Comparison of Endocannabinoid and Orexin Hedonic Hotspots for Sensory Pleasure in the Ventral Pallidum

by

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Abstract

The ventral pallidum (VP) is part of the mesolimbocortical pathway involved in reward 'liking' and 'wanting.' Recent studies demonstrate that anandamide and orexin are involved in food intake and reward, and that the VP receives direct endocannabinoid and orexin signals from the nucleus accumbens and lateral hypothalamus. To test whether endocannabinoids and orexin mediate hedonic circuits in the VP, we assess behavioral taste reactivities ('liking') and appetitive food intake ('wanting') after drug microinjection. To indicate the spread of drug, the behavioral functions are mapped onto the Fos plume map; neurochemical maps of 'liking' and 'wanting' can thus be constructed. Based on the functional mapping procedure, our data indicates that hedonic hotspots may be located in the posterior VP, in which microinjections of anandamide and orexin amplify the natural 'liking' reactions to sweetness.

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Why do most individuals favor sweet over bitter flavors? What is the fundamental factor that determines pleasure and aversion? These questions are important to address, since a variety of eating disorders and compulsory behavior disorders (e.g. drug addiction, compulsive gambling) are associated with disrupted sensations of 'liking' and 'wanting'. While 'liking' refers to the pleasurable sensation and hedonic impact of an unconscious reward, 'wanting' refers to the motivation for reward that comprises unconscious incentive salience or desires for cognitive goals (Berridge & Kringelbach, 2008). 'Liking' and 'wanting' are two dissociable elements of reward as exemplified in the incentive-sensitization theory of drug addiction. To shed light on the psychological brain functions in behavioral disorders, the understanding of those two major psychological components of reward—'liking' and 'wanting'—is the first priority.

Ventral Pallidum

The reward circuitry of the mammalian brain consists of synaptically interconnected neurons associated with the medial forebrain bundle (Hernandez et al., 2006). Within this mesolimbocortical reward system, the ventral pallidum (VP) is identified as a convergent point for limbic reward signals. Thus, it earns its name as a "limbic final common pathway" for mesolimbocortical processing of many rewards (Smith et al., 2009). The original notion

of VP serving only as a motor expression is remodified to incorporate its reward and motivation functions. Not only does VP translate the limbic motivation signals into motor output, it also mediates reward and motivation in a more complex limbic-related anatomical circuitry. First, it receives reward signals from various brain structures such as orbitofrontal, prefrontal and infralimbic cortex, amygdala, lateral hypothalamus, ventral tegmental area, parabrachial nucleus, and subthalamic nucleus. Then, it conversely projects the signals back to almost all of its input structures. Further, it relays the output signals to the corticolimbic loops via direct projections to medial prefrontal cortex, and dense projections to mediodorsal nucleus of thalamus, which eventually transmits the signals to prefrontal cortex (Smith & Berridge, 2005; Smith et al., 2009). Therefore, by manipulating neurotransmitter release in the VP, the brain can decide when to intensify the impact of rewarding external cues (Pećina et al., 2006).

Cannabinoids

Endocannabinoids are a group of retrograde neurotransmitters that mediate their effects through cannabinoid receptors. Naturally there are exogenous and endogenous cannabinoids. Exogenous cannabinoids (such as delta-9-tetrahydrocannabinol (Δ9-THC)) are active constituents of marijuana, which has been used for centuries as recreational drugs and medicinal agents. So far, two major endocannabinoids derived from arachidonic acid have been identified: anadamide and 2-arachidonoylglycerol (2-AG) (Yates & Barker, 2009;

Muccioli, 2010).

The functions of cannabinoids are shown to result from the actions on cannabinoid receptors CB1 and CB2 (Maileux & Vanderhaeghen, 1992; van der Stelt & Marzo, 2003; Villares, 2007). CB1 and CB2 are Gi/o-coupled receptors that can inhibit the second messenger adenylyl cyclase and stimulate mitogen-activated protein kinase. CB1 is predominantly expressed in the brain, and CB2 is primarily found on the cells of the immune system. Even though CB1 is the major receptor involving in the neuroactive functions of cannabinoids, CB2 receptors have also been reported to occur on glial cells and neurons in the ventral striatum (Gong et al., 2006). Cannabinoids have been documented to contribute to a range of physiological systems, including cognitive function, control of eating behaviors, regulation of appetite, neuroprotective and immune suppressive properties (Lupica et al., 2004; Pagotto et al., 2006; Fernández-Ruiz et al., 2010; Roser et al., 2010).

Cannabinoids have been shown to have rewarding effects and an ability to stimulate increases in food intake (Pagotto et al., 2006). Systemic administration of Δ9-THC is shown to cause an increased intake of sweet foods such as sucrose (Koch & Matthews, 2001; Jarrett et al., 2005). Anandamide can also stimulate eating behavior and food intake (Pagotto et al., 2006). Recently the roles of cannabinoids in the regulation of appetite and body weight have raised great interest (Kirkham, 2005). It is now confirmed that endocannabinoids, acting at brain CB1 cannabinoid receptors, stimulate appetite and ingestive behaviors partly through

interactions with orexigenic signals (Crespo et al., 2008). Both the nucleus accumbens and hypothalamic nuclei are sensitive sites for the hyperphagic actions of these substances.

Studies have indicated that the functional interaction and integration between cannabinoids and other neurotransmitters are important in reward. In this aspect, CB1 receptors are located on GABAergic presynaptic axons in the nucleus accumbens shell (Mátyás et al., 2006) and are often co-localized with opioid receptors at the same synapses in the striatum. In addition, by activating presynaptic cannabinoid CB1 receptors cannabinoids can reduce glutamate release in the dorsal and ventral striatum (nucleus accumbens) and alter synaptic plasticity, thereby modulating neurotransmission in the basal ganglia and in the mesolimbic reward system. Interactions have been demonstrated between the endogenous cannabinoid and opioid systems in 'liking' reward: CB1 and μ-opioid receptor type 1 (MOR1) co-localize in the same presynaptic nerve terminals and signal through a common receptor-mediated G-protein pathway, suggesting the additive effects of endogenous opioid and cannabinoid systems in the regulation of appetite (Tallett et al., 2008). Both cannabinoids and opioid can induce taste hedonics of reward within the ventral striatum (Kelley et al., 2002).

Orexin

The orexins (also called hypocretins) are recently discovered hypothalamic neuropeptides that are synthesized by neurons located mainly in the perifornical area of the

posterolateral hypothalamus, a region classically implicated in feeding behavior (Tsujino & Sakurai, 2009). Two kinds of orexins, orexin A (OXA) and orexin B (OXB), have so far been found. They act through two subtypes of receptors (OX1R and OX2R) that belong to the G protein-coupled receptors superfamily. Both receptors have been shown to be widely distributed in brain regions where orexin neuron projection has been discovered (Laburthe et al., 2007)

Orexin A and orexin B appear to play a role in various distinct functions such as energy homeostasis, appetite and regulation of feeding, reward, gastric secretion, arousal and the sleep-wake cycle (Tsujino & Sakurai, 2009). In the regulation of food intake, studies demonstrate that cannabinoid and OXA share a common mechanism. Hypothalamic orexigenic circuits are involved in cannabinoid CB1 receptor antagonism-mediated reduction of appetite (Crespo et al., 2008). Orexin deficiency causes abnormalities in energy homeostasis and reward systems. Studies suggest that the orexin system interacts with systems that regulate reward and energy homeostasis to maintain proper vigilance states. Recent studies suggest that OXA has a more robust stimulatory effect on energy expenditure relative to OXB. Further, OX1R predominately mediates behaviors known to influence energy expenditure (Teske et al., 2010). Therefore, orexin's role in mediating reward and energy homeostasis may be a potentially important therapeutic target for treatment of obesity and drug addiction (Tsujino & Sakurai, 2009).

Hedonic Hotspots

Recent studies have shown that pleasure 'liking' reactions are coordinated by a network of hedonic hotspots distributed across subcortical brain regions, including the rostral-dorsal quadrant of the nucleus accumbens shell, the posterior half of VP, and the parabrachial nucleus in the pons (see Figure 1). It is the weighted combination of sensory signals in those distributed hotspots that forms the functional integrated circuit regulating pleasure. An analogy, according to Berridge & Kringelbach (2008), is several scattered islands that form a single archipelago. While each hotspot is capable of enhancing hedonic 'liking' reactions when opioid, endocannabinoid, or related neurochemical receptors in these hedonic hotspots are activated, together, they obey the hierarchical and unanimous rules to control pleasure generation in the brain.

In 2005, Smith and Berridge endeavored to find the actual spot within the VP that mediates the hedonic impact of 'liking' and incentive motivation of 'wanting' by microinjecting drugs into the VP that could affect neurotransmitter release. They measured affective orofacial 'liking' during a taste reactivity test because the facial expressions in response to sweet and bitter flavors are similar between rodents and primates. The also scored 'wanting' by the amount of rat chow eaten and total duration of eating behavior in a food intake test.

The results showed that the μ-opioid agonist $_{D}$-Ala-*N*-Me-Phe-Glycol-enkephalin

(DAMGO) enhanced the pleasurable sensation of 'liking' the flavor of sucrose when microinjected in the posterior VP, but conversely suppressed hedonic impact when microinjected in the anterior and central region. In a similar fashion, DAMGO stimulated the 'wanting' of food when microinjected in the posterior and central VP, but suppressed eating duration when microinjected at the anterior region. On the other hand, when bicuculline methylbromide, a $GABA_A$ antagonist, was injected into the VP, it failed to enhance the sucrose 'liking' reaction at any site of this region, but instead enhanced the 'wanting' behavior at all sites of the VP. This result demonstrated that 'liking' and 'wanting' are related but separable since the same drug (either DAMGO or bicuculline methylbromide) could elicit different activation sites for pleasure sensations ('liking') and eating behavior ('wanting') in the VP. Moreover, the discovery that there was a gradual increase in 'liking' reactions following injections towards the posterior VP created a 'liking' anteroposterior gradient, and verified the existence of a 1 mm^3 opioid hedonic hotspot located in the posterior VP (Smith & Berridge, 2005).

