2007

Behavioral and Neural Characterization of Conditioned Flavor-Taste Preferences

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I would like to dedicate this dissertation to my wife, Julie Rose Golden, and to the loving memory of my brother, Vincent Scott Gardiner. Their strong belief in my abilities existed even when I did not believe in myself. I can only hope they know the positive influence they have on my every decision, every day.

I also dedicate this work to my sons, Vincent Scott Golden and Tighe Rubin Golden. You are only young once, and if you work it out right, it will last you a lifetime.
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<tbody>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AOB</td>
<td>accessory olfactory bulb</td>
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<tr>
<td>AON</td>
<td>anterior olfactory nucleus</td>
</tr>
<tr>
<td>BLA</td>
<td>basolateral amygdala</td>
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<tr>
<td>BSA</td>
<td>1% bovine serum albumin</td>
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<td>CeA</td>
<td>central nucleus of the amygdala</td>
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<tr>
<td>CFNP</td>
<td>conditioned flavor-nutrient preference</td>
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<td>CFTP</td>
<td>conditioned flavor-taste preference</td>
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<td>CS</td>
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<tr>
<td>CTA</td>
<td>conditioned taste aversion</td>
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<tr>
<td>DAB</td>
<td>0.05% 3,3-diaminobenzidine tetrahydrochloride</td>
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<td>DCS</td>
<td>D-cycloserine</td>
</tr>
<tr>
<td>DG</td>
<td>dentate gyrus</td>
</tr>
<tr>
<td>dH2O</td>
<td>deionized distilled water</td>
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<td>F</td>
<td>fructose</td>
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<tr>
<td>GUS CTX</td>
<td>gustatory cortex</td>
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<td>KA</td>
<td>Kool-Aid</td>
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<tr>
<td>LHB</td>
<td>lateral habenula</td>
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<tr>
<td>LiCl</td>
<td>lithium chloride</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NTS</td>
<td>nucleus of the solitary tract</td>
</tr>
<tr>
<td>NR</td>
<td>N-methyl-D-aspartate glutamate-glycine receptor</td>
</tr>
<tr>
<td>MeAv</td>
<td>ventral region of the anterior medial nucleus of the amygdala</td>
</tr>
<tr>
<td>MeAd</td>
<td>dorsal region of the anterior medial nucleus of the amygdala</td>
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<tr>
<td>MePv</td>
<td>ventral region of the posterior medial nucleus of the amygdala</td>
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<tr>
<td>MeAd</td>
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<tr>
<td>PB</td>
<td>0.1 M phosphate-buffer</td>
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<tr>
<td>PBN</td>
<td>parabrachial nucleus</td>
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<td>PBS</td>
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<td>PIRIa</td>
<td>anterior piriform cortex</td>
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<tr>
<td>PIRIp</td>
<td>posterior piriform cortex</td>
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<tr>
<td>PRH</td>
<td>perirhinal cortex</td>
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<td>S</td>
<td>saccharin</td>
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<td>SD</td>
<td>Sprague-Dawley</td>
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<td>US</td>
<td>unconditioned stimulus</td>
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<td>VLO/LO</td>
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ABSTRACT

Many animals, including humans, choose their source of nutrition based on the nutritive value and the flavor (i.e., odor, taste and texture) of available foods. Sweet taste is one of the more potent orosensory stimuli that contributes to food choice. Laboratory animals develop preferences for neutral or aversive tastes and flavors by associating them with the taste of sugars and non-caloric sweeteners. Learning how these preferences develop would aid in understanding how and why specific foods are selected over others. Given the wide availability and variety of sweetened foods and beverages in modern society, the formation and persistence of learned food preferences by consumers may contribute to health issues such as diabetes and obesity.

Conditioned flavor-taste preference (CFTP) learning is a form of associative learning in which a rat comes to prefer a neutral flavor paired with a preferred taste. Experimentally, one flavor (the conditioned stimulus or CS+; e.g., cherry or grape Kool-Aid) is paired with the sweet and highly preferred taste of fructose (F; the unconditioned stimulus or US) while a second flavor (the CS-) is paired with the less preferred taste of saccharin (S) on 1-bottle conditioning days (CS+/F or CS-/S). The acquisition of the learned preference is then assessed with a 2-bottle preference test in which both flavors mixed with saccharin (CS+/S and CS-/S) are presented simultaneously. While CFTP learning is well known, it has not been well characterized. The olfactory and gustatory associative brain regions necessary for CFTP learning are unknown. Dopamine receptors have been implicated, but otherwise it is not known which neurotransmitters or receptors mediate CFTP.

In order to identify the associative neural substrates that are involved in CFTP learning, three approaches were taken; behavioral, pharmacological and molecular assays.

To precisely characterize the behavioral acquisition and expression of a CFTP, lickometers were used to determine the pattern of drinking in rats. During 1 or 2-bottle preference tests, total intake, bout size, bout number, lick rate and first minute licks were analyzed. The pattern of drinking was examined under 3 conditions:

1. During expression of unconditioned preferences for 8% fructose over 0.2% saccharin. The unconditioned preference for fructose over saccharin was slow to develop, but was seen in significantly greater total intake, bout size, and first minute licks for fructose by the fourth preference test.
2. During the pairing of Kool-Aid flavors with either 8% fructose or 0.2% saccharin. CS-/S total intake and bout size was significantly greater than CS+/F during conditioning, but a preference for the CS+ flavor was seen in the third and fourth 2-bottle preference test days with significantly greater total intake and bout size of CS+/S vs. CS-/S.
3. During long-term presentations of Kool-Aid mixed with two different concentrations of saccharin (0.2% vs 0.05%) as the unconditioned stimuli. Total intake, bout size
and bout number were significantly greater for the flavor mixed with the high concentration of saccharin over the low concentration of saccharin. During conditioning. During 2-bottle preference tests when both flavors were mixed with the low concentration of saccharin, total intake and bout size were significantly greater for the CS+.

The increases in lick rate and bout size observed in all 3 experiments suggest that fructose is more palatable than saccharin, and a high concentration of saccharin is more palatable than a low concentration. A change in relative palatability of the Kool-Aid flavors is conditioned by association with the high palatability tastes; greater intake of the conditioned flavor is mediated by increased bout size. These results suggest that flavor preference learning interacts with both orosensory processes and satiety processes (i.e. prolonged bout size) to elevate intake of the preferred flavor.

The N-methyl-D-aspartate (NMDA) glutamate receptor (NR) is a candidate mediator in olfactory and taste learning (Barkai and Saar, 2001; Jimenez and Tapia, 2004). To determine if NR is involved in CFTP, systemic MK-801, a non-competitive NR antagonist, was administered prior to conditioning and prior to expression. To determine the glycinergetic contribution to NR activation in CFTP, systemic D-cycloserine, an agonist at the NR glycine-binding site, was administered prior to conditioning and reversal learning. While vehicle-treated rats acquired a preference for CS+/S over CS-/S, CFTP learning was completely blocked in MK-801-treated rats. The effect of MK-801 was specific to CFTP acquisition, because follow-up experiments demonstrated that MK-801 did not induce a conditioned taste aversion, cause state-dependent learning, or affect CFTP expression. In a second approach, rats were injected with DCS (15 mg/kg) 60 min prior to daily conditioning. In contrast to MK-801, administration of DCS prior to conditioning enhanced CFTP learning (but not reversal conditioning). These results demonstrate that NR neurotransmission is critical for CFTP learning. Furthermore, enhancement of CFTP learning by DCS suggests that endogenous levels of glycine or D-serine may be a limiting factor in CFTP learning.

To determine the activation of neural populations during associative CFTP learning, c-Fos immunohistochemistry was used to illuminate the differential patterns of cellular activation. To eliminate the potential confounds of food restriction, restricted drinking sessions and potential postingestive effects that may effect c-Fos activation, rats were conditioned using a highly preferred concentration (0.2% saccharin) and a lesser preferred concentration of saccharin (0.05% saccharin) as the unconditioned stimuli. In order to standardize exposures, stimuli were applied by intraoral infusion. C-Fos immunolabeling was visualized within the brain after an intraoral infusion of either CS+ or CS- flavors (e.g. grape and cherry Kool-Aid) in combination with greater or lesser preferred taste US (e.g. 0.2% or 0.05% saccharin). Neuronal activation was assessed in forebrain sites (e.g. gustatory cortex, amygdala, and lateral hypothalamus). There was no difference in intraoral intake between all experimental groups in both conditioned and unconditioned rats, and extensive c-Fos activation was evoked in the olfactory, gustatory and learning relays of all experimental groups. Analysis of the differential patterns of c-
Fos immunolabeling among unconditioned rats revealed a significant increase in c-Fos immunolabeling in the basolateral nuclei of the amygdala after intraoral infusions of dH2O compared to unconditioned rats after intraoral infusions of CS+/0.2% saccharin or CS-/- 0.055 saccharin. Therefore, the basolateral amygdala may be involved in the unconditioned response to sweetened flavors, or in the association of flavor with sweet tastes. Among conditioned rats, there was a trend towards greater in c-Fos immunolabeling in the lateral habenula of rats after intraoral infusions of CS+ vs the CS- or saccharin alone. Therefore, the lateral habenula, which is part of the accumbens-ventral tegmental area reward pathway, may be involved in the discrimination of learned preferences.
BACKGROUND AND SIGNIFICANCE

Conditioned flavor-taste preference is a robust model of associative learning that is well known, although not well characterized. More specifically, it is the preference formed after the association of a neutral flavor (the conditioned stimulus or CS) and a palatable or preferred taste (the unconditioned stimulus or US). CFTP can be distinct from other forms of preference learning, such as flavor-nutrient conditioning or odor-reward conditioning.

