence supports the notion that normal functioning of VP is needed for positive hedonic 'liking' in humans. Lesion or GABA inhibition in caudal VP replaced rats' hedonic 'liking' reactions to sucrose with aversive 'disliking' reactions, as well as a loss of food appetite. Similarly, in two clinical cases, human patients with localized lesion in VP (and ventral GP) experienced depressed mood (described as anhedonia by the authors), flat affect, hopeless feelings, social isolation, and profound lack of motivation (Miller, et al., 2006; Vijayaraghavan, et al., 2008). The impairment of neurotransmission in VP, even when VP is anatomically intact, can also contribute to reduction in positive emotions and elevation of negative emotions. For example, in PET scan studies by Zubieta et al., the deactivation of μ-opioid receptors in human VP was shown to be associated with 'the increases in negative affect ratings and the reductions in positive affect ratings during the sustained sadness state' (Zubieta, et al., 2003).

VP role in 'disliking'/disgust

Although the experimental design was focused on affective reactions to food tastes, the results may have broader implications for understanding emotional disgust and moral disgust. There are findings from human studies that suggest the basal ganglia is involved in disgust circuit, and the malfunction of basal ganglia, which includes VP and other structures, is linked to disgust-associated disorders, such as OCD, Huntington's disease, and perhaps Parkinson's disease (Adolphs, 2002; Adolphs, et al., 2003; Olatunji, et al., 2010; Phillips, et al., 1997; Sprengelmeyer, et al., 1996; Sprengelmeyer, et al., 1997; Surguladze, et al., 2010; Suzuki, et al., 2006). For example, patients with Huntington's disease or OCD, which neuronal degeneration or abnormal neural activity occur in basal ganglia, had difficulty recognizing facial expression of disgust (Sprengelmeyer, et al., 1996; Sprengelmeyer, et al., 1997). Although no study has yet examined VP's role in disgust-associated disorders, it is likely the normal functioning of VP is require for the processes of disgust if it stands true that food disgust and emotional disgust share the same neural circuit in the brain. The strongest evidence perhaps come from the study by Calder et al (2007), in which they showed the neural activity in anterior human VP was associated with

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individual sensitivity to images of disgusting food. It will be of particular importance for future studies to clarify VP's role in emotional disgust and disgust-associated psychopathology.

VP role in addiction

VP may similarly contribute to the disorders relating to drug addiction in humans. As discussed in the introduction chapter, a reduction of VP GABA transmission and an elevation of VP opioid transmission have been shown to associate with drugs of addiction in animals (Mickiewicz, Dallimore, & Napier, 2008; Tzschentke, 1998; Waraczynski & Demco, 2006). In human studies, VP has been reported to respond to drug-predictive cues in drug users, suggesting that VP is involved in drug reward processes (A. R. Childress, et al., 2008). In one clinical study, VP lesion was shown to decrease the motivation of a chronic drug addict to consume drug (Miller, et al., 2006). The patient reported 'the disappearance of drug cravings' and 'no longer experiencing pleasure from drinking alcohol'. Additionally, the imbalance of neurotransmission in VP has been suggested to link to drug use in humans (Frankel, Alburges, Bush, Hanson, & Kish, 2007, 2008). Although relatively little is know about the causal role VP plays in drug addiction in humans, it is clear that the VP is involved in drug reward processing in human.

VP transfers reward value into action

VP has also been suggested to mediate the evaluation of reward and the transition of the reward value into appropriate reactions (Kelley, Baldo, & Pratt, 2005; K. S. Smith, et al., 2009; Waraczynski & Demco, 2006; Zahm, 2006). Previous studies have shown that the malfunction or loss of function of VP would cause impairments in behavioral tasks that require reward evaluation, such as visual discrimination, reversal learning, or conditioned place preference for food reward (Everitt, et al., 1987; Ferry, Lu, & Price, 2000; McAlonan, Robbins, & Everitt, 1993b; Robbins, et al., 1989). Clinical reports support the notion that VP in involved reward evaluation by showing that the presentation of 'unseen' drug, sex, or monetary cues elicited rapid responses in VP in fMRI studies (A. R. Childress, et al., 2008; Pessiglione, et al., 2007). The level of VP activation is correlated with subjective rating of the same drug or sex cues when they are presented in a visible form (A. R.

Childress, et al., 2008), suggesting that VP encodes reward values. Also, the activation of VP to the monetary cues is correlated with the effort the subjects spent in order to acquire the 'unseen' monetary reward (Pessiglione, et al., 2007), suggesting that VP activation is not only associated with the reward value but also the motivation to act. Together, these results suggest that VP codes the affective/motivational evaluation of reward and may be capable of transforming the reward value into action in humans.