In addition to the finding of an opioid hedonic hotspot, an endocannabinoid hotspot has also been found in the medial shell of the nucleus accumbens (Mahler et al., 2007). By microinjecting anandamide into the dorsal nucleus accumbens shell, the hedonic 'liking' response to sucrose more than doubled compared to that of the vehicle. Based on the Fos plume mapping, a 1.6 mm^3 endocannabinoid hotspot was revealed as located in the dorsal

half of the medial shell. Remarkably, this endocannabinoid hotspot overlapped with the previously found 1 mm^3 opioid hotspot in the dorsal rostral quadrant of nucleus accumbens medial shell (Smith & Berridge, 2005). This discovery implies that endocannabinoid and opioid signals might interact with each other and stimulate hedonic 'liking' through the same subcortical circuitry. 'Liking' might further increase the reward value of food since both anandamide and 2-AG stimulate food intake in the nucleus accumbens (Mahler et al., 2007; Williams & Kirkham, 2002). From those studies, the endocannabinoid hotspot in the nucleus accumbens serves as a sufficient cause, which amplifies the 'liking' signals, for pleasure generation in the brain (Berridge & Kringelbach, 2008).

So far, there are three known hedonic hotspots in the brain's subcortical structures that can cause pleasure, including the rostral-dorsal quadrant of nucleus accumbens shell, the posterior half of the VP, and the parabrachial nucleus in the pons. The hedonic 'liking' reactions to sweetness are greatly enhanced when opioid agonists are microinjected into the posterior half of the VP (Smith & Berridge, 2005). An endocannabinoid hotspot for sensory pleasure is also identified in the dorsal half of the nucleus accumbens shell (Mahler et al., 2007). Nevertheless, the role of endocannabinoids in mediating pleasure in the VP still remains elusive. It is likely that endocannabinoids may also enhance sucrose 'liking' in this area of the brain since the VP is the direct recipient of nucleus accumbens and endocannabinoid projections, and endocannabinoids have been reported to induce taste

hedonics and food intake in the brain (Kelley et al., 2002; Harkany et al., 2003). Moreover, studies have shown that orexin mediates food intake and drug reward in the lateral hypothalamus, which projects orexin not only to the nucleus accumbens but also to the VP, the convergent point for the limbic reward system. According to Crespo et al. (2008), hypothalamic orexigenic circuits are correlated with the cannabinoid CB1 receptor in food intake reward. Thus, orexin may play a role in reward regulation in the VP, and endocannabinoids and orexin may interact with each other during pleasure generation in the brain.

The main purpose of this study is to find whether endocannabinoids and orexin increase hedonic 'liking' when injected into the VP and to further compare the two 'liking' hotspots with Fos plume neurochemical maps. We hypothesize that microinjections of anandamide and orexin into the posterior VP will enhance the 'liking' facial expressions of rats during taste reactivity testing when exposed to sweet oral infusions. To test this hypothesis, two independent variables—the site of injections and type of drugs, are operationalized by measuring the location of upregulated immediate early gene expression following localized drug microinjections (Fos plumes). The corresponding dependent variables—'liking' and 'wanting'—are tested by the off-line video scoring of hedonic facial expressions (taste reactivity testing) and eating behavior (food intake testing) following microinjection. By integrating the results of behavioral functions and drug activation and spread through analysis

of Fos plumes, we should be able to construct a neurochemical map of 'liking' and 'wanting,' and further compare the endocannabinoid and orexin hotspot locations in the VP.

Methods

Subjects

Seventeen male Sprague Dawley rats (Harlan) weighing 350-550 g at the beginning of the experiment were used as our animal models. Thirteen rats participated in behavioral testing (experiment 1), while the remaining four were used for Fos-like protein immunohistochemistry (experiment 2). They were housed either doubly or triply in opaque tub cages covered with corn-cob bedding on a 12:12 h light-dark cycle. Room temperature was around 21℃, and rats were given *ad libitum* access to food (Purina Rat Chow; Purina Mills, St. Louis, MO) and water (tap water). All experimental procedures fell within ethical guidelines as determined by the University of Michigan's University Committee on Use and Care of Animals (UCUCA).

Experimental Design

Rats were divided into the behavioral group (experiment 1) and Fos analysis group (experiment 2) at the beginning of the experiment (see Figure 2). The behavioral group and Fos analysis group were run in parallel with the same experimental conditions so that we could map the results of behavioral effects ('liking' and 'wanting') in the VP based on the spread of Fos activation after drug microinjection. Both groups had the same surgical

coordinates for the intracranial cannulae and received drug treatment. While the behavioral group was scored by taste reactivity testing followed by food intake testing, the Fos analysis group was studied by generating Fos plumes, which measures zones of local neuronal activation around the injected site immediately after the microinjection. The day that the drug was microinjected was equivalent to test day one for behavioral testing. This split-and-recombine design facilitated the neuroanatomical and neurochemical mapping of 'liking' and 'wanting.'

The experiment encompassed both within-subjects and between-subjects designs. The split-group permitted a more accurate measurement of drug effects within-subjects since the placement of the oral and intracranial cannulae could differ slightly from subject to subject. The maximal drug effect could also be achieved without diminishing drug efficacy based on the "uncertainty principle," which states that the behavioral maximum and Fos maximum cannot be measured simultaneously in a repeated-measures experiment (Mahler et al., 2007). Moreover, the recombine-group allowed between-subject data integration of both behavioral and Fos analyses. Anandamide and orexin effects on the appetitive reactions ('liking') and on food intake ('wanting') could be projected onto the observed Fos plumes.

Experiment 1: Taste Reactivity and Food Intake Behavioral Testing

Surgery. All rats from the behavioral group were implanted with two oral cannulae [PE-100 tubing] for taste reactivity testing and two intracranial cannulae (23 gauge; stainless

steel) for drug microinjection into the VP. Before the surgery, rats were handled for 10 minutes, treated with 0.2 ml of atropine sulfate, and then anesthetized with 80 mg/kg ketamine HCl and 10 mg/kg xylazine. Bilateral oral cannulae were implanted starting from within the mouth lateral to the first maxillary molar, traveling beneath the zygomatic arch, and finally exiting the dorsal part of the head (Grill & Norgren, 1978; Grill & Berridge, 1985).

After oral cannulae implantation the rat was placed onto a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA) for the intracranial cannula surgery. Chronic bilateral microinjection guide cannulas were implanted into the VP (centered around Mediolateral, ± 3 mm; Anteroposterior, -0.8 mm; Dorsoventral, -5.5 mm (Paxinos & Watson, 1998) and anchored with four bone screws and acrylic cement. Stylets were inserted into the intracranial cannulae to prevent occlusion and infection. Rats were allowed at least seven days for recovery before the taste reactivity testing.

Drugs and VP microinjections. Anandamide (0.1 ml) (Tocris Bioscience, City, ST) was diluted to dose with artificial cerebrospinal fluid (ACSF) (0.7 ml). Similarly, Tocrisolve (Tocris) (0.1 ml) vehicle was diluted with ACSF (0.7 ml). The concentration of orexin A is 10 nM, and the vehicle used for orexin is ACSF. To acclimate rats to the testing environment, three days of habituation with mock water infusion were arranged before the actual test days. 0.2 µl ACSF was microinjected on the final habituation day to familiarize rats to the

microinjection procedure.

During microinjections, 16 mm stainless-steel microinjector tips (29 gauge) were inserted into the bilateral guide cannulae. PE-20 tubing connected the microinjector tips to a syringe pump, and rats were bilaterally injected with either drug (anandamide or orexin) or vehicle (Tocrisolve or ACSF) at an infusion rate of 0.20 µl/min. Microinjector tips were left in place for 1 min after the injection to allow for diffusion away from the injection center. Stylets were reinserted into the microinjection guide cannulae and the rat was transferred to the testing chamber for taste reactivity testing.

Only one drug or vehicle was given per test day, and each test date was at least 48 h apart. To prevent the damage of brain tissue (gliosis), a maximum of four drug injections was conducted within the same behavioral group subject. The drug or vehicle order was counterbalanced to avoid order effects.

Taste Reactivity Testing. The taste reactivity testing followed 30 min after the drug microinjection since previous studies from the pilot group indicated that orexin reaches its maximum effect after a 30 min interval and anandamide-induced hedonic enhancement was similar between 15 and 45 min after microinjection (Mahler et al., 2007). Before the testing, a 1 ml syringe with 1.0 %, 0.03 M sucrose solution was connected to one side of oral cannulae via a hollow tube (PE-50 connected to a PE-10 delivery nozzle). After the set-up, the rat was placed onto a transparent floor surrounded by transparent cylindered walls. An angled mirror

was situated underneath the transparent floor so that experimenters could video record the taste reactivity response. During the 1 min taste reactivity testing session, a total of 1 ml of sucrose was infused gradually into the rat, and the video camera captured the close-up orofacial expressions of the rats' response.

Food Intake Testing. The food intake testing occurred immediately after the taste reactivity testing. The experimental setup included a 23 × 20 × 45 cm transparent tub cage covered with corn-cob bedding that was similar to the home cage, and a video camera placed in front of the cage that recorded the rat's behavior. Each cage contained a single rat, and premeasured food pellets and water were available *ad libitum*. After 60 min of testing, leftover food pallets and water were measured again in order to calculate the amount of food (g) or water (ml) intake.

Taste Reactivity Video Scoring. The hedonic, aversive, and neutral orofacial expressions captured during taste reactivity testing were scored off-line in slow motion (1/30 frame by frame to $1/10^{th}$ actual speed) based on an established behavioral classification and time bin scoring procedures (Grill & Berridge, 1985; Berridge, 2000). Hedonic responses included rhythmic midline tongue protrusions, lateral tongue protrusions, and paw licks. Aversive responses included gapes, head shakes, face washes, forelimb flails, and chin rubs. Neutral responses, which are less consistently linked to hedonic/aversive taste valuation, included passive dripping of solution out of mouth, ordinary grooming, rhythmic mouth

movements, rearing, yawning, head scratching, doing nothing, and off-screen. The analysis was conducted using Noldus Observer 8.0 software, and all scorers were blind to the microinjection condition.

Based on a time bin scoring procedure, the various taste reactivity components were tallied either as discrete events (one event equals one occurrence) or 2s/5s time bins (up to 2s/5s continuous bout duration equals one occurrence) to balance responses of relative frequency (Berridge, 2000). Discrete events included hedonic lateral tongue protrusions, aversive gapes, head shakes, forelimb flails, chin rubs, and neutral yawning. Other components with shorter bouts were tallied as 2s time bins with minimum continuous bout duration of 1/2 second, while those with longer bouts were tallied as 5s time bins with minimum continuous bout duration of 1s. Based on this criterion, hedonic tongue protrusions were counted as 2s time bins, and hedonic paw licks, aversive forelimb flails, neutral passive dripping, ordinary grooming, rhythmic mouth movements, rearing, head scratching, doing nothing, and off-line were counted as 5s time bins. The total of hedonic ('liking'), aversive ('disliking'), and neutral orofacial responses were summed for each rat.