CFTP is a simple association of flavor (a mixed oronasal taste and odor stimulus) and taste (e.g. sweet or bitter tastants); postingestive effects need not play a role (see Table 1). Neutral or isoprefereed flavors are used as CSs. Two differentially preferred tastants are used as the US (published studies have used sweet tastes almost exclusively). CFTP requires a small number of pairings. CFTP causes an increase in CS+ intake in the absence of the US in both 1-bottle acceptance and 2-bottle preference tests. CFTP resists extinction, but can be modified with reversal of the CS/US pairing.

CFTP is distinct from conditioned flavor nutrient preferences

CFTP is distinct from conditioned flavor nutrient preferences (CFNP), which are formed by the pairing of a neutral flavor (CS+) with a caloric nutrient (US) and a different neutral flavor with a non-nutritive source for multiple trials. The nutrient US can be delivered either orally or through intragastric infusions but not by sham-feeding (Sclafani 1991), thus implicating a postingestive site of action. The intragastric approach eliminates the flavor of the nutrient as a potential confound in food conditioning studies, but also ignores the potential role taste has in the conditioning of food preferences. CFTP learning might be as relevant as CFNP learning: overconsumption of preferred foods by humans may be due in part to the hedonically-driven association of taste and food flavor (Sclafani, 2001).

CFTP versus other models

CFTP has several advantages over other models of odor and taste conditioning, such as conditioned taste aversion (CTA), flavor-nutrient conditioning, or odor-reward conditioning. CFTP is nearly as robust as CTA learning. It takes a few more trials (e.g. 4-8 pairings) to acquire a CFTP compared to single-trial CTA, but it resists extinction as long if not longer than CTA (Golden and Houpt, 2005). While CTA is an excellent model of gustatory learning, it models aversive learning. Preference learning, as in CFTP, may be a more relevant model of positively-motivated learning tasks.

In odor-reward conditioning, rats demonstrate a preference for an odor paired with a drug of abuse (e.g. cocaine administered systemically, intracerebroventricularly, or directly into the nucleus accumbens while being exposed to a distinctive odor) (Barr and Wang, 1992). This model demonstrates the formation of an odor preference with a central
reward, which might mediate motivational aspects of odor conditioning and simulate the hedonic character of a preferred US, but it does not address the association between two natural sensory inputs. In contrast, CFTP is a robust demonstration of the simple association of olfactory and gustatory pathways, although CFTP is also mediated by dopaminergic pathways.

The sensory inputs in many models of associative olfactory learning are not as narrowly defined as in CFTP learning. For example, in mating-induced odor preferences (e.g., in voles) multiple sensory modalities are involved (i.e., vision, olfaction, audition, touch and motor function) (Curtis and Wang, 2005; Insel et al., 1995). In the CFTP model, there are only two sensory modalities contributing to the formation of a flavor-taste preference.

CS and US characteristics

In any associative learning paradigm, the CS+ and CS- must be 1) neutral in stimulating the rat’s response but 2) easily distinguished from each other by the rat. The CS used in my protocol is Kool-Aid, which is used by the Scalfani lab and others for flavor conditioning in rats (see Table 1). Two flavors of Kool-Aid solution, grape and cherry, are presented to rats. When mixed with 0.2% saccharin, these 2 flavors are isopalatable (i.e. equal intake in a 2-bottle preference test). Thus they are considered neutral relative to each other prior to pairing with an additional US. The two sensory components of the Kool-Aid flavors most relevant to CFTP learning are taste and olfaction; the resulting grape and cherry flavors are highly salient and easily distinguished by odor (at a distance) or by flavor (mixed odor and taste during ingestion).

Other than flavor, there is little difference between the two Kool-Aids. The main common tastant used in Kool-Aid is citric acid; a low percentage of NaCl is also an ingredient. Maltose dextrin, a highly preferred “polycose” tastant in rats, is also an ingredient of grape and other (but not cherry) Kool-Aid flavors. Specific differences between the two flavors, however, are controlled for by counter-balancing grape and cherry across groups as the CS+ and the CS-.

To form a taste-induced preference, the US should 1) potently stimulate the rat’s response (i.e. increased preference and intake) and 2) stimulate the response through taste and not by other properties of the US. It is well known that rats prefer 8% fructose over 0.2% saccharin in short-term 2-bottle tests (Scalfani et al., 1994). I have also found a higher intake of fructose in comparison to saccharin initially during short-term 1 and 2-bottle presentations and a higher lick rate of the fructose after multiple exposures (see Chapter 1). Increased intake and lick rate are often used as measures of palatability in rats. Furthermore, saccharin may have aversive properties compared to fructose. Rats will avoid drinking high concentrations of saccharin. While the exact taste perception is unknown in rats, saccharin has a bitter component to humans and rats generalize a CTA against saccharin to bitter solutions (e.g., quinine)(Nowlis et al., 1980).

Sweet taste alone is sufficient to induce flavor preferences, as shown when flavors are paired with sham-feeding of sucrose solutions (Nissenbaum and Scalfani, 1987). There are additional non-gustatory distinctions between the fructose US+ and saccharin
US-, however. Sugars and saccharin have an odor component (Capaldi et al., 2004; Rhinehart-Doty et al., 1994) although it is unlikely that rats prefer the odor of fructose per se over the odor of saccharin per se. There are also postingestive differences: 8% fructose is caloric and hyperosmotic compared to noncaloric and hyposmotic 0.2% saccharin. Postingestive factors do not appear to contribute to fructose-conditioned preferences, however, because gastric infusions of fructose do not induce robust flavor preferences in short-term sessions (Ackroff and Sclafani, 2004; Sclafani et al., 1993).

Differences between the US+ and US- are not operative during expression tests, because expression of the preference is tested with both the CS+ and CS- mixed with saccharin (i.e., rats cannot use differences between saccharin and fructose to express a CS preference.) Rats are never presented with a simultaneous choice between saccharin and fructose, so they never have to discriminate between them directly.

Neural substrates of CFTP

The reward basis for CFTP learning has been probed pharmacologically (Baker et al., 2003; Baker et al., 2004; Yu et al., 1999; Yu et al., 2000a, b). In Yu et al. (Yu et al., 2000b), dopamine D₁ and D₂ receptor antagonists did not block the acquisition of CFTP learning using sucrose- or saccharin-paired flavors in sham-fed rats. However, both dopamine D₁ and D₂ antagonists blocked expression of a previously acquired CFTP (Yu et al., 2000a, b). A later study found that dopamine D₁ and D₂ receptor antagonists blocked both the acquisition and expression of CFTP conditioned by the sweet taste of fructose in real-feeding rats (Baker et al., 2003). The general opioid antagonist, naltrexone, does not significantly attenuate the acquisition and expression of CFTP in either sham-feeding or real-feeding rats (Baker et al., 2004; Yu et al., 1999). Thus dopaminergic pathways have been implicated in CFTP learning, apparently related to the rewarding properties of the gustatory US and the acquisition and expression of rewarding properties by the flavor CS+. However, these studies did not examine the associative mechanism for the formation of the flavor preference itself.

A number of studies have used excitotoxic and aspiration lesions in critical gustatory, regulatory and associative learning brain regions to examine their role in CFNP learning (Sclafani et al., 2001; Touzani and Sclafani, 2002a, 2005, 2002b). However, to my knowledge, only one experiment has examined the effect of excitotoxic lesions (in the amygdala) on CFTP learning. Two groups of rats, a lesioned group infused with ibotenic acid in phosphate buffer into a large area of the amygdala and a control group infused with the buffer alone, were conditioned for a flavor preference with one Kool-Aid flavor paired with a fructose/saccharin mixture and an alternate flavor paired with saccharin alone. Preference for the CS+ was measured using total intake in 2-bottle tests. Control rats displayed a robust preference for the CS+ flavor, while amygdala-lesioned rats failed to display a preference for the CS+ in 2-bottle preference testing (Touzani and Sclafani, 2005).
Experimental Approach

Three approaches were taken in order to identify the associative neural substrates that are involved in CFTP learning. To precisely characterize the behavioral aspects of a CFTP, I used lickometers to determine the pattern of the drinking in rats during access to the unconditioned stimuli alone, 8% fructose or 0.2% saccharin, during the pairing of Kool-Aid flavors with either 8% fructose or 0.2% saccharin and the pairing of Kool-Aid flavors with either 0.2% saccharin or 0.05% saccharin. To characterize the role of N-methyl-D-aspartate (NMDA) glutamate-glycine receptor (NR) in CFTP, systemic injections of NR agonists and antagonists were used to determine if NR were necessary in CFTP learning. To characterize the where in the brain the associative process of CFTP learning was occurring, c-Fos immunoreactivity was used to determine the activation of neural populations before, during and after associative CFTP learning.