VP role in social affiliation

Finally, VP has been shown to be critically involved in social affiliation, and the dysfunction of VP may be manifest in social affiliation-related disorders. For example, vasopressin V1a receptors in VP have been found to be higher in monogamous prairie vole, and vasopressin receptor gene transfer into the VP facilitates pair-bond formation (Pitkow, et al., 2001; Wang & Aragona, 2004). Importantly, variability in the human V1a receptor affects pair-bonding behavior in men (Walum, et al., 2008). In primates, both the formation and the maintenance of pair bonding are associated with VP activation, which were assessed with structural magnetic resonance imaging and position emission tomography(Bales, et al., 2007). In humans, VP has been reported to be activated when subjects view photographs of their rejected beloved and when a different group of subjects (who they claimed to be in love with the same partners for more than 20 years) view photographs of their beloved (Aceveda, Aron, Fisher, & Brown, 2008; Fisher, Aron, & Brown, 2006). Other than formation of social bonds, VP is also involved in sexual arousal and maternal behavior. In human imaging studies, the activity of VP (together with ventral globus pallidus) was reported to increase during male sexual arousal, as well as in response to subliminal sexual images (Rauch, et al., 1999). Although no clinical studies so far have examined the role of VP in affiliation-related disorders, such as autism, two patients with VP lesion were reported to have experienced social withdrawal and social isolation, suggesting that the normal functioning of VP may be important for formation of social bonds (Miller, et al., 2006; Vijayaraghavan, et al., 2008).

Future Directions

The findings of this dissertation raise several questions that require future studies to clarify. Broadly, I provide evidence that the inhibition or lesion of posterior ventral pallidum (together with sublenticular extended amygdala), at a site that highly overlaps with opioid, orexin, and anandamide hotspots, increased aversive 'disliking' affect to a natural sweet taste. This result suggests that 'liking' and 'disliking' are both mediated by the same anatomical substrate in VP. However, in the medial NAc shell, although the opioid and anandamide hedonic hotspot is located in the anterior part, GABA inhibition increased aversive 'disliking' to sweet taste, as well as defensive paw-treads but only in posterior part. This result suggests that 'liking' and 'disliking' may be mediated by different neuronal substrates in NAc shell. Together, these findings raise several interesting questions in terms of 'liking' and 'disliking' circuits. For example, in what specific subarea is the GABA aversive 'disliking' site located in the NAc shell? Do the NAc 'disliking' site and VP 'disliking' site interact to mediate reward 'disliking'? Or to a broader extent, do 'liking' and 'disliking' circuits interact to mediate the affective aspect of reward? A variety of methodologies including lesion, antagonist microinjections, immunochemical Fos labeling, and electrophysiological measures of neuronal activation may help answer these questions.

Another major question brought up by the experiments relating to the orexin hedonic hotspot and endocannabinoid hotspot is whether and how homeostasis affects hedonic 'liking' processes. As discussed earlier, both orexin and endocannabinoids are activated by hunger and are capable of enhancing hedonic 'liking' reactions when microinjected in NAc shell or VP. Future research using homeostasis manipulation, such as fasting or feeding, and taste reactivity to test the changes of reward 'liking' may shed light on the mechanisms between homeostasis and 'liking' aspect of reward.

Another question raised by the findings of orexin hedonic hotspot and anandamide hedonic hotspot in VP is whether and how opioid, orexin, and anandamide interact to mediate hedonic 'liking'. Although there is evidence that

suggests that the three neurotransmitter signals interact to mediate reward, as discussed earlier, relatively little is known about the mechanisms underlying their interactions with regard to hedonic 'liking' aspect of rewards. For example, is the normal functioning of opioid system required for orexin-enhanced 'liking' to sucrose? Do anandamide hotspots in NAc shell and in VP interact to mediate 'liking'? Or do anandamide hotspots interact with opioid hotspots to mediate 'liking' processes? The detailed mechanisms of these interactions in reward 'liking' are another promising field.