Histology. The behavioral group was sacrificed after the last day of behavioral testing. Rats were deeply anesthetized with sodium pentobarbital and their brains removed. Brains were preserved in 10% paraformaldehyde for 3 days, transferred to a 30% sucrose solution for 1-3 days (until the brains sank), coronally sectioned into 60 μm slices, immersed in 0.2 M,

pH 7.4, sodium phosphate buffer (NaPB) solution, and then mounted on slides. Slides were then stained using cresyl violet dye to confirm cannula placement. Thus, three dorsoventral, mediolateral, and anteroposterior coordinates of the microinjection tip were retrieved.

Experiment 2: Fos Plume Analysis of Local Impact

All rats from the Fos analysis group were handled for 2 days for 10 min each day. They followed the same surgical and drug microinjection procedure as the behavioral group except the implantation of the bilateral oral cannulae. For the drug and vehicle animals, only the bilateral intracranial cannulae with the same coordinate as the behavioral group was implanted. For the normal sham animals, neither the oral nor the intracranial cannulae were implanted.

Fos-Like Protein Immunohistochemistry. The Fos analysis group was deeply anesthetized with sodium pentobarbital (0.2 g/kg) 90 min after VP microinjections with anandamide and Tocrisolve. The 90 min was chosen based on the previous finding that translation of *c*-fos mRNA to Fos protein is maximal between 60 and 120 min (Muller et al., 1984). After being transcardially perfused, the retracted brain was preserved in 4% paraformaldehyde for a day and then transferred to 30% sucrose for 3 days. Then, the brain was coronally sectioned into 40 μm slices and immersed in 0.1 M, pH 7.4, NaPb solution. Brain slices from the Fos group were prepared for Fos-like protein immunohistological staining.

On the first day of the immunohistological staining, one set of brain sections was stored in the cryoprotectant, while the alternative set underwent the immunohistology staining. The set that underwent the staining was first rinsed with 0.1 M NaPB two times, followed by another rinse with 0.1 M NaPB plus 0.2% Triton X-100. After the third rinse, the brain slices were pre-blocked in 5% normal donkey serum (NDS) and NaPB plus 0.2% Triton X-100. Brain slices were then transferred into a primary antibody solution (5% NDS and goat anti-c-Fos (1:10, Invitrogen)) and incubated overnight at 4ºC. Notice that during all rinses, pre-blocks, and antibody incubations, the tissue was gently agitated on a laboratory shaker. Rinses all lasted for 10 min, and pre-blocks all lasted for 30 min.

After the primary incubation, brain slices were again rinsed with 0.1 M NaPB plus 0.2% Triton X-100 three times, and then pre-blocked in 5% NDS and NaPB plus 0.2% Triton X-100 plus 2 drops of Image-FX Signal Enhancer (Invitrogen). Then, the tissue was covered to protect from light, and incubated in secondary solution (5% NDS, donkey anti-goat Alexa Fluor 488 (excitation, 488 nm; emission, 519 nm; Invitrogen), and 2 drops of signal enhancer) for 2 h. After incubation, tissues were rinsed twice in 0.1 M NaPB plus 0.2% Triton, followed by a final rinse with 0.1 M NaPB. Brain sections were then mounted, air-dried, and coverslipped with ProLong Gold antifade reagent (Invitrogen).

Fos Plume Maps of Drug-induced Local Activation Spread. Fos plumes were imaged on a Leica optical microscope using a L5 filtercube (excitation band at 480-505 nm and an

emission band at 505-545 nm). A seven-armed grid with boxes spaced 125 μm apart emanating radially in seven directions (45, 90, 135, 180, 225, 270, 315°) was placed on each image, with the center of the grid slightly under the microinjection center (as identified by the presence of damage by the microinjector tip). The number of immunofluorescent cells in each 125 ×125 μm block was tabulated and used to calculate an estimated plume size.

Similar to previous studies, we compared the anandamide Fos plumes to either the vehicle-induced baseline Fos expression or the normal "virgin tissue" that was not damaged by surgical intrusion or gliosis (Pećina & Berridge, 2000, 2005; Smith & Berridge, 2005). Each anandamide Fos plume was mapped as intense (greater than or equal to 3x) or moderate (greater than or equal to 2x and below 3x) zones based on different elevation degrees of Fos expression compared to the vehicle and normal level; the spread of each level of expression (3x and 2x) from the injection center to the last box in each radial arm was averaged to derive the radius, assuming a spherical shape of functional drug spread. Concentric hexagon symbols based on intense and moderate Fos plume radii represented the spread of drug activation around the microinjection site.

Mapping Procedure of Microinjection Effects for Localization of Function.

Neurochemical maps were generated by recombining the behavioral group data (hedonic responses, aversive responses, and food intake) with the Fos data. Each behavioral function was compared with its baseline vehicle activity, and a color representing the percentage

change was assigned to each hexagon symbol of the microinjection site. Thus, each plume symbol in a map illustrated three identities: the location of microinjection site for each behavioral group rat, the intensity of behavioral effects of drugs on 'liking,' 'disliking,' and 'wanting' on that rat, and the size of the local neuronal activation elicited by drug (Mahler et al., 2007). Bilateral cannulae microinjection sites were plotted on corresponding coronal slices and the bilateral cannulae for each rat were collapsed into one unilateral map of sagittal and horizontal slices. Such three-dimensional depiction provided information of Fos plume position in the brain and the location of functional hotspots (Paxinos & Watson, 1998).

Statistics. All statistics were run on SPSS 17.0 software and α was always set at $p \leq 0.05$. To describe anandamide and orexin behavioral effects as percentage increase or decrease over vehicle levels, a constant value of 1 (hedonic and aversive responses) and 0.1 (food intake) was added to every datum to avoid the problem of having zero in the denominator. Paired samples t-tests were used to test drug effects on hedonic, aversive, and eating behavior. Between-subjects ANOVAs were used to determine anatomical location effects of microinjection sites (hotspot vs non-hotspot).

Results

Fos Plume Mapping

Fos plumes indicated the spread and intensity of cellular activation as a result of drug administration. Previous studies reasoned that drug-induced local Fos activation might either

be stimulated directly by neurons with corresponding receptors, or indirectly by adjacent neurons that in turn activate nearby Fos-expressing neurons via local circuits (Mahler et al., 2007). Such reasoning along with the spherical spreading of drugs helped to generate a Fos plume map that indicated the likely areas of behavioral effects after drug microinjections.

To achieve a statistically significant Fos plume radius, the typical Fos plume analysis requires 3-5 drug-injected animals, 4-6 vehicle-injected animals, and 3-5 normal (sham) control animals (Mahler et al., 2007; Reynolds & Berridge, 2008). Due to limited time constraint, only 4 animals were run for the Fos immunohistochemistry: 2 with anandamide microinjections, and 2 with Tocrisolve microinjections. Additional 2 sham animals were contributed from my mentor Chao-yi Ho with my consistent Fos analysis for number of activated cells. Orexin Fos plume radii were also given by my mentor to facilitate the Fos plume mapping of my orexin behavioral data.

Microinjections of anandamide produced an inner intense zone with a >3x elevation of Fos expression, and an outer moderate zone with a >2x elevation of Fos activation over both vehicle and normal VP levels. For the anandamide group compared with the normal animals, the mean Fos plume radius was 0.063 ±0.021 mm for the 3x elevation, and 0.175 ± 0.035 mm for the 2x elevation. On the other hand, the vehicle group induced a greater level of Fos expression compared to the normal group since vehicle injection pressure and cannula-related damage were all contributing factors for cellular activation. Therefore, the average Fos plume

radius of 3x elevation over Tocrisolve was 0.047 ± 0.011 mm, and the average Fos plume radius of 2x elevation over Tocrisolve was 0.163 ± 0.031 mm. The Fos plumes using the vehicle group expression as the basal line cast a ceiling on the anandamide/orexin induced Fos expressions. To avoid the underestimation of the drug effect, we chose to compare the drug elevation over the normal Fos plume radius based on the Mahler paper (Mahler et al., 2007).

Orexin radii were calculated using the same analysis method mentioned above. Compared to normal sham animals, the average Fos plume radius with orexin microinjection was 0.065 mm for 3x elevation, and 0.160 mm for 2x elevation. The results were similar to the anandamide radii derived above (see Figure 3).

Anandamide Enhances Sucrose Hedonic Impact

Among the 13 behavior group rats, 2 rats were excluded from the data analysis due to cannula misplacement outside of the VP. The remaining 11 rats showed a significant increase, $t(10) = 3.22$, $p = 0.009$, in hedonic responses to sucrose when microinjected with anandamide, $M = 31.09$, $SE = 1.43$, compared to the vehicle level, $M = 22.36$, $SE = 2.35$. In comparison, aversive responses to sucrose after anandamide microinjection, $M = 1.27$, $SE = 0.51$, were significantly reduced, $t(10) = -3.06$, $p = 0.012$, compared to the vehicle level, $M = 4.63$, $SE = 1.11$. Anandamide microinjections in the VP caused an average 26% increase in the number of positive hedonic reactions to sucrose compared to control levels (vehicle = 100%; see

Figure 4), and an average 72% decrease in the number of negative aversive reactions to sucrose compared to control levels (vehicle = 100%; see Figure 5). Anandamide amplified the total number of positive hedonic reactions elicited by sucrose, especially the tongue protrusions component, $t(10)= 2.48$, $p=0.032$, while it significantly decreased the total aversive reactions to sucrose as mentioned above.

Orexin Enhances Sucrose Hedonic Impact

Only 9 of 13 rats in the behavioral group were used for orexin taste reactivity analysis, as 2 rats had cranial cannula placements outside of the VP and 2 rats did not receive orexin on test days. Similar to the anandamide effect, the remaining 9 rats showed a significant increase, $t(8) = 2.89$, $p = 0.020$, in hedonic responses to sucrose when microinjected with orexin, $M = 32.33$, $SE = 1.47$, compared to the vehicle, $M = 25.89$, $SE = 2.33$. Nevertheless, unlike anandamide, orexin had no effect on aversive responses, $M= 2.44$, $SE = 0.90$, $t(8)= -0.62$, $p=0.551$, compared to that of the vehicle, $M= 3.33$, $SE = 1.43$. Orexin microinjections in the VP caused overall increases of 24% in the number of positive hedonic reactions to sucrose compared to control levels (vehicle = 100%; Figure 6), but had no effect (the 26% decrease was not significant based on the paired t-test) on the number of negative aversive reactions to sucrose compared to control levels (vehicle = 100%; Figure 7). Orexin selectively amplified the total number of positive hedonic reactions elicited by sucrose, especially the lateral tongue protrusion component, $t(8) = 3.23$, $p = 0.012$.