1. Microstructure of CFTP. To examine the microstructure of the drinking patterns during CFTP learning, cages equipped with lickometers that recorded number of licks in 6-s bins at each of two bottles. In addition to total intake, we analyzed the number of bouts of licking during a test session, the size of bouts, the pattern of cumulative licks, and the number of licks in the first minute of drinking. An increase in bout size but not number is consistent with a change in palatability of the CS+ flavor solution, as it is similar to the pattern of licking seen with highly palatable taste solutions (Sclafani and Glendinning, 2003). Caloric density of the solution also has an effect on the drinking pattern. Non-caloric solutions tend to have a greater number of bouts with shorter lengths in comparison to caloric solutions that have a smaller number of bouts with an increased bout length (Smith, 2000; Smith et al., 1987). The results from these studies established the baseline for observing the effects of future manipulations on CFTP learning under my protocol.

2. Role of NMDA receptors. To examine the role of N-methyl-D-aspartate (NMDA) glutamate-glycine receptor (NR) in CFTP learning, systemic NR agonists and antagonists were used to modify NR function. The NR is a candidate molecule that potentially underlies the formation of the CFTP. NR is widely distributed throughout the CNS, but is particularly dense in highly plastic regions involved in learning (e.g. cortex, limbic system, and cerebellum) (Monyer et al., 1994). NR activation and the resulting calcium influx play a critical role in neural plasticity in vitro as a synaptic coincidence detector (e.g. in LTP) (Cain, 1997).

NR has previously been identified as a participant in olfactory and taste learning (Barkai and Saar, 2001; Jimenez and Tapia, 2004). For example, during conditioned taste aversion learning (CTA), exposure to a novel taste (but not a familiar taste) results in increased phosphorylation of the NR2B subunit in the insular cortex, which parallels the high saliency of novel tastes in CTA learning (Rosenblum et al., 1997). NR activity is required in CTA learning, because antagonism of the NR in the insular cortex by injection of the competitive NR antagonist 2-amino-5-phosphono pentanoic acid (APV) impedes CTA learning (Rosenblum et al., 1997).
NR activity in the amygdala is also required for odor-potentiated CTA. Pretreatment with the competitive NR antagonist APV impaired potentiated taste-odor aversion learning (i.e., saccharin presented with an almond odor and paired with LiCl injection), although in odor-alone and taste-alone conditioning, aversion learning was not impaired (Willner et al., 1992).

Two approaches were taken. First, a necessary role for the NR was established using systemic injections of MK-801, a non-competitive antagonist. Systemic MK-801 has been used to attenuate other forms of learning, such as olfactory discrimination learning for water reward in weanling (Griesbach et al., 1998) and adult rats (Quinlan et al., 2004). A complication of systemic MK-801 treatment that must be controlled for in behavioral experiments, however, is the induction of “non-specific” or aversive side effects at high doses (Sharp et al., 2001).

Second, the contribution of activity at the glycine-binding site of the NR was assessed by administration of D-cycloserine (DCS), a high-affinity glycine agonist. In addition to glutamate binding, NR channel opening requires agonist binding to a glycine-binding site. Endogenous ligands for the glycine-binding site of the NR include glycine of neural origin or D-serine synthesized by astrocytes. For example, neuronal migration in the developing cerebellum is dependent on serine racemase in glia to convert L-serine to D-serine, which together with glutamate potentiates NR activity in granule neurons (Kim et al., 2005).

By stimulating NR at the time of learning, exogenous DCS can potentiate learning in behavioral studies. For example, DCS has been shown to enhance spatial learning in a water maze (Riekkinen and Riekkinen, 1997) and to accelerate extinction in rats after fear conditioning with footshock (Walker et al., 2002). Potentiation of NR activity and learning by DCS treatment has been interpreted as indicating that endogenous levels of glycine or D-serine are less than optimal and thus limit NR activation; exogenous DCS can then raise NR activation to an optimal level for learning.

3. c-Fos expression in CFTP. To identify where in the brain the association between olfactory and taste stimuli was occurring, we examined the differential patterns of c-Fos activation following CFTP learning and expression. Using c-Fos expression as a marker for neural activation has many advantages. c-Fos mRNA and protein are quickly synthesized in response to transynaptic activity throughout the brain. Thus, c-Fos can serve as a marker of neuronal responses to sensory stimuli (i.e., differential patterns correlated with training experience indicate that learning has occurred). The delayed activation of c-Fos in relation to the behavioral stimulus offers the ability to dissect immediate behavioral responses that may be temporary from long term behavioral changes that are protein-synthesis dependent. The neuronal response is quantifiable by counting the number of c-Fos positive nuclei and the phenotype of the cells can be confirmed with double-labeling using other histochemical probes. This technique also offers the ability to visualize multiple activated brain areas that have been previously identified as a part of the neural network mediating ingestive behavior. Finally, there is a large body of previous work that has used c-Fos to elucidate gustatory and olfactory
pathways to aid in the interpretation of my data. However, using c-Fos immunohistochemistry to visualize neural activation has some disadvantages. Not all cells express c-Fos when activated, so not all activity is revealed. The temporal resolution is modest, as a ~1-h delay is required for the synthesis of c-Fos. Finally, c-Fos is a postmortem procedure.
**Table 1.** Experimental protocol for fructose/saccharin conditioned flavor-taste preferences. Four days of 2-h, 1-bottle presentations followed by a 2-bottle preference test. This cycle is repeated three or four times.

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<td><strong>Conditioning day 1</strong></td>
<td>Cherry or grape Kool-Aid with 8% fructose</td>
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<tr>
<td><strong>Conditioning day 2</strong></td>
<td>Empty bottle</td>
<td>Grape or cherry Kool-Aid with 0.2% saccharin</td>
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<tr>
<td><strong>Conditioning day 3</strong></td>
<td>Cherry or grape Kool-Aid with 8% fructose</td>
<td>Empty bottle</td>
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<tr>
<td><strong>Conditioning day 4</strong></td>
<td>Empty bottle</td>
<td>Grape or cherry Kool-Aid with 0.2% saccharin</td>
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<td><strong>Preference test day 5</strong></td>
<td>Cherry or grape Kool-Aid with 0.2% saccharin</td>
<td>Grape or cherry Kool-Aid with 0.2% saccharin</td>
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CHAPTER 1

MICROSTRUCTURE OF FRUCTOSE, SACCHARIN AND CONDITIONED FLAVOR-TASTE PREFERENCES.

1. Introduction

Unconditioned taste biases and learned associations between flavor and taste cues are both important factors in the choice of one flavor or taste over another. Conditioned flavor-taste preference (CFTP) is a form of associative learning in which an animal comes to prefer a conditioned stimulus (CS; e.g., a Kool-Aid flavor), after it has been paired with an unconditioned stimulus (US; e.g., fructose or saccharin). The acquisition and developing expression of sweet taste and associated flavor preferences over time can be examined by studying the microstructure of ingestion.

Increased gross intake of one solution over another in 2-bottle tests indicates that a preference either exists or has been conditioned. Other more sophisticated methods have been used to assess the emergence of a conditioned flavor-nutrient preference including lickometer analysis (Sclafani et al., 1998), taste reactivity scoring (Myers and Sclafani, 2003, 2001b), the sham feeding preparation (Myers and Sclafani, 2001a) and the progressive ratio task (Sclafani and Ackroff, 2006). Rats display more hedonic taste reactivity responses (e.g., tongue protrusions, mouth movements) for a previously neutral flavor (CS+) that has been paired with gastrointestinal infusions of glucose in comparison to a previously neutral flavor (CS-) that has been paired with gastrointestinal infusions of water (Myers and Sclafani, 2001b). In contrast, a later experiment showed that increased intake of bitter or sour solutions paired with an gastrointestinal infusion of glucose did not reflect an increase in palatability as shown by the absence of change in hedonic taste reactivity responses (Myers and Sclafani, 2003).

Sham-feeding tests allow for the taste or flavor of a solution to stimulate intake without the benefit of postingestive effects. Following the conditioning of a flavor-nutrient preference, the non-reinforced CS+ flavored saccharin solution was compared in six 30 min/day 1-bottle sham feeding tests to 16% fructose in identical 1-bottle tests. During real feeding test prior to the six days, the intake of the CS+ and the 16% fructose solution were the same. However, during the six days of sham feeding, the intake of the non-reinforced CS+ solution slowly fell, while the by the sixth day the 16% fructose intake had more than doubled (Myers and Sclafani, 2001a). This suggests that as conditioned satiety was extinguished, the orosensory stimulus alone drove the increased intake.
Progressive ratio licking is a relatively direct measure of the alteration in salience of a flavor. Rats will have a higher breaking point showing they are willing to work more for a solution that is highly preferred in comparison to a lesser-preferred solution. After CFNP learning, rats show a preference for the CS+ in 2-bottle tests and lick more for the CS+ than the CS- in progressive ratio tests (Sclafani and Ackroff, 2006).

Finally, lick analysis similar to the approach used in this study has shown that the emergence of a flavor preference is revealed in the modification of lick patterns. In a series of 23-h, 1-bottle tests, rats were presented with water, 2% sucrose, 2% maltose dextrin, 1% sucrose + 1% maltose dextrin, 0.2% saccharin and 1% sucrose + 1% maltose dextrin + 0.2% saccharin (Sclafani et al., 1998). Rat intake of the 1% sucrose + 1% maltose dextrin mixture was significantly greater than each of its components alone. However, the addition of 0.2% saccharin significantly increased total intake even more. This increase in intake was driven by an increase in bout size, but not bout number or lick rate.