It will also be interesting to know how glutamate affects 'liking' and 'disliking' in the VP. While inhibitory GABA signal in the VP mainly conveys information from the NAc, excitatory glutamatergic afferents pass on signals from the cortex, amygdala, and hippocampus to the VP (Fuller, et al., 1987; Groenewegen, et al., 1999; Haber & Knutson, 2010; Maurice, et al., 1997; Zaborszky, et al., 1997). Although relatively little is known about the role of glutamate in VP, there are studies that suggest VP glutamate is involved in taste reward processing. For example, microinjections of NMDA or AMPA antagonists in the ventral striatum/VP in pigeons enhanced eating (Da Silva, Marino-Neto, & Paschoalini, 2003). Also, VP neurons increased firing rate in response to sucrose infusion, suggesting that the magnification of glutamatergic inputs may contribute to the excitation of VP neurons (Tindell et al., 2006). Interestingly, μ-opioid agonist DAMGO, which facilitates hedonic 'liking' in the posterior VP, has been shown to modulate glutamatergic input by potentiating the excitatory effect (increasing signal to noise ratio), and GABAergic input by negating the inhibitory effects (decreasing signal to noise ratio) (Napier & Mitrovic, 1999). Taken together, these results suggest that DAMGO may enhance 'liking' via facilitating glutamate effects and suppressing GABA effects in the VP, which in turn result in an increase of firing rate of the VP neurons. It requires future studies to clarify whether VP glutamate is involved in 'liking' aspect of rewards. Interesting, an interaction between GABA and glutamate may exist in VP. For example, a single dose of PCP (NMDA antagonist) changes the BZD GABA binding in VP. While results from the current dissertation suggest that GABA flips 'liking' into 'disliking', it is still unclear whether glutamate is involved in the

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affective aspect of reward, or how excitatory glutamatergic signal and inhibitory GABAergic signal in the VP interact to mediate 'liking' and 'disliking' of reward.

Conclusion

The question of how pleasure and aversion are generated in the brain is to a considerable extent about how hedonic and aversive gloss are painted onto mere sensations. This dissertation uses modern experimental tools, such as microinjections, Fos plume mapping techniques, in combination with behavioral taste reactivity test, to reveal the first evidence of an aversive 'disliking' site mediated by GABA neurotransmission in posterior VP (Figure 5-1). In addition, two other neurotransmitter signals (orexin and anandamide) are identified to mediate hedonic 'liking' aspect of reward at a site that highly overlaps with the opioid hedonic hotspot that was previously identified (Figure 5-1). Therefore, these studies provide a demonstration of how 'liking' and 'disliking' are processed in limbic system, and provide potential mechanisms for reward- related disorders in humans, such as obesity, depression, additions, etc.
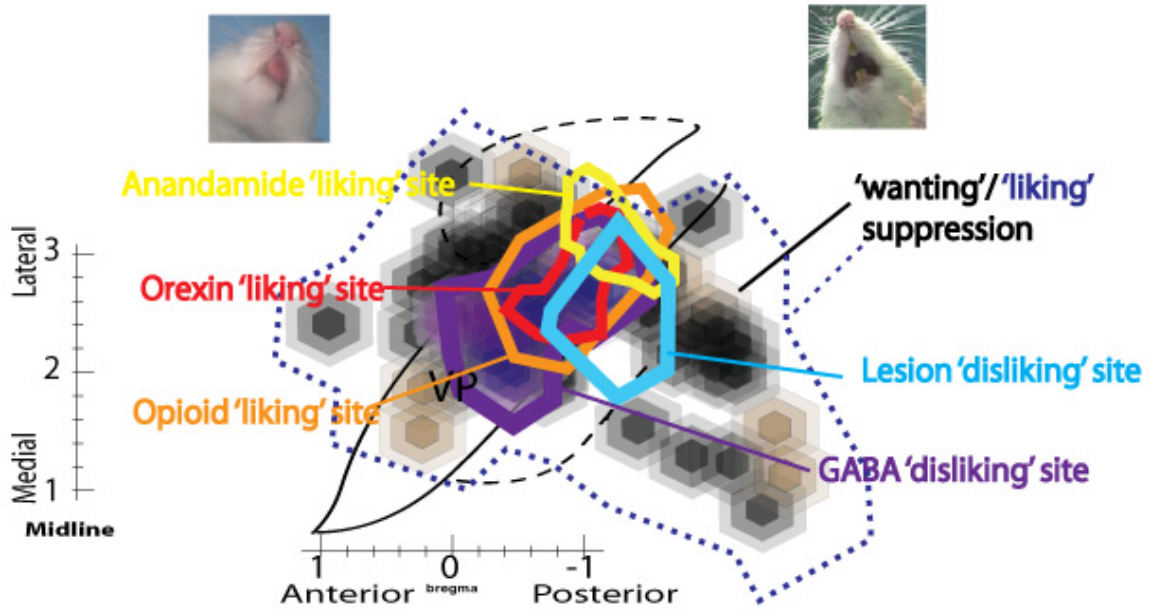
Figure 5-1 Summary of hedonic hotspots and aversive sites. GABA/lesion 'wanting' suppression site and GABA/lesion 'liking' suppression site encompasses the largest area, including the caudal VP, sublenticular amygdala, and lateral hypothalamus. Within this larger loss of 'wanting' and loss of 'liking' area, opioid hedonic hotspot (orange), anandamide hedonic hotspot (yellow), and orexin hedonic hotspot (red) highly overlap with GABA 'disliking' area and lesion 'disliking' area.

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