Hedonic Maps for 'Liking' Enhancement and Hotspot Focus in Posterior VP

A neurochemical map was generated by superimposing the 'liking' enhancement from the behavioral group onto the spread of Fos plumes for drug microinjections. This split-and-recombine design showed that both anadamide and orexin enhanced positive hedonic responses of rats, and that there seemed to be intensive hedonic increase zones located at the posterior VP for both anandamide and orexin. Since the number of anandamide microinjected subjects was more than that of orexin, there was a more concentrated hedonic hotspot found for anandamide. The current anandamide and orexin hotspots were outlined on top of the Fos plume maps for hedonic 'liking' (see Figure 8, 9). The total numbers of hedonic responses were compared between areas inside and outside the hotspot. One-way ANOVA was used to compare the hedonic 'liking' difference between the hotspot group and the non-hotspot group.

Two of the anandamide rats showed a more than 60% increase in the number of positive hedonic responses compared to the tocrisolve vehicle level (120% and 180% increases respectively). A one-way ANOVA test comparing the percentage of hedonic enhancement between such hotspot sites and sites outside of the hotspot demonstrated that 'liking enhancement' was significantly higher in this potential hotspot, $F(1, 11) = 51.20$, $p = 0.000$. The hotspot group exhibited a 129% increase in total hedonic responses to sucrose, while the non-hotspot group yielded only a 15% increase. A particular effective endocannabinoid

hotspot for enhancing 'liking' reactions to sucrose might exist in the posterior VP (see Figure 8).

Similar to anandamide group, a hotspot outline was chosen for the orexin group. Nevertheless, since the number of orexin subjects was less than that of anandamide group, a 30% hedonic increase was chosen as a minimum hotspot standard for the orexin group. Three of the orexin rats showed a relative intense 'liking' enhancement compared to the non-hotspot group: 32%, 35%, and 157% respectively. One-way ANOVA test demonstrated that 'liking' enhancement was significantly greater within the hotspot, $F(1, 9) = 6.01$, $p = 0.037$. On average, hotspot group had a 61% increase in total hedonic responses to sucrose, while the non-hotspot group almost showed no increase (1% in 'liking' enhancement). The current orexin hedonic hotspot outline sets up a stage for further miniature hotspot identification (see Figure 9).

Anandamide Had No Effect on Eating Behavior and Food Intake

Microinjections of anandamide into the VP had no effect on the food intake amount compared to that of the vehicle (see Figure 10). The total food intake for the rats microinjected with anandamide, $M = 1.43$ g, $SE = 0.47$, $t(10) = -0.03$, $p = 0.974$ was almost equivalent to the control, $M = 1.45$ g, $SE = 0.53$.

Orexin Had No Effect on Eating Behavior and Food Intake

Microinjections of orexin into the VP had no effect on the food intake amount compared

to that of the vehicle (see Figure 11). The total food intake for the rats microinjected with orexin , M= 1.22 mg, SE = 0.56, $t(8) = 0.15$, $p = 0.883$, was almost identical to the control, M= 1.11 mg, SE = 3.33.

Discussion

Our preliminary results support the hypothesis that microinjections of anandamide and orexin into the posterior VP enhance the 'liking' facial expressions of rats during oral infusion of sweet solutions. The hedonic 'liking' enhancement is specifically evident in the posterior part of the VP, overlapping with a previously found 1 mm^3 opioid hedonic hotspot. Nevertheless, both drugs seemed to have no effect on 'wanting' motivation during food intake. Therefore, our findings suggest that endocannabinoids and orexin may transmit 'liking' signals within the same VP hedonic circuit, along with the opioid hotspot and other neurochemicals, regulating natural sensory reward in the brain.

Anandamide Enhances Taste 'Liking' and Hedonic Hotspot within the Posterior VP

Based on the Fos plume mapping, anandamide stimulates 'liking' enhancement in the VP. The average 26% increase of hedonic 'liking' responses to sucrose in the VP demonstrates that endocannabinoids can enhance the 'liking' value of a natural reward, thus verifying that VP is a sufficient cause for pleasure generation in the brain and that it contains the hedonic hotspot responsible for the observed endocannabinoid enhancement of 'liking' for sweetness (Berridge & Kringelbach, 2008). Conversely, anandamide significantly decreases the aversive

responses elicited by sucrose. The negative aversive responses to sucrose might be due to subject's maladjustment to the oral cannula even after three days of habituation or mild distress during the experimental testing. As expected, with anandamide microinjections this aversion is significantly reduced as reflected by the decrease of rats' aversive orofacial responses during the taste reactivity test. The anandamide-induced 'liking' enhancement might help to explain the "rich get richer" form of reward amplification: that is, subjects dramatically amplify the most pleasant reward, while other less liked stimuli remain relatively unchanged. A similar analogy to this concept in the real world is humans' selection of sweet or high fat foods over less-palatable foods (Koch & Matthews, 2001).

Furthermore, the positive hedonic responses climax at the posterior VP localized hotspot, which is consistent with the previously found opioid effect: DAMGO causes increased hedonic 'liking' reactions to sucrose only in the posterior VP, but conversely suppressed 'liking' reactions in the anterior and central VP (Smith & Berridge, 2005). Based on the neurochemical anandamide Fos plume map, we operationally define an endocannabinoid hedonic hotspot at in the posterior VP, where an > 120% 'liking' enhancement to sucrose is observed (see Figure 8). Altogether, the result indicates that the posterior VP contains a particularly potent hotspot for endocannabinoid magnification of hedonic sweet reward.

This endocannabinoid hotspot overlaps with the $1mm^3$ opioid hotspot located in the posterior VP, which parallels the finding in nucleus accumbens (Smith & Berridge, 2005;

Mahler et al., 2007). Therefore, endocannabinoids and opioids may interact with each other in the same hedonic circuit in both the nucleus accumbens and VP if colocalization occurs in hotspot neurons. Postsynaptic anandamide may travel retrogradedly to presynaptic axon terminals and activate CB1 receptors on the membrane. Opioids released by the presynaptic neurons might simultaneously recruit endocannabinoid release at the postsynaptic spiny neuron (Piomelli, 2003; Cota et al., 2006; Kirkham, 2008). Such possibility of the receptor mechanism illuminates how endocannabinoid and opioid neurochemical signals in the VP interact to enhance 'liking' reactions to natural sensory pleasure.

Orexin Enhanced Taste 'Liking' and Hedonic Hotspot within the Posterior VP

Similar to endocannabinoids, orexin also enhances hedonic 'liking' in the VP with average 24% intensity to the vehicle. Such finding substantiates the VP role as a sufficient cause of pleasure, where the reward function is enhanced by activation of a brain system (Berridge & Kringelbach, 2008). When microinjecting orexin specifically in the posterior hotspot region, the positive hedonic impact is intensely enhanced (an average 60% increase compared to 1% 'liking' enhancement of the vehicle). A preliminary orexin hedonic hotspot is operationally outlined in the posterior VP (see Figure 9). By comparing the current result to those that are previously published, the current hotspot outline overlaps with the previous findings (Ho & Berridge, 2009), rendering our experimental results reliable. On the other hand, orexin does not significantly decrease aversive responses elicited by sucrose as shown

in the endocannabinoid case. The reason might be due to sucrose's role as a prototypical sweet reward, which does not induce great aversive responses in neither the vehicle nor the orexin animals. Thus, the aversive decrease in orexin rats is rendered insignificant.

The finding that orexin enhances 'liking' for sweet taste supports orexin's role in hunger and reward, in which a "pleasure gloss" is presumably painted on the food stimulus to increase the appetite. The projection of hypothalamic orexin neurons to the posterior VP hedonic hotspot may even account for why palatable foods taste even better in the hunger state, a hedonic plasticity shift called "alliesthesia" (Ho & Berridge, 2009).The anatomical overlap between the orexin, endocannabinoid, and opioid hotspots in the posterior VP further suggests that those neurotransmitters may mediate pleasure through the same subcortical hedonic circuit. Together with the direct projection of endocannabinoid and opioid signals from their overlapped nucleus accumbens hotspot, the role of VP as a "limbic final common pathway" for reward mediates the hedonic and motivational signaling in the subcortical brain.

Food Intake and 'Wanting'

Central endocannabinoid and orexin systems have been implicated in inducing appetitive motivation and food intake. Cannabinoid receptor agonists have shown to induce hyperphagia, and orally administered cannabinoids have been found to promote eating by increasing the incentive value of food (Williams & Kirkham, 2002). Similarly, administration of orexin stimulates food consumption, and orexin signaling in VTA induces high-fat food

intake (Cason et al., 2010). Recent studies establish a role for endocannabinoid and orexin in reward-based feeding by increasing the appetitive aspects of feeding motivation.

Nevertheless, contrary to previous studies demonstrating that orexin increases feeding, neither anandamide nor orexin promote appetitive 'wanting' for food in our experiment. This inconsistency with literature implies that some factors might interfere with the food intake testing. As Thorpe & Kotz (2005) suggested in their previous papers, age and weight of experimental subjects are crucial factors in the feeding test. They have consistently observed less spontaneous activity in older animals. In this aspect, our experimental subjects are older and slightly overweight compared to normal. Another factor is the time course of drug effect. In fact, in the previous food intake testing conducted in our lab, Smith and Mahler both performed testing directly after the drug microinjection instead of having a 30 minute taste reactivity testing first (Smith & Berridge, 2005; Mahler et al., 2007). Mahler et al. (2007) also pointed out that the anandamide effect on hedonic enhancement is robust within 15-45 min after drug microinjection. Our one hour food intake testing was conducted 30-90 min after anandamide microinjection. Thus, the drug effect might not be maintained throughout the course of the experiment and might even diminish towards the end of the food intake test. Future research with a separate testing group for food intake might help us to elucidate the actual 'wanting' effect after drug microinjection. If food intake result remains insignificant, then there is dissociation between 'liking' and 'wanting' as suggested in the incentive

sensitization theory of drug addiction: Sensitized 'wanting' compels drug pursuit in drug addict regardless of cognitive expectancy of reward 'liking' (Robinson & Berridge, 2003).