The present study characterized the microstructure of ingestion 1) during 1- and 2-bottle presentations of fructose and saccharin, to assess the unconditioned response to two taste solutions of varying palatability (see Table 2). And 2) during the acquisition and expression of a CFTP by pairing palatable taste solutions with flavors of Kool-Aid in order to examine conditioned responses to flavors in rats with learned preferences (see Tables 3 and 4).

We employed two CFTP protocols. In the first (Baker et al., 2003; Baker et al., 2004; Golden and Houpt, 2007), one flavor (CS+; e.g., grape or cherry Kool-Aid) was paired in mixture with the sweet and highly preferred taste of 8% fructose (F) while a second flavor (CS-) was paired in mixture with the less-preferred taste of 0.2% saccharin (S) on 1-bottle conditioning days (CS+/F or CS-/S). The acquisition of the learned preference is then assessed with a 2-bottle preference test in which both flavors mixed with saccharin are presented simultaneously (CS+/S vs. CS-/S) (see Table 3). In the second protocol, one flavor (CS+) was paired in mixture with a concentrated 0.2% saccharin solution (US+) while a second flavor (CS-) was paired in mixture with a less-preferred dilute 0.05% saccharin solution (US-) on 2-bottle conditioning days (CS+/US+ vs. CS-/US-). This procedure is similar to one used by Holman (1975) who used 0.32% and 0.065% saccharin mixed in almond and banana extract. Again, acquisition was assessed with a 2-bottle preference test in which both flavors mixed with the dilute concentration of saccharin are presented simultaneously (CS+/US- vs. CS-/US-) (see Table 4). These two conditioning protocols differ in that fructose has a greater postigestive effect on drinking than either concentration of saccharin. Therefore, the CFTP protocol with fructose and saccharin used 2-h conditioning sessions to minimize the postigestive effects of fructose, while the CFTP protocol with saccharin alone used 23-h conditioning sessions.

Rats were tested in cages equipped with lickometers that recorded number of licks in 6-s bins at each of two bottles. In addition to total intake, we analyzed the number of bouts of licking during a test session, the size of bouts, the pattern of cumulative licks, average lick rate within bouts and the number of licks in the first minute of drinking.
2. Methods

2.1 Subjects

Male albino Sprague–Dawley rats (290–375 g, Charles River Laboratories, Wilmington, MA, USA) were housed individually in the Smith “hotel” apparatus under a 12:12-h light–dark cycle with Purina rat chow and water initially available ad libitum. All conditioning and testing took place in the rat’s home cage during the first half of the lights-on phase.

2.2 Description of the “Hotel” Apparatus

The “hotel” apparatus has been described in detail previously (Smith, 2000). Briefly, eight cages constitute a single “hotel”. One eating and two drinking ports are attached to opposite short walls of the rectangular polycarbonate cage. Licks are measured by the completion of a contact circuit each time the rat’s tongue touches the recessed sipper tube (~0.5 cm) of the lickometer. As the rat enters the feeding compartment its head breaks an infrared beam between an infrared light emitting diode (IR LED) and a photo detector and a computer records the beam breaks. Food consumption was not analyzed in these experiments, however.

2.6 Drinking Analysis

The number of licks within each 6-s bin was recorded by the computer, resulting in 1,200 six-second bins for each 2-h testing period in Experiments 1 and 2, and 13,800 bins for the 23-h of testing per day in Experiment 3. The number of licks in the first minute of testing and the cumulative licks across the test sessions were analyzed.

In addition, the total number of bouts, the length of bouts and lick rate within bouts across the test sessions were analyzed. The bout criteria for drinking has been described previously (Smith, 2000). Briefly, a potential drinking bout started when a rat made 3 licks in a six second bin. The bout had to contain at least 30 licks to be considered as a valid bout. The bout was considered to have ended if the rat stopped drinking for 50 six-second bins (5 min).

Total intakes were measured by weighing the bottles before and after each conditioning and testing session to the nearest 0.1 g. Significant differences were detected by two-way ANOVA with solution (CS+ vs. CS-) and test day as repeated measures, using the Newman-Keuls test for post hoc comparisons.

2.4 Experiment 1: Fructose vs. Sodium Saccharin

To determine the microstructure of an unconditioned preference, food-restricted rats (n = 6) were given a daily 2-h session with 1-bottle presentation of either a 8% fructose (F) or 0.2% sodium saccharin solution (S); after every four 1-bottle sessions, rats were given a 2-h, 2-bottle preference test (see Table 2).

Six days before testing began, the rats were placed on a food restriction schedule (11-15g rat chow / day) that maintained their body weights at approximately 90% of their
initial ad libitum weight through the entire experiment. Daily food ration (Powdered Purina Chow; PC Type 5001) was provided immediately after the end of the 2-h test sessions. Water was available ad libitum at all times. Rats were initially trained to approach the bottles rapidly by being presented with 8% maltose dextrin (BioServ, Frenchtown, NJ, USA) during a 2-h 1-bottle session. This training procedure was repeated daily for 5 days until all rats approached the sipper tubes with short latency (< 1 min). Maltose dextrin intake was not analyzed.

Rats received four 1-bottle sessions (2 h/day) with the 8% fructose (BioServ, Frenchtown, NJ, USA) solution presented on days 1 and 3, and the 0.2% sodium saccharin (Sigma, St. Louis, MO, USA) solution presented on days 2 and 4. This pattern of 4 days of 1-bottle sessions was repeated a total of 4 times in experiment 1. Thus rats received a total of 8 fructose sessions and 8 sodium saccharin sessions. During 1-bottle sessions, rats were presented with the solution bottle and an empty bottle; the positions of the solution bottle and empty bottle were alternated daily.

To measure taste preference, rats were given a 2-bottle preference test (2 h/day) with the fructose and sodium saccharin solutions after every fourth 1-bottle session.

2.5 Experiment 2: Acquisition and Expression of CFTP

Rats (n = 7) were conditioned to acquire a flavor-taste preference by pairing a one Kool-Aid flavor with a more preferred taste solution and a second Kool-Aid flavor with a less preferred taste solution (see Table 3). Rats were food restricted and were trained to approach the sipper tubes using 8% maltose dextrin as described in experiment 1. Water was available ad libitum at all times.

The conditioning solutions were 8% fructose or 0.2% sodium saccharin flavored with 0.05% unsweetened grape or cherry Kool-Aid (Kraft Foods North America, Inc., Rye Brook, NY, USA). Half of the rats received cherry-flavored fructose solution and grape-flavored sodium saccharin solution; the flavors were reversed for the remaining rats. In the 2-bottle preference tests, the cherry and grape flavors were each presented in a 0.2% sodium saccharin solution without fructose. The fructose-paired flavor is referred to as the CS+ and the sodium saccharin-paired flavor as the CS- because 8% fructose is preferred to 0.2% saccharin (Sclafani and Ackroff, 1994). CS+/F refers to the flavored fructose solution used in conditioning, and CS+/S refers to the same flavor in sodium saccharin solution used during 2-bottle preference tests. The CS-/S refers to the flavored sodium saccharin solution used in conditioning and preference testing.

Rats received four 1-bottle conditioning sessions (2 h/day) with the CS+/F solution presented on days 1 and 3, and the CS-/S solution presented on days 2 and 4. This pattern of 4 days of 1-bottle conditioning sessions was repeated a total of 4 times in experiment 2 to establish a CFTP. Thus rats received a total of 8 CS+/F sessions and 8 CS-/S sessions in experiment 2. During 1-bottle sessions, rats were presented with the solution bottle and an empty bottle; the positions of the solution bottle and empty bottle were alternated daily.

To measure the CFTP, rats were given a 2-bottle preference test (2 h/day) with the CS+/S and CS-/S solutions after every 4 conditioning sessions. The positions of the
grape- and cherry-flavored solutions were also alternated with each 2-bottle test. During the 2-bottle preference test the CS+/S and CS-/S solutions differed only in flavor; therefore increased intake of CS+ is a measure of a conditioned response acquired from the prior association of the flavor with fructose.

2.6 Experiment 3: saccharin/saccharin mediated CFTP

To characterize the microstructure of a conditioned flavor-taste preference, rats (n = 6) were given a daily 23-h session with a 2-bottle presentation of a Kool-Aid flavor paired with a more preferred 0.2% sodium saccharin solution (CS+) and a second Kool-Aid flavor paired with a less preferred 0.05% sodium saccharin solution (CS-) (see Table 4). Sodium saccharin was used as the sole unconditioned stimulus to avoid postdigestive effects during the long-term conditioning. After every four 1-bottle sessions, rats were given a 23-h, 2-bottle preference test. Food and water was available ad libitum at all times.