Future Directions

More experimental subjects in the future can help us elucidate more detailed anandamide and orexin hotspots in the posterior VP. First, not all of the VP regions are covered by microinjection placements in our studies. With more microinjections in the posterior and anterior VP, more focused hedonic hotspots in the posterior VP and a potential "cold spot" in the anterior VP could be found (Mahler et al., 2007). Secondly, due to limited time constraint, all of our current Fos plume images from the sham group are surgically-operated separately and only 4 animals are run for the anandamide and vehicle group (the ideal image number should be 3-6 animals each group). In addition, some of the Fos images are subject to background interference. Non-specific binding impedes the accurate positive Fos counts, and may decrease the accuracy of the plume radii derived. Thus, a higher number of subjects completing the testing regimen can facilitate our Fos plume analysis, and construct a higher resolution neurochemical map. Last but not least, to gain a better drug effect on food 'wanting,' running a separate group for food intake is expected. By choosing appropriate subjects and a testing time course with maximal drug effect, drug induced 'wanting' enhancement might be observed. The two components of reward—'liking' and 'wanting' might be related but dissociable in the endocannabinoid and orexin hedonic

circuit of the VP.

In addition to the limitation of subject numbers discussed above, additional methodological change might illuminate the gray area of the hedonic circuit. In addition to our findings of an endocannabinoid and orexin hotspot in the VP, other opioid hedonic hotspots have been found within the subcortical regions of the brain, such as nucleus accumbens and the parabrachial nucleus in the pons (see Figure 1). It will be interesting in the future to search if there are more endocannabinoid and orexin hotspots even in the cortical brain such as the orbitofrontal cortex, which has been suggested in functional magnetic resonance imaging (FMRI) studies in cocaine users (Berridge & Kringelbach, 2008). Furthermore, among those subcortical brain structures that generate pleasure, the VP is the only brain region that is necessary for pleasure. Neuronal death caused by excitotoxic lesion abolishes all hedonic 'liking' and further replaces it with aversive 'disliking' even to sucrose (Cromwell & Berridge, 1993). The VP's crucial role deserves researchers' special attention, and future use of selective receptor agonists/antagonists and lesions can further verify VP's necessary and sufficient role in pleasure causation.

Pleasure is generated by hedonic circuits in the brain that paints a "pleasure gloss" onto objective external sensations, and transforms them into pleasurable feelings of 'liking' (Pećina et al., 2006).This study will shed new insight in the neuronal generation of pleasure sensations in the brain. The localization and functional verification of the endocannabinoid

and orexin hedonic hotspots will provide new strategies to guide pharmacological approaches for future diagnosis and medical therapy of diseases. For example, a medicine mediating the endocannabinoid and orexin hedonic hotspot in the posterior VP might alter food palatability in patients suffering from obesity. With a potential finding of increased food intake after microinjection of anandamide and orexin A in the posterior VP, a new therapy may be developed to decrease the sensitization of drug incentive in drug addicts. By manipulating this endocannabinoid and orexin hotspot in the posterior VP to modulate 'liking' and 'wanting,' the motivational disorders (anorexia and obesity), drug addiction (cocaine and heroin addiction), and mood disorders (e.g. schizophrenia and anxiety) could gain therapeutic benefits in the future (Berridge & Kringelbach, 2008; Patching & Lawler, 2009).

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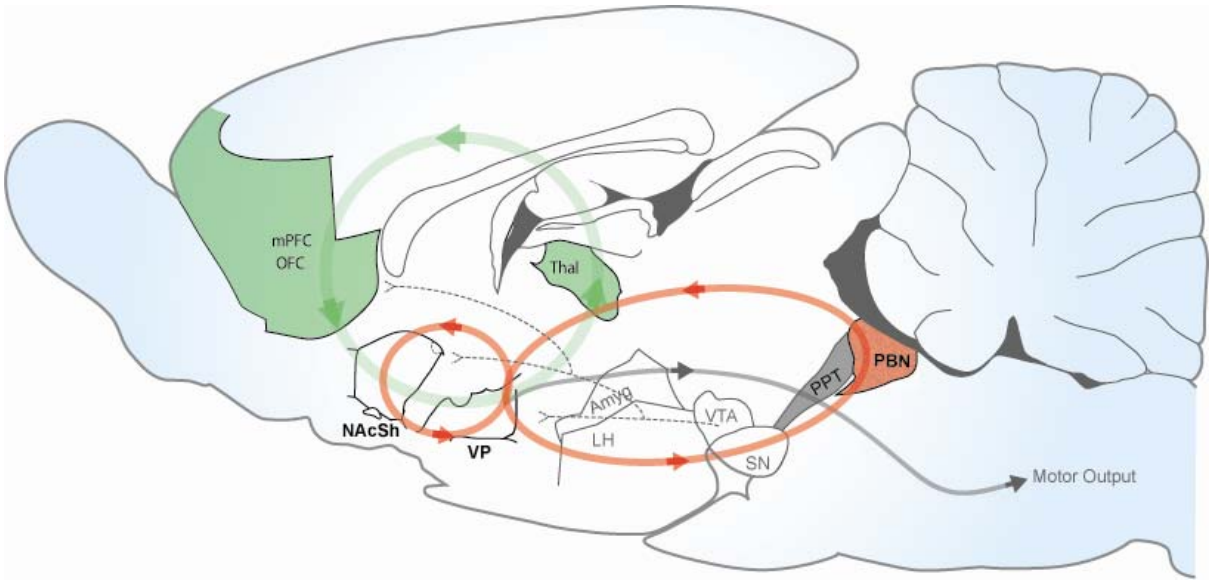

*Figure 1.*Hedonic hotspots and hedonic circuits. Pleasure generation is coordinated by a network of hedonic hotspots distributed across subcortical brain regions, including the rostral-dorsal quadrant of the nucleus accumbens shell, the posterior half of ventral pallidum, and the parabrachial nucleus in the pons. Each hotspot is capable of enhancing core 'liking' reactions to sweetness activated by opioid, endocannabinoid, orexin, or other neurochemical signals. Reprinted from Berridge & Kringelbach (2008). Based on Kringelbach (2005), Pecina et al. (2006), and Smith & Berridge (2007).

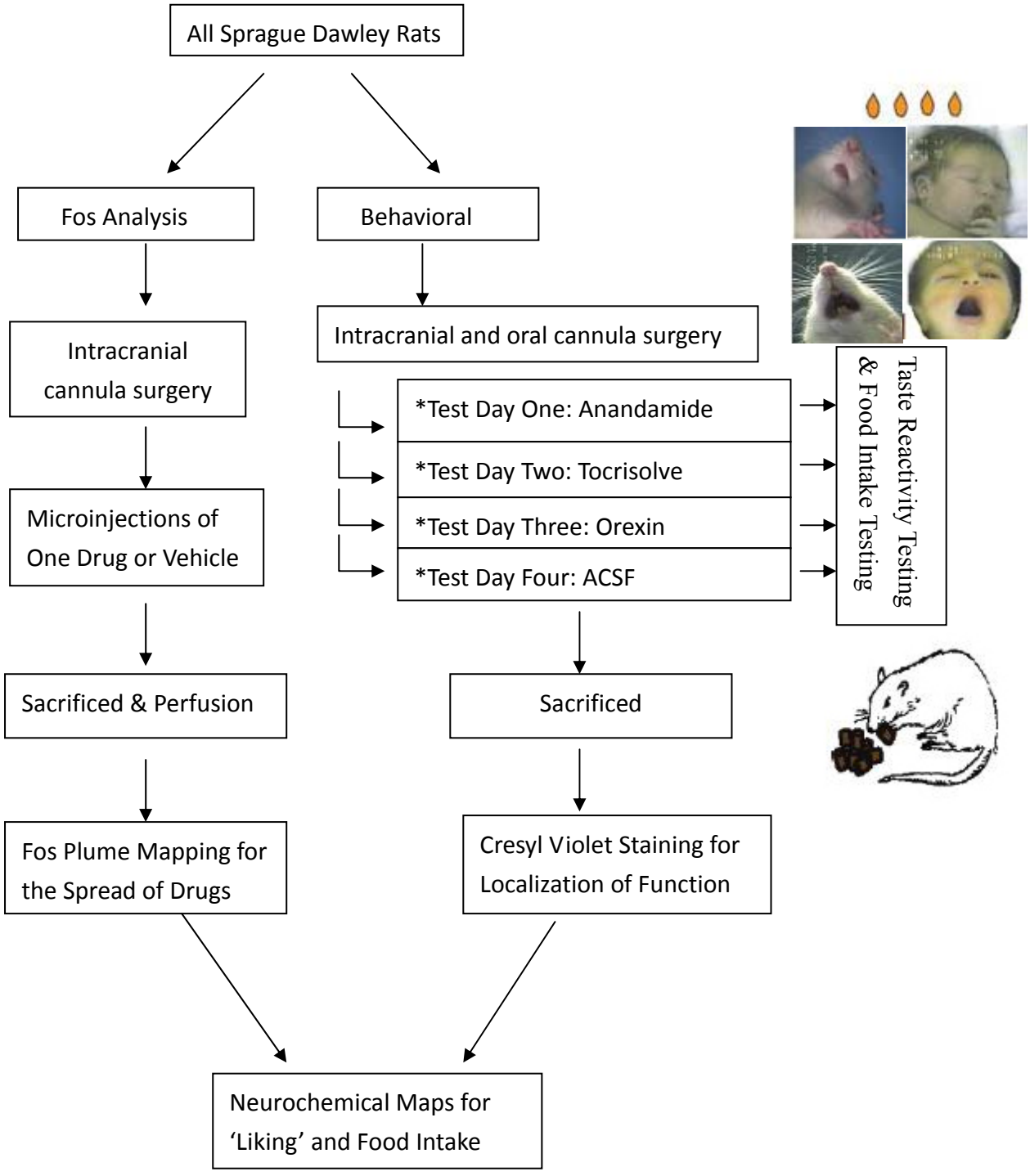
All Sprague Dawley Rats
Fos Analysis
Behavioral
Intracranial cannula surgery
Intracranial and oral cannula surgery
*Test Day One: Anandamide
*Test Day Two: Tocrisolve
*Test Day Three: Orexin
*Test Day Four: ACSF
Taste Reactivity Testing & Food Intake Testing
Microinjections of One Drug or Vehicle
Sacrificed & Perfusion
Sacrificed
Fos Plume Mapping for the Spread of Drugs
Cresyl Violet Staining for Localization of Function
Neurochemical Maps for 'Liking' and Food Intake

Figure 2. The flow diagram for experimental procedures. By mapping the localization of functions ('liking' from the taste reactivity testing and 'wanting' from the food intake testing) of the behavioral group onto the spread of Fos plumes taken from the Fos analysis group, this split-and-recombine design facilitates the construction of neuroanatomical and neurochemical maps of sucrose 'liking' and 'wanting.' The order of drug administration during the four test days is random to exclude statistical bias.

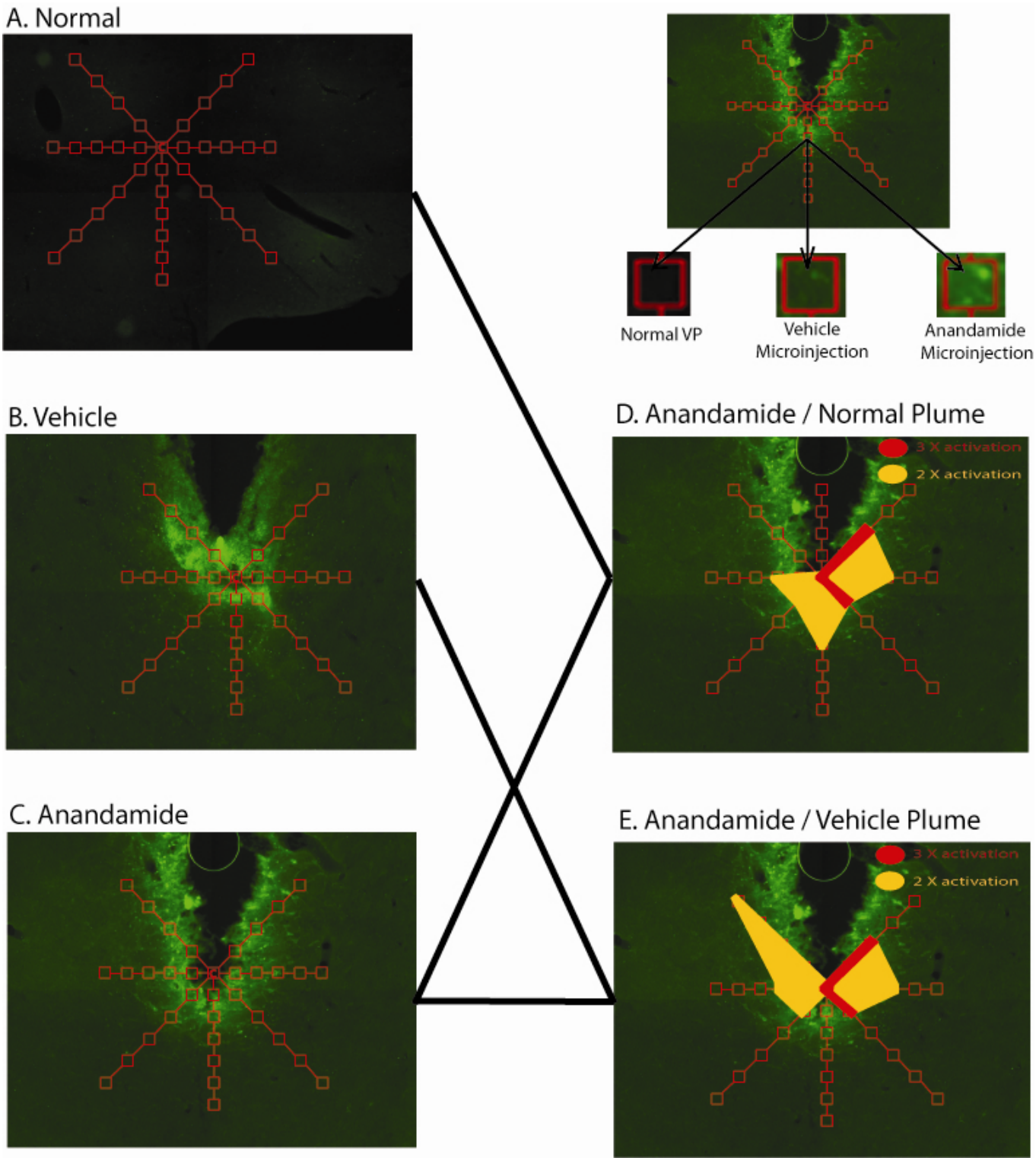
A. Normal
Normal VP
Vehicle Microinjection
Anandamide Microinjection
B. Vehicle
D. Anandamide / Normal Plume
3 X activation
2 X activation
C. Anandamide
E. Anandamide / Vehicle Plume
3 X activation
2 X activation

Figure 3. The Fos plume images. ***A***, the normal sham animal; ***B***, the Tocrisolve vehicle animal; and ***C***, the anandamide microinjected animal. The tip of the microinjector is labeled as center, and a Fos grid is imposed on the Fos plume image to facilitate Fos cell counts in seven directions: 45, 90, 135, 180, 225, 270, 315°. Examples of Fos densities from uninjected normal VP tissue, and after vehicle or anandamide microinjection are shown in small sampling boxes on the upper right corner. The Fos plume is color coded based on the Fos expression elevation above the ***D***, normal or ***E***, vehicle level after anandamide microinjection (red, absolute increase of >3X; orange, absolute increase of > 2X).

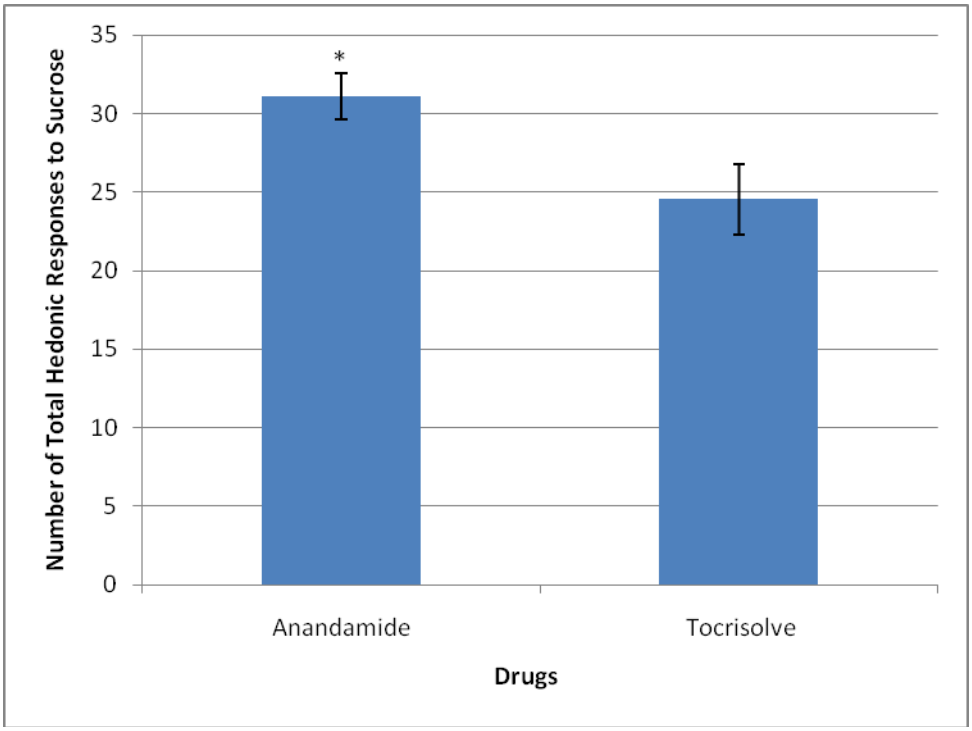

*Figure 4.*There was a significant difference in the number of total hedonic responses to sucrose on anandamide-injected days versus tocrisolve-injected days. Anandamide microinjections in the VP caused an average 26% increase in the positive hedonic reactions to sucrose compared to that of the vehicle, $t(10) = 3.22$, $p = 0.009$.

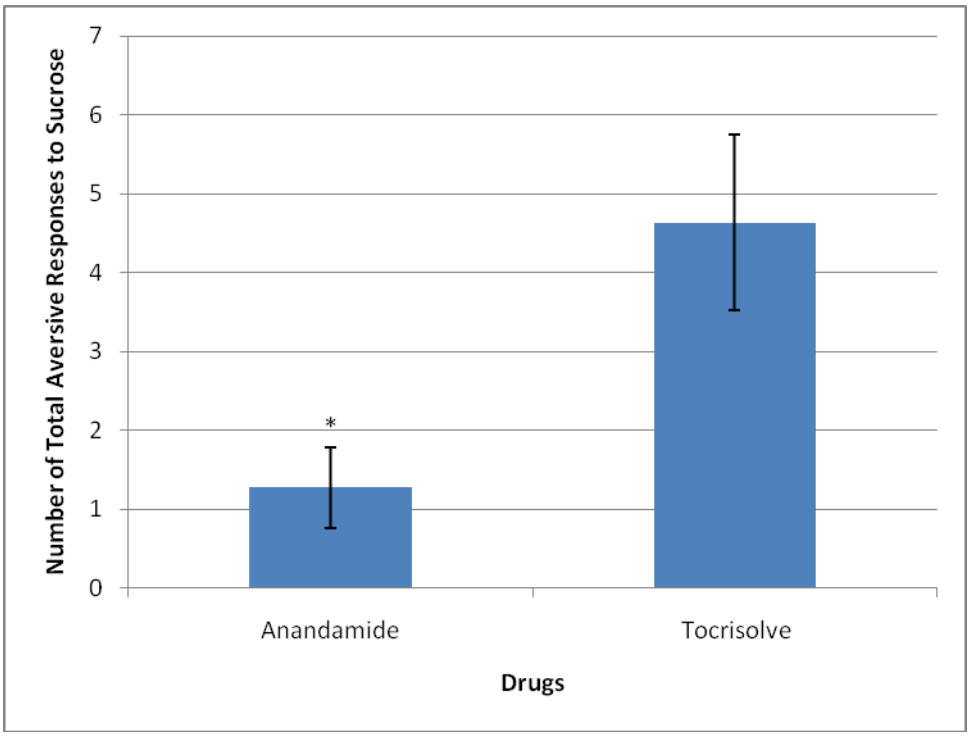

Figure 5. There was a significant difference in number of total aversive responses to sucrose on anandamide-injected days versus tocrisolve-injected days. Anandamide microinjections in the VP caused an average 72% decrease in negative aversive reactions to sucrose compared to that of the vehicle, $t(10) = -3.06$, $p = 0.012$