The conditioning solutions were 0.2% sodium saccharin or 0.05% sodium saccharin flavored with 0.05% unsweetened grape or cherry Kool-Aid. Half of the rats received the cherry-flavored 0.2% sodium saccharin solution and grape-flavored 0.05% sodium saccharin solution; the flavors were reversed for the remaining rats. In the 2-bottle preference tests, the cherry and grape flavors were each presented in a 0.05% sodium saccharin solution without fructose. The 0.2% sodium saccharin-paired flavor is referred to as the CS+ and the 0.05% sodium saccharin-paired flavor as the CS- because 0.2% saccharin is preferred to 0.05% sodium saccharin while the 0.05% flavored sodium saccharin solution is isopalatable to water (Golden & Houpt; see chapter 3). CS+/US+ refers to the flavored 0.2% sodium saccharin solution used in conditioning, and CS+/US- refers to the same flavor in the 0.05% sodium saccharin solution used during 2-bottle preference tests. The CS-/US- refers to the flavored 0.05% sodium saccharin solution used in conditioning and preference testing.

Rats received four 2-bottle conditioning sessions (23 h/day) with the CS+/US+ solution and the CS-/US- solution. This pattern of four days of 2-bottle conditioning sessions was repeated a total of three times. Thus rats received a total of twelve 2-bottle conditioning sessions. The positions of the solution bottles were alternated daily.

To measure the CFTP, rats were given a 2-bottle preference test (23 h/day) with the CS+/US- and CS-/US- solutions after every 4 days of 1-bottle access. The positions of the grape- and cherry-flavored solutions were also alternated with each 2-bottle test. During the 2-bottle preference test the CS+/US- and CS-/US- solutions differed only in flavor; therefore increased intake of CS+ is a measure of a conditioned response acquired from the prior association of the flavor with the higher concentration of saccharin.
3. Results

3.1 Experiment 1: Fructose vs. Sodium Saccharin

Across 1-bottle sessions, there was no significant difference between fructose and saccharin in total intake, bout size and number and first minute licks. Across the 16 days of 1-bottle presentations of fructose or saccharin, two-way ANOVAs showed that there was no significant effect of either solution or day and no significant interaction on 2-h intake, number of bouts or bout size (Figure 1A, B, C). For lick rate, there was a significant effect of solution [F(1,5)=96.63, p=0.0002], a significant effect of days [F(7,35)=3.45, p=0.007] and a significant interaction [F(7,35)=4.22, p<0.002] (Figure 1D). Lick rate was significantly greater for the fructose solution by the thirteenth day of conditioning and was significantly higher than the first day of the same solution. For the number of licks in the first minute of drinking, there was no effect of solution, no effect of days and no significant interaction (Figure 1E).

The cumulative lick curves for fructose and saccharin were very similar on the first day of 1-bottle presentations, with a gradual steady intake of both solutions throughout the 2-h presentation (Figure 2A). However, cumulative lick curves for fructose and saccharin on the last day of 1-bottle presentations were different during the initial 15 minutes of drinking (Figure 2B). The fructose cumulative lick curve shows rapid intake during the first 15 minutes while the saccharin cumulative lick curve shows a more gradual intake during the same time period. After the first 15 minutes, the cumulative lick curves for both solutions show a similar gradual intake throughout the remainder of the 2-h presentation.

During 2-bottle preference tests, total intake and bout size for fructose were greater than saccharin by the last preference test day, although there was no difference in bout number and lick rate. The number of first minute licks for saccharin significantly decreased by the second preference test day and remained low for the remainder of testing (Figure 3). Across the four 2-bottle preference test days, two-way ANOVAs showed that there was no effect of test solution, a significant effect of days [F(3,15)=17.09, p=0.00004] and no interaction for 2-h intake (Figure 3A). For the number of bouts there was no effect of test solution, a significant effect of days [F(3,15)=5.96, p=0.007] and no interaction (Figure 3B). For bout size, there was a significant effect of test solution [F(1,5)=11.55, p=0.02], no effect of test day and no interaction (Figure 3C). For lick rate, there was a significant effect of test solution [F(1,5)=13.29, p=0.01], no effect of days and no interaction (Figure 3D). For the number of licks in the first minute of drinking, there was a significant effect of test solution [F(1,5)=92.33, p=0.0002], no effect of test day and no interaction (see Figure 3E). Total intake and bout size of fructose was greater than saccharin by the fourth preference test day (p=0.055). There was no difference in lick rate between fructose and saccharin during 2- bottle testing (Figure 3D). Post hoc comparisons showed that the number of licks at the fructose bottle in the first minute remained constant (~250 licks/minute),
while the number of licks at the saccharin bottle decreased after the first test and were significantly lower on test days 10, 15 and 20 (~125 licks/minute; see Figure 3E).

The cumulative lick curves for fructose and saccharin during the first three 2-bottle preference tests were consistent with the pattern of total intake. For the first three preference test days there was little difference between fructose and saccharin intake (Figure 4A, B, C). However, during the fourth 2-bottle preference test fructose intake predominated, with the majority of intake within the first 30 minutes of the test followed by more gradual intake throughout the remainder of the 2-h presentation. This is in contrast to the saccharin intake, which was low, although steady, throughout the 2-h presentation (Figure 4D).

3.2 Experiment 2: Acquisition and Expression of CFTP

CS-/S total intake was significantly greater than CS+/F during the sixth conditioning day. CS-/S bout size was significantly greater than CS+/F during the majority of conditioning days, although there was no difference in bout number. However, lick rate for the CS+/F was significantly greater than CS-/S through most conditioning days (Figure 5). Two-way ANOVA across the 16 days of conditioning with CS+/F and CS-/S revealed a significant effect of solution [F(1,6)=25.25, p=0.002], a significant effect of days [F(7,42)=6.52, p=0.00003] and a significant interaction [F(7,42)=2.38, p=0.04] for total intake (Figure 5A). For bout number, there was no effect of solution, no effect of days and a significant interaction [F(7,42)=2.79, p=0.02] (Figure 5B). For bout size, there was a significant effect of solution [F(1,6)=17.56, p=0.006], a significant effect of days [F(7,42)=4.27, p=0.001] and no interaction (Figure 5C). For lick rate, there was a significant effect of solution [F(1,6)=80.57, p=0.0001], a significant effect of days [F(7,42)=10.25, p=0.0000] and a significant interaction [F(7,42)=13.71, p=0.0000] (Figure 5D). For licks in the first minute of drinking, there was no effect of solution, a significant effect of days [F(7,42)=8.92, p=0.000001] and no interaction (Figure 5E). Intake and bout size for CS-/S increased within 3 days of conditioning, while CS+/F intake only increased after 5 days of conditioning with no change in bout size. However, lick rate was significantly greater for the CS+/F in comparison to the CS-/S (Figure 5D). There was no difference between CS+/F and CS-/S in licks during the first minute of drinking, although the number of first minute licks for both solutions was significantly greater for the last day of conditioning in comparison to the first day (Figure 5E).

The cumulative lick curves for CS+/F and CS-/S were very similar on the first days of conditioning, with rapid intake of both solutions for the first 60 minutes and more gradual intake of the CS+/F throughout the remainder of the 2-h presentation. During the last days of conditioning, however, rats rapidly consumed the CS+/F solution at a high average rate throughout the first 15-20 minutes; intake rate of the CS-/S solution was much slower during that time, but remained steady for the remainder of the 2-h test (Figure 6).

CS+/S total intake and bout size were significantly greater for preference test days 3 and 4, although there was no difference in bout number, lick rate and first minute licks
(Figure 7). Two-way ANOVA across the four 2-bottle preference tests with CS+/S and CS-/S revealed a significant effect of solution \([F(1,6)=8.33, p=0.03]\), no effect of days, and a significant interaction \([F(3,18)=5.58, p=0.007]\) for total intake (Figure 7A). For bout number, there was no effect of solution, a significant effect of days \([F(3,18)=3.53, p=0.04]\), and no interaction (Figure 7B). For bout size, there was no effect of solution, no effect of days, and a significant interaction \([F(3,18)=6.67, p=0.003]\) (Figure 7C). For lick rate and the number of licks in the first minute of drinking, there was no effect of solution, no effect of days and no interaction (Figure 7D, E). CS+/S intake and bout size was significantly higher during the third and fourth preference tests (Figure 7A, C). There was no difference between CS+/S and CS-/S for lick rate (Figure 7D) or number of licks during the first minute of drinking (Figure 7E).

The cumulative lick curves for CS+/S and CS-/S during the 2-bottle preference tests were consistent with the pattern of total intake, with similar patterns for the two solutions during the first two preference tests. During the third and fourth preference tests, however, rats rapidly consumed the CS+/S solution at a high average rate throughout the 2-h test; intake rate of the CS-/S solution was much slower (Figure 8; for an example of an individual rat’s lick patterns see figure 9).

### 3.3 Experiment 3: saccharin/saccharin mediated CFTP

Although total intake, bout size and bout number was significantly greater for CS+/US+ over CS-/US- in, there was no significant difference in lick rate between the two solutions during conditioning (Figure 10). Two-way ANOVA across the 12 days of conditioning with CS+/US+ and CS-/US- revealed a significant effect of solution \([F(1,5)=214.77, p=0.00003]\), no effect of days and no interaction for intake (Figure 10A). For the number of bouts, there was a significant interaction of solution \([F(1,5)=143.17, p=0.00007]\), no effect of days and a significant interaction \([F(11,55)=5.77, p=0.000004]\) (Figure 10B). For bout size, there were significant effects of solution \([F(1,5)=147.19, p=0.00007]\), no effect of days \([F(11,55)=2.35, p=0.02]\) and no interaction (Figure 10C). For lick rate, there was no effect of solution, no effect of days and no interaction (Figure 10D). Intake, number of bouts, and bout size for CS+/US+ were significantly higher compared to CS-/US- on almost all days of conditioning (Figure 10 A, B, C).

The cumulative lick curves for the first and last conditioning days showed a consistent average intake of the CS+/US+ solution, with most intakes occurring during the lights-off period (Figure 11).

Although total intake and number of bouts were significantly greater for CS+/US- over CS-/US-, there was no significant difference in bout size and lick rate between the two solutions during preference testing (Figure 12). Two-way ANOVA across the 3 days of 2-bottle preference testing with CS+/US- and CS-/US- showed a significant effect of solution \([F(1,5)=58.95, p=0.0006]\), a significant effect of days \([F(2,10)=6.08, p=0.02]\), and no interaction for total intake (Figure 12A). For the number of bouts, there was a significant effect of solution \([F(1,5)=64.45, p=0.0005]\), no effect of days and no interaction (Figure 12B). For bout size or lick rate, there was no effect of solution, no effect of days and no interaction (Figure 12C, D). CS+/US- total intake and number of
bouts were significantly greater in comparison to the CS-/US- across all three preference test days (Figure 12A, B). There was no difference in bout size and lick rate between the two solutions during preference testing (Figure 12C, D). The cumulative lick curves were consistent with the overall intake of the solutions (Figure 13).

4. Discussion

This study was the first to use lickometers to examine the associative processes involved in the microstructure of ingestion during the acquisition and developing expression of sweet taste preferences and conditioned flavor-taste preferences over time. As expected, rats expressed a preference for the highly preferred sweet taste of 8% fructose over 0.2% sodium saccharin (Sclafani and Ackroff, 1994), developed a preference for a flavor paired in mixture with 8% fructose over a flavor paired in mixture with 0.2% sodium saccharin (Baker et al., 2003; Baker et al., 2004; Golden and Houpt, 2007; Sclafani and Ackroff, 1994) and developed a preference for a flavor paired in mixture with a concentrated (0.2%) sodium saccharin solution over a flavor paired with a dilute (0.05%) sodium saccharin solution that is isopalatable with water in rats (Holman, 1975). This study establishes that the addition of a Kool-Aid flavor the unconditioned stimuli allowing for rapid discrimination of fructose versus saccharin. This study also suggests that preferred flavors conditioned with a caloric US elicit a greater bout size in comparison to the less preferred flavors and that preferred flavors conditioned with a non-caloric US elicit a greater bout number in comparison to the less preferred flavors. These findings suggest that as the saliency of the taste and the caloric content increases, different processes are utilized that alter the ingestive response.

During 1-bottle presentations of fructose and saccharin there were no differences in the total intake, number of bouts, or bout size between solutions. Furthermore, no differences in total intake, number of bouts, or bout size were seen during the first three 2-bottle tests of fructose vs. saccharin. Only after 16 days of the 1-bottle presentations and three 2-bottle test days, was a preference for the fructose solution expressed during the fourth 2-bottle presentation. This preference was shown as an increase in both total intake and the bout size for fructose.

Although a preference for fructose was not evident from total intake during the first three 2-bottle tests, the analysis of the number of licks in the first minute of drinking revealed the expression of a preference for the fructose solution from the second 2-bottle presentation on. Interestingly, the first minute licks for the fructose solution remained unchanged during 2-bottle preference tests; rather it was the number of first minute licks for the saccharin that decreased starting on the second 2-bottle preference test day. The discrepancy between greater number of first minute licks vs. total intake of fructose and saccharin might reflect the initial evaluation of the solutions by the rats vs. their integrated drinking response across the 2-h test. The initial response to the two solutions in the first minute was to consume more fructose. As drinking continued, however, the postingestive accumulation of the more concentrated and caloric fructose solution might induce satiety that decreases overall intake relative to the saccharin solution and masks
the initial preference. Indeed, examination of the cumulative lick curves show the emergence of rapid initial fructose intake (within the first 15 minutes) followed by a plateau of slower licking, typical of satiating solutions (see Figure 2B). Conversely, saccharin intake shows a more constant rate of licking throughout the 2-h 1-bottle and 2-bottle tests.

Lick patterns vary depending on the unconditioned palatability and concentration of the solutions being presented. Non-caloric solutions tend to elicit a greater number of bouts than do caloric solutions and the opposite is true for bout length. The average bout size and the time between bouts for sucrose is much greater than either saccharin or water in long-term tests (Smith et al., 1987). As the concentration of sucrose increases from 0.03M to 1.0M bout size increases, bout number decreases and the lick rate within a bout increases (Smith, 2000). Palatability of one unconditioned preference can be altered by the addition of a second taste. For example, the lick patterns for a mixture of 1% sucrose and 1% maltose dextrin are different than for 2% solutions of each component alone. Adding 0.2% saccharin to the sucrose and maltose dextrin greatly increases bout size and length (Sclafani et al., 1998). These findings suggest that as the saliency of the taste and caloric content increases, different processes are utilized that alter the ingestive response.

Addition of a Kool-Aid flavor to the unconditioned stimuli changed both total intake and bout size. Although intake of both solutions increased across the 1-bottle conditioning days, CS-/S intake was significantly increased in comparison to the CS+/F by the fourth conditioning day (Figure 5A). The higher intake of CS-/S was correlated with larger bout size (Figure 5C), but with a higher lick rate of CS+/F across the 2-h trial compared to CS-/S (Figure 5D).

Interestingly, in experiment 1 1-bottle intakes of fructose and saccharin were never different, while in experiment 2 rats showed a differential response to the CS-/S and CS+/F in 1-bottle tests after relatively few trials. The addition of Kool-Aid may have served as a salient cue to the relative satiating potential of the CS-/S and CS+/F solutions, which allowed the rats to modify their total intake appropriately.

Although in 1-bottle tests rats consumed more CS-/S than CS+/F, when the satiating effects of fructose were removed in the 2-bottle tests, a preference for the CS+ was revealed after 12 conditioning days in both total intake and bout size. Analysis of cumulative licks for the four 2-h 2-bottle preference tests revealed little difference between the CS+/S and CS-/S on test days 1 and 2. However, on test days 3 and 4 there was a high rate of continuous licking at the CS+/S bottle, while licking at the CS-/S bottle plateaued almost immediately. Because rats did not immediately consume larger volumes of CS+/S during the first and second preference tests, this suggests that not only did the rats learn to identify and prefer the flavor that had previously been paired in mixture with fructose, the rats also learned that the solution was not satiating when mixed with saccharin.

The CFTP protocol using fructose and saccharin as unconditioned stimuli has several complications. The taste of fructose is preferred to saccharin, but 8% fructose is also nutritive and of higher osmotic concentration than 0.2% saccharin. The caloric content of fructose is not believed to contribute to flavor-taste conditioning because it
will not condition a preference when pumped directly into the gut (Sclafani and Ackroff, 1994). However, its postingestive effects cannot be ruled out (especially during long-term access). Also, rats must be food restricted and conditioned daily in multiple brief trials. Thus, the final experiment was an effort to increase the efficiency of the protocol and to remove the potential confounds of food restriction, restricted drinking sessions and potential postingestive effects. Rats had 23-h access to 2 bottles using a high concentration and low concentration of saccharin as taste stimuli. Rats expressed a preference for the CS+/US+ or CS+/US- to the CS-/US- during both 2-bottle conditioning and testing on all days as shown in total intake and bout number. Cumulative lick curves showed most drinking starting at dark onset (1800 hours) with drinking of the CS+/US+ or CS+/US- throughout the night during conditioning and testing respectively. However, licking at the CS-/US- bottle plateaued very early during both conditioning and testing.

Analysis of bout size revealed a higher bout size for the CS+/US+ than CS-/US- during conditioning days, but bout size was never significantly different for CS+/US- in comparison to the CS-/US- during preference testing. Thus, when saccharin at two different concentrations was used as the US, bout size correlated with saccharin concentration, while bout number correlated with preference. Conversely, when conditioning used fructose vs. saccharin, bout size correlated with preference. This suggests that flavor preference learning can interact with both orosensory processes and satiety processes to elevate intake of the preferred flavor.

Myers and Sclafani (2001a) suggest that an increase in the total intake of a caloric solution in sham feeding could be the extinction of learned inhibitory control over meal size that is based on the previous experience of the flavor being paired with satiating postingestive effects. Although this conclusion was based on a study of CFNP, our results are consistent with this conclusion. As in CFNP learning, we observed an increase in intake, and an increase in lick rate or bout size or bout number when the CS+ flavor that had previously been paired with 8% fructose is presented in 0.2% saccharin solution. The gradual emergence of the increased intake during expression tests suggests extinction of fructose-conditioned satiety.
**Table 2.** Experimental protocol for fructose/saccharin unconditioned taste preferences. Four days of 2-h, 1-bottle presentations followed by a 2-bottle preference test. This cycle is repeated four times.