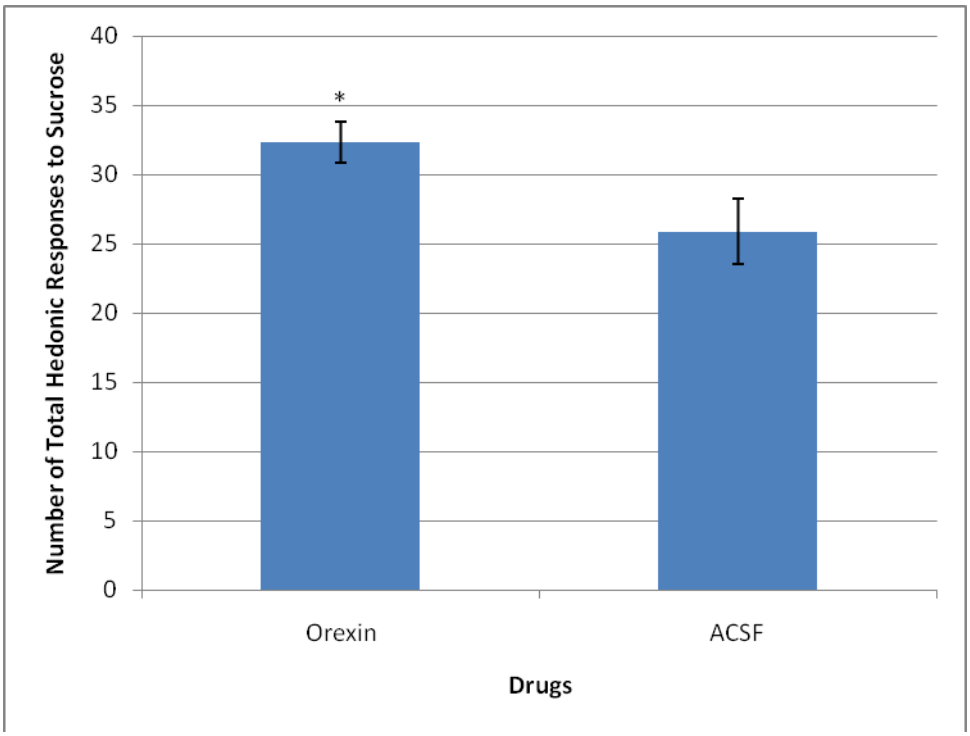

Figure 6. There was a significant difference in the number of total hedonic responses to sucrose on orexin-injected days versus ACSF-injected days. Orexin microinjections in the VP caused an average 24% increase in positive hedonic reactions to sucrose compared to that of the vehicle, $t(8) = 2.89$, $p = 0.020$.

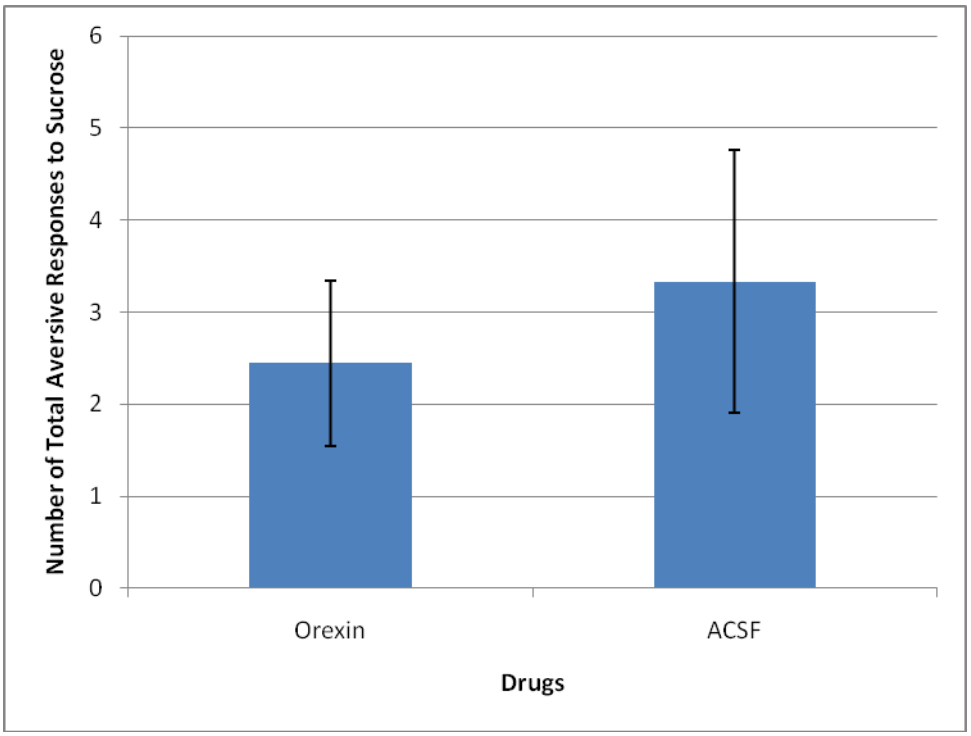

Figure 7. There was no significant difference in the number of total aversive responses to sucrose on orexin-injected days versus ACSF-injected days. Orexin microinjections in the VP had no significant effect on aversive reactions to sucrose compared to that of the vehicle, $t(8)= -0.62$, $p=0.551$.

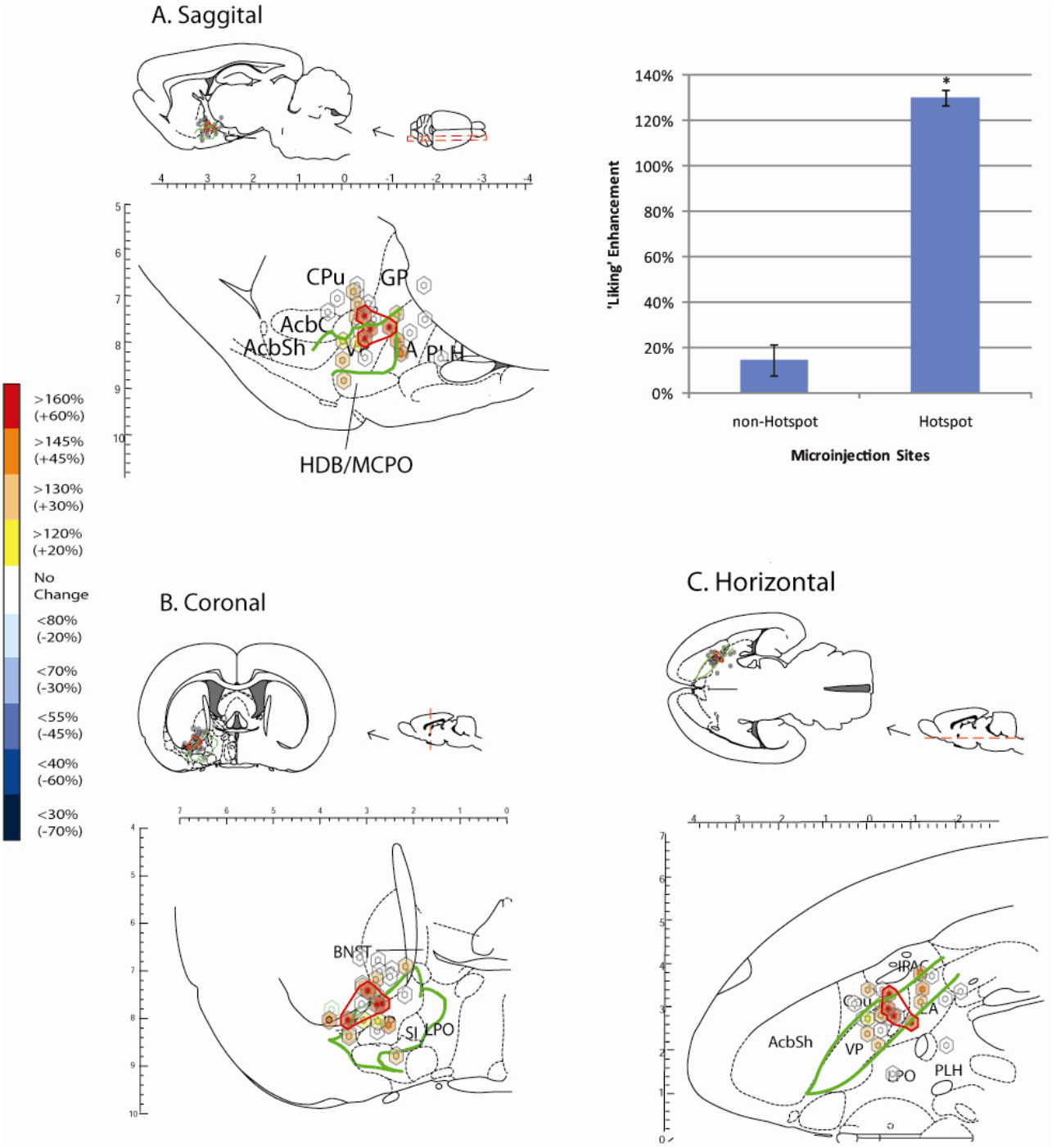
A. Saggital
CPu
GP
AcbC
AcbSh
VP
A
PLH
HDB/MCPO
>160% (+60%)
>145% (+45%)
>130% (+30%)
>120% (+20%)
No Change
<80% (-20%)
<70% (-30%)
<55% (-45%)
<40% (-60%)
<30% (-70%)
'Liking' Enhancement
140%
120%
100%
80%
60%
40%
20%
0%
non-Hotspot
Hotspot
Microinjection Sites
B. Coronal
BNST
SI
LPO
C. Horizontal
IRAC
Cu
LA
AcbSh
VP
LPO
PLH

Figure 8. Fos plume neurochemical map showing the anandamide 'liking' enhancement in the VP. Colors denote intensity of positive hedonic increases as expressed as within-subject percentage changes from vehicle microinjection at the same site (vehicle = 100%). The symbol size shows the radius of intense Fos plumes (3X elevation above normal; 0.063 mm radius), surrounded by semitransparent halos that show the radius of moderate Fos plumes (2X elevation above normal; 0.175 mm radius). Red lines delineate the potential hedonic hotspot, while green lines outline the VP structure throughout several brain slices. Notice that bilateral VP sites from left and right brains are collapsed onto a unilateral single map of the VP. Bar graph above shows that 'liking' enhancement compared to the vehicle is significantly higher within the endocannabinoid hotspot than outside the hotspot, $F(1, 11) = 51.20$, $p = 0.000$. ***A***. Saggital view. ***B***. Coronal view. ***C***. Horizontal view.