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<tr>
<td><strong>Solutions</strong></td>
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<tr>
<td><strong>Day 1</strong></td>
<td>8% fructose</td>
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<tr>
<td><strong>Day 2</strong></td>
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<td>0.2% saccharin</td>
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<tr>
<td><strong>Day 3</strong></td>
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<tr>
<td><strong>Day 4</strong></td>
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<td>0.2% saccharin</td>
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<tr>
<td><strong>Preference test day 5</strong></td>
<td>0.2% saccharin</td>
<td>8% fructose</td>
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Table 3. Experimental protocol for fructose/saccharin conditioned flavor-taste preferences. Four days of 2-h, 1-bottle presentations followed by a 2-bottle preference test. This cycle is repeated four times.

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<tr>
<td><strong>Conditioning</strong></td>
<td><strong>day 1</strong></td>
<td><strong>day 2</strong></td>
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<tr>
<td><strong>day 1</strong></td>
<td>Cherry or grape Kool-Aid with 8% fructose</td>
<td>Empty bottle</td>
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<tr>
<td><strong>day 2</strong></td>
<td>Empty bottle</td>
<td>Grape or cherry Kool-Aid with 0.2% saccharin</td>
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<td><strong>day 3</strong></td>
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<tr>
<td><strong>Preference</strong></td>
<td>Cherry or grape Kool-Aid with 0.2% saccharin</td>
<td>Grape or cherry Kool-Aid with 0.2% saccharin</td>
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<td><strong>test day 5</strong></td>
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Table 4. Experimental protocol for saccharin/saccharin conditioned flavor-taste preferences. Four days of 23-h, 2-bottle presentations followed by a 2-bottle preference test. This cycle is repeated three times.

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<tr>
<td><strong>Conditioning</strong></td>
<td><strong>day 1</strong></td>
<td><strong>day 1</strong></td>
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<tr>
<td><strong>day 1</strong></td>
<td>Cherry or grape Kool-Aid with 0.2% saccharin</td>
<td>Grape or cherry Kool-Aid with 0.05% saccharin</td>
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<td><strong>Conditioning</strong></td>
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<td><strong>day 2</strong></td>
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<td><strong>Conditioning</strong></td>
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<td><strong>day 4</strong></td>
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<td><strong>Preference</strong></td>
<td><strong>test day 5</strong></td>
<td><strong>test day 5</strong></td>
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<tr>
<td><strong>test day 5</strong></td>
<td>Cherry or grape Kool-Aid with 0.05% saccharin</td>
<td>Grape or cherry Kool-Aid with 0.05% saccharin</td>
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</table>
Figure 1. Rats did not express a preference for either fructose or saccharin during exposure on alternating days. Total intake, bout number, bout size, lick rate and licks in the first minute were measured after a 2-h, 1-bottle presentation. There was no difference between fructose (black circles) and saccharin (white circles) for A. total intake, B. number of bouts or C. bout size, during the sixteen 1-bottle presentations. D. Lick rate was significantly greater for the fructose by day 13 of conditioning and significantly higher from the first day for fructose itself. Lick rate for the saccharin was significantly higher on conditioning day 9 in comparison to day 1. E. Analysis of licks during the first minute of drinking show that rats did not show a preference for fructose or saccharin during conditioning. * p < 0.05 vs. saccharin; † p < 0.05 vs. first day of the same solution.
Figure 2. Cumulative lick curves for fructose (heavy line) and saccharin (light line) were similar on the first and last days of 1-bottle presentations, with rapid intake within the first 15-30 minutes and more gradual intake throughout the remainder of the 2-h presentation.
Figure 3. Total intake and bout size of fructose was greater than saccharin by the fourth day of preference testing, but there was no difference for bout number. A. On the fourth preference test after 16 conditioning days, all rats consumed more fructose (black circles) than saccharin (white circles) (p = 0.055). B. Across the four 2-bottle preference test days, there was no difference for the number of bouts between fructose and saccharin. C. On the fourth preference test after 16 conditioning days, the bout size was greater for fructose in comparison to saccharin. D. Across the four 2-bottle preference test days, there was no difference for lick rate between fructose and saccharin. E. The number of first minute licks was significantly greater for fructose over saccharin by the second day of preference testing. The number of first minute licks was significantly lower for saccharin by the fourth day of preference testing in comparison to the first day of preference testing. * p < 0.05 vs. saccharin; † p < 0.05 vs. first day of the same solution.
Figure 4. Cumulative lick curves for fructose (heavy line) and saccharin (light line) during the first three 2-bottle preference tests were consistent with the pattern of total intake; in the fourth 2-bottle preference test fructose intake predominated, with the majority of intake within the first 30 minutes of the test.
Figure 5. During CFTP acquisition, total intake and bout size was significantly greater for the CS-/S (white circles) solution in comparison to the CS+/F (black circles) solution, but there was no difference for bout number. Total intake, bout number, bout size, lick rate and licks in the first minute were measured after a 2-h, 1-bottle presentation. A. Total intake for the CS-/S solution increased within 3 days of conditioning, while the CS+/F solution intake increased only after 5 days of conditioning. B. Across the sixteen 1-bottle conditioning days, there was no significant difference for the number of bouts between the CS-/S solution and the CS+/F solution. C. Bout size for the CS-/S solution increased within 3 days of conditioning and remained significantly greater across the remaining 1-bottle conditioning days. D. Lick rate was significantly greater for the CS+/F in comparison to the CS-/S across sixteen 1-bottle conditioning days. E. There was no difference between the CS+/F and CS-/S solutions on number of licks during the first minute of drinking during conditioning, although both solutions significantly increased in comparison to the first day of drinking. * p < 0.05 vs. preceding test of other solution; † p < 0.05 vs. first day of the same solution.
Figure 6. Cumulative lick curves for the CS+/F and CS-/S solutions were very similar on the first days of conditioning, with rapid intake of both solutions for the first 60 minutes and more gradual intake of the CS+/F solution throughout the remainder of the 2-h presentation. During the last days of conditioning, rats rapidly consumed the CS+/F solution at a high average rate throughout the first 15-20 minutes. Intake rate of the CS-/S solution was much slower during that time, but remained steady for the remainder of the 2-h test.
Figure 7. Total intake and bout size of CS+/S (black circles) was significantly greater than CS-/S solution (white circles) by the third day of preference testing, but there was no difference for bout number. A. On the third preference test after 12 conditioning days, all rats consumed significantly more of the CS+/S than the CS-/S. B. Across the four 2-bottle preference test days, there was no significant difference for the number of bouts between the CS+/S than the CS-/S. C. On the third preference test day after 12 conditioning days, bout size was significantly greater for the CS+/S in comparison to the CS-/S. Bout size of the CS+/S solution for the third preference test day was also significantly greater in comparison to the same solution on the first preference test day. D. There was no significant difference in lick rate during preference testing. E. There was no significant difference in first minute licks during preference testing. * p < 0.05 vs. CS-/S; † p < 0.05 vs. first day of the same solution.
Figure 8. Cumulative lick curves for the CS+/S and CS-/S solutions during the 2-bottle preference tests were consistent with the pattern of total intake. During the third and fourth preference test days, rats rapidly consumed the CS+/S solution while the intake rate of the CS-/S solution remained slow.
Figure 9. An example of an individual rat’s lick patterns for the CS+/S and CS-/S solutions during A. the first preference test day and B. the fourth preference test day.
Figure 10. During CFTP acquisition, total intake, bout number and bout size was significantly greater for the CS+/US+ (black circles) in comparison to the CS-/US- (white circles). Total intake, bout number, bout size and lick rate were measured after 23-h, 2-bottle conditioning. A. Total intake for the CS+/US+ was significantly greater in comparison to the CS-/US- across all twelve conditioning days. B. Across the twelve 2-bottle conditioning days, the number of bouts for the CS+/US+ was significantly greater in comparison to the CS-/US-. Bout number for the CS+/US+ was significantly greater on conditioning days 7, 8, 9, 12 and 13 and was significantly lower for the CS-/US- on conditioning days 2-14 in comparison to the same solution on conditioning day 1. C. Bout size for the CS+/US+ was significantly greater in comparison to the CS-/US- during ten of the twelve conditioning days. D. There was no significant difference in lick rate during 23-h, 2-bottle conditioning. * p < 0.05 vs. CS-/US-; † p < 0.05 vs. first day of the same solution.
Figure 11. Cumulative lick curves for the first and last conditioning days showed a consistent average intake of the CS+/US+ solution, with most intake occurring during the lights-off period.
Figure 12. During CFTP preference testing, total intake and bout number was significantly greater for the CS+/US- (black circles) in comparison to the CS-/US- (white circles), but there was no difference in bout size. Total intake, bout number, bout size and lick rate were measured after 23-h, 2-bottle preference testing. A. Total intake for the CS+/US- was significantly greater in comparison to the CS-/US- across all three 2-bottle preference test days. B. Across the three 2-bottle preference test days, the number of bouts for the CS+/US- was significantly greater in comparison to the CS-/US-. C. There was no difference in bout size between the CS+/US- and the CS-/US- during preference testing days. D. There was no significant difference in lick rate across the three days of preference testing. * p < 0.05 vs. CS-/US-
Figure 13. Cumulative lick curves were consistent with preference test day total intake of the CS+/US- and CS-/US- solutions.
CHAPTER 2

NMDA RECEPTOR IN CONDITIONED FLAVOR-TASTE PREFERENCE LEARNING: BLOCKADE BY MK-801 AND ENHANCEMENT BY D-CYCLOSERINE.