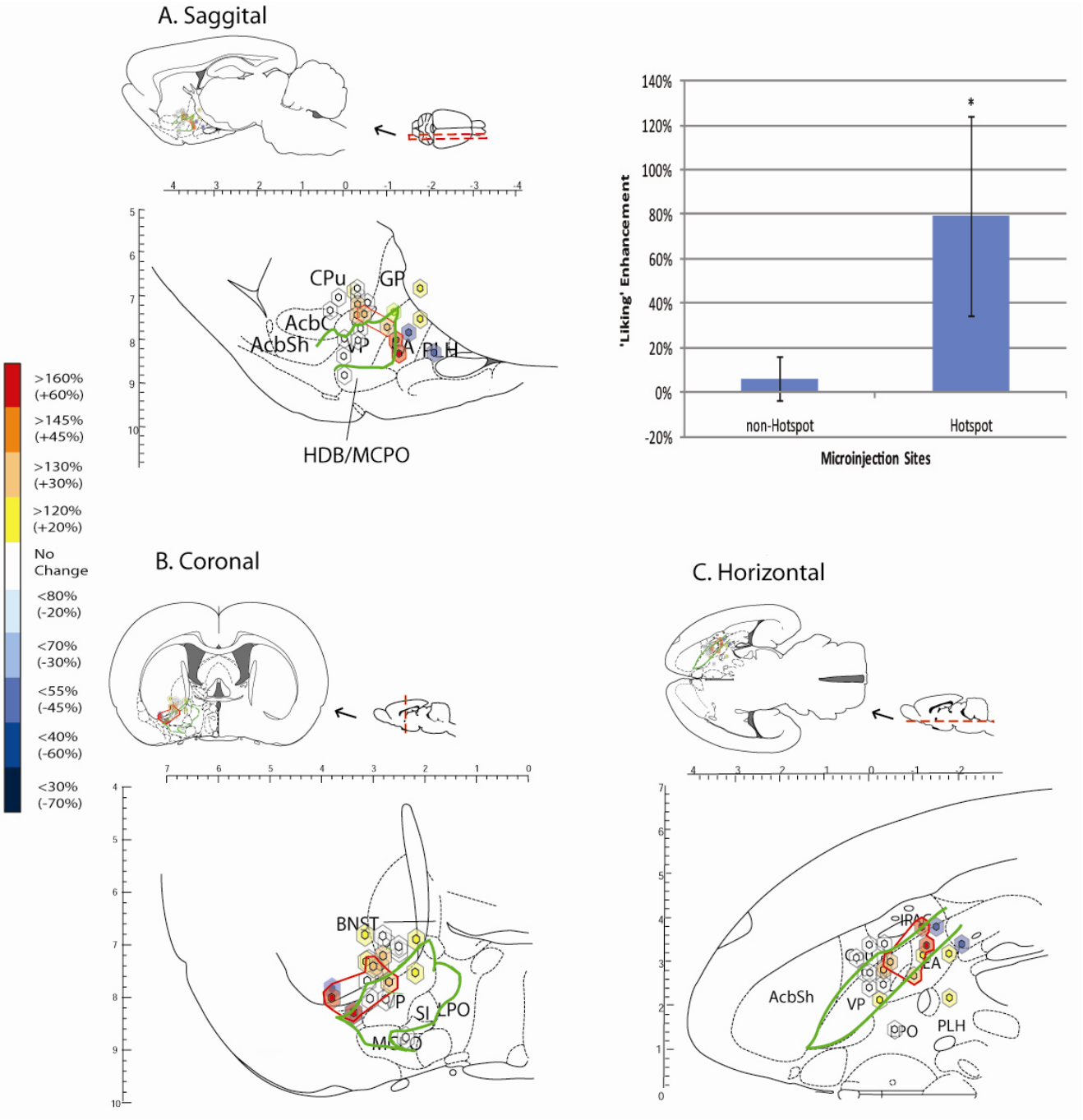
A. Saggital
CPu
GP
AcbC
AcbSh
VP
BA
PLH
HDB/MCPO
'Liking' Enhancement
140%
120%
100%
80%
60%
40%
20%
0%
-20%
*
non-Hotspot
Hotspot
Microinjection Sites
>160% (+60%)
>145% (+45%)
>130% (+30%)
>120% (+20%)
No Change
<80% (-20%)
<70% (-30%)
<55% (-45%)
<40% (-60%)
<30% (-70%)
B. Coronal
BNST
VP
SI
LPO
C. Horizontal
IPAC
CA
AcbSh
VP
PO
PLH

Figure 9. Fos plume neurochemical map showing the orexin 'liking' enhancement in the VP. Colors denote intensity of positive hedonic increases as expressed as within-subject percentage changes from vehicle microinjection at the same site (vehicle = 100%). The symbol size shows the radius of intense Fos plumes (3X elevation above normal; 0.065 mm radius), surrounded by semitransparent halos that show the radius of moderate Fos plumes (2X elevation above normal; 0.160 mm radius). Red lines delineate the potential hedonic hotspot, while green lines outline the VP structure throughout several brain slices. Notice that bilateral VP sites from left and right brains are collapsed onto a unilateral single map of the VP. Bar graph above shows that 'liking' enhancement compared to the vehicle is significantly higher within the endocannabinoid hotspot than outside the hotspot, $F(1, 9) = 6.01$, $p = 0.037$. ***A***. Saggital view. ***B***. Coronal view. ***C***. Horizontal view.

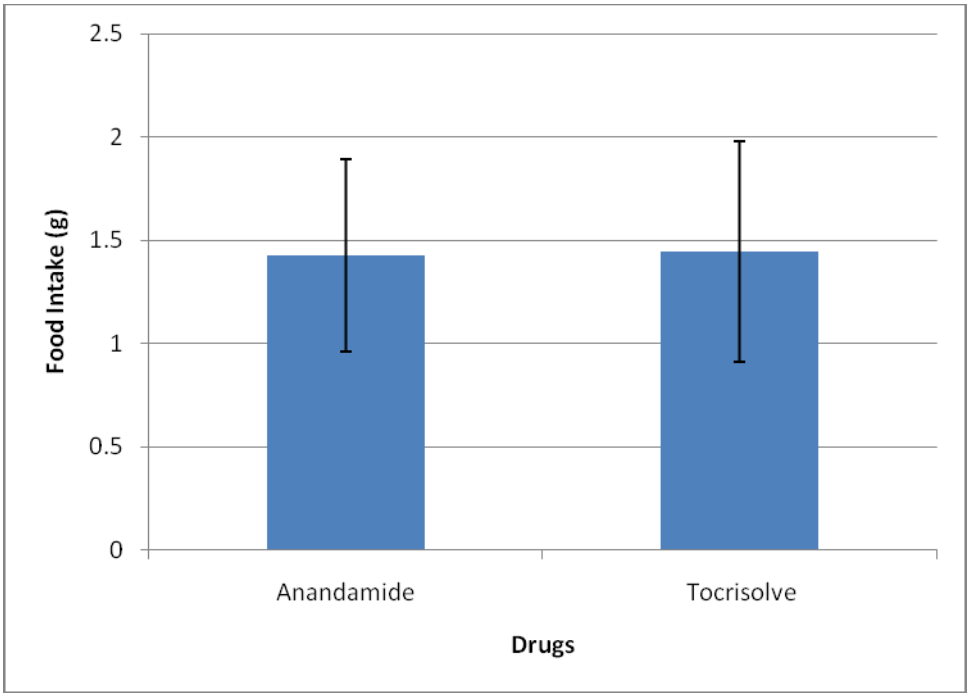

Figure 10. There was no significant difference in the amount of food intake on anandamide-injected days versus tocrisolve-injected days, $t(10) = -0.03$, $p = 0.974$.

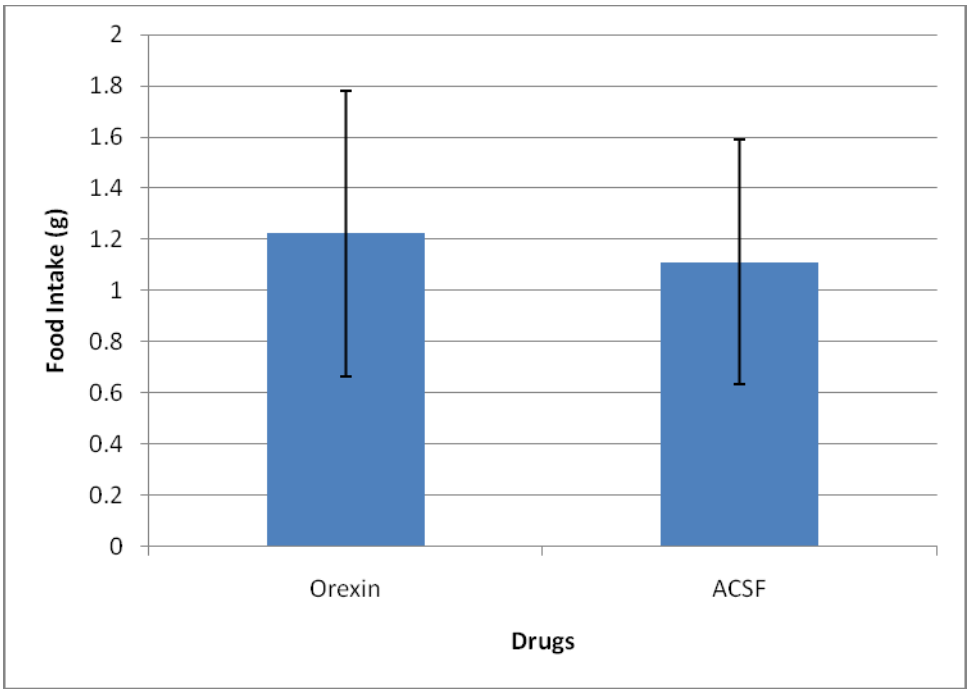

Figure 11. There was no significant difference in the amount of food intake on orexin-injected days versus ACSF-injected days, $t(8) = 0.15$, $p = 0.883$.