1. Introduction

This chapter has been published as Golden, GJ, Houpt, TA. NMDA receptor in conditioned flavor-taste preference learning: blockade by MK-801 and enhancement by D-cycloserine. Pharmacology, Biochemistry and Behavior 2007;86:587-596.

Conditioned flavor-taste preference (CFTP) is a form of associative learning in which an animal comes to prefer a neutral flavor after it has been paired with a preferred taste. CFTP learning is mediated by orosensory stimuli (i.e. CFTP can be acquired by rats sham-drinking the flavors and tastants, in which the postingestive effects are minimized (Sclafani and Ackroff, 1994)), rapidly acquired after only a few trials, and very resistant to extinction (Baker et al., 2003; Baker et al., 2004). In one model of CFTP learning (Baker et al., 2003; Baker et al., 2004), one flavor (the conditioned stimulus or CS+; e.g., cherry or grape Kool-Aid) is paired in mixture with the sweet and highly preferred taste of fructose (F; the unconditioned stimulus or US) while a second flavor (the CS-) is paired in mixture with the less preferred taste of saccharin (S) on 1-bottle conditioning days (CS+/F or CS-/S). The acquisition of the learned preference is then assessed with a 2-bottle preference test in which both flavors mixed with saccharin are presented simultaneously (CS+/S vs. CS-/S).

Although CFTP learning is common and robust, its neural substrates are not well characterized. The specific olfactory and gustatory relays and the associative brain regions necessary for CFTP learning are unknown. While chemical mediators of reward properties of the CS and US have been identified (e.g. dopamine receptors; (Baker et al., 2003; Baker et al., 2004; Yu et al., 1999; Yu et al., 2000a, b)), the associative mechanisms underlying the formation of the CFTP itself have not been explored.

One candidate molecule is the N-methyl-D-aspartate (NMDA) glutamate-glycine receptor (NR). NR is widely distributed throughout the CNS, but is particularly dense in highly plastic regions involved in learning (e.g. cortex, limbic system, and cerebellum) (Monyer et al., 1994). NR activation and the resulting calcium influx play a critical role in neural plasticity in vitro as a synaptic coincidence detector (e.g. in LTP) (Cain, 1997).

NR has previously been identified as a participant in olfactory and taste learning (Barkai and Saar, 2001; Jimenez and Tapia, 2004). For example, during conditioned taste aversion learning (CTA), exposure to a novel taste (but not a familiar taste) results in increased phosphorylation of the NR2B subunit in the insular cortex, which parallels the
high saliency of novel tastes in CTA learning (Rosenblum et al., 1997). NR activity is required in CTA learning, because antagonism of the NR in the insular cortex by injection of the competitive NR antagonist 2-amino-5-phosphono pentanoic acid (APV) impairs CTA learning (Rosenblum et al., 1997).

NR activity in the amygdala is also required for odor-potentiated CTA. Pretreatment with the competitive NR antagonist APV impaired potentiated taste-odor aversion learning (i.e., saccharin presented with an almond odor and paired with LiCl injection), although in odor-alone and taste-alone conditioning, aversion learning was not impaired (Willner et al., 1992).

The present study was conducted in order to establish a role for NR in CFTP learning. Two approaches were taken. First, a necessary role for the NR was established using systemic injections of MK-801, a non-competitive antagonist. Systemic MK-801 has been used to attenuate other forms of learning, such as olfactory discrimination learning for water reward in weanling (Griesbach et al., 1998) and adult rats (Quinlan et al., 2004). A complication of systemic MK-801 treatment that must be controlled for in behavioral experiments, however, is the induction of “non-specific” or aversive side effects at high doses (Sharp et al., 2001).

Second, the contribution of activity at the glycine-binding site of the NR was assessed by administration of D-cycloserine (DCS), a high-affinity glycine agonist. In addition to glutamate binding, NR channel opening requires agonist binding to a glycine-binding site. Endogenous ligands for the glycine-binding site of the NR include glycine of neural origin or D-serine synthesized by astrocytes. For example, neuronal migration in the developing cerebellum is dependent on serine racemase in glia to convert L-serine to D-serine, which together with glutamate potentiates NR activity in granule neurons (Kim et al., 2005).

By stimulating NR at the time of learning, exogenous DCS can potentiate learning in behavioral studies. For example, DCS has been shown to enhance spatial learning in a water maze (Riekkinen and Riekkinen, 1997) and to accelerate extinction in rats after fear conditioning with footshock (Walker et al., 2002). Potentiation of NR activity and learning by DCS treatment has been interpreted as indicating that endogenous levels of glycine or D-serine are less than optimal and thus limit NR activation; exogenous DCS can then raise NR activation to an optimal level for learning.

To assess the effects of MK-801 and DCS on CFTP, rats were given injections of the drugs prior to a daily, 2-h pairing of Kool-Aid flavors mixed with either highly preferred fructose (CS+/F) or less-preferred saccharin (CS-/S). CFTP was assessed after every 4 conditioning days in 2-h, 2-bottle preference tests with both Kool-Aid flavors containing saccharin (CS+/S vs. CS-/S). We found that MK-801 blocked acquisition of CFTP, but not expression of a previously learned CFTP. To rule out non-specific or aversive effects that might oppose or mask CFTP learning, control experiments demonstrated that MK-801 under our conditions did not reduce unconditioned intake, induce a conditioned taste aversion (CTA), or lead to state-dependent learning.

Conversely, DCS administered prior to conditioning potentiated the acquisition of CFTP, by both accelerating the rate of learning and increasing the magnitude of CFTP
expression. Because CFTP learning is very resistant to extinction, we therefore tested the effects of DCS pretreatment on reversal conditioning rather than on unreinforced extinction trials. In contrast to its effects on acquisition, DCS did not enhance the rate or magnitude of reversal CFTP learning.

2. Methods

2.1 Subjects

Male albino Sprague–Dawley rats (290–375 g, Charles River Laboratories, Wilmington, MA, USA) were housed individually in polycarbonate cages under a 12:12-h light-dark cycle with Purina rat chow and water available ad libitum. All testing took place in the rat’s home cage during the first half of the lights-on phase. Six days before testing began, the rats were placed on a food restriction schedule (15-20g rat chow / day) that maintained their body weights at approximately 90% of their initial ad libitum weight through the entire experiment. Water was available ad libitum at all times. Rats were initially trained to drink 8% maltose dextrin (BioServ, Frenchtown, NJ, USA) during a 2-h one-bottle session. This conditioning procedure was repeated daily for 5 days until all rats approached the sipper tubes with short latency (< 1 min). The daily food ration was given after each conditioning session.

2.2 Conditioning Solutions

The conditioning solutions were 8% fructose (BioServ, Frenchtown, NJ, USA) or 0.2% sodium saccharin (Sigma, St. Louis, MO, USA) flavored with 0.05% unsweetened grape or cherry Kool-Aid (Kraft Foods North America, Inc., Rye Brook, NY, USA). Half of the rats in each group received cherry-flavored fructose solution and grape-flavored saccharin solution; the flavors were reversed for the remaining rats. In the 2-bottle preference tests, the cherry and grape flavors were each presented in a 0.2% saccharin solution without fructose. The fructose-paired flavor is referred to as the CS+ and the saccharin-paired flavor as the CS- because 8% fructose is preferred to 0.2% saccharin (Sclafani and Ackroff, 1994). CS+/F refers to the flavored fructose solution used in conditioning, and CS+/S refers to the same flavor in saccharin solution used during 2-bottle preference tests. The CS-/S refers to the flavored saccharin solution used in conditioning and preference testing.

Intakes were measured by weighing the bottles before and after each session to the nearest 0.1 g. Significant differences were detected by ANOVA, using the Newman-Keuls test for post hoc comparisons. Data are presented as mean intake ± standard error of the mean.

2.3 Acquisition and Expression of CFTP

Rats received 4 one-bottle conditioning sessions (2 h/day) with the CS+/F solution presented on days 1 and 3, and the CS-/S solution presented on days 2 and 4. This pattern of 4 days of one-bottle conditioning sessions was repeated a total of 3 times in experiment 1 and 4 times in experiments 4 and 5 to establish a CFTP. Thus rats
received a total of 6 CS+/F sessions and 6 CS-/S sessions in experiment 1 and 8 CS+/F sessions and 8 CS-/S sessions in experiments 4 and 5 (see Table 5). During one-bottle sessions, rats were presented with the solution bottle and an empty bottle; the positions of the solution bottle and empty bottle were alternated daily.

To measure the CFTP, rats were given a 2-bottle preference test (2 h/day) with the CS+/S and CS-/S solutions. The positions of the grape- and cherry-flavored solutions were also alternated with each 2-bottle test. During the 2-bottle preference test the CS+/S and CS-/S solutions differed only in flavor; therefore increased intake of CS+ is a measure of a conditioned response acquired from the prior association of the flavor with fructose.

2.4 Experiment 1: MK-801 and CFTP Acquisition

Naive rats (n=16) were placed on food restriction and trained with 2-h maltose dextrin access as described above. Rats were divided into two groups. The rats in the first group (MK-801 group, n = 8) received a MK-801 injection (Sigma, St. Louis, MO, USA; 100 µg in 1ml/kg ip) while rats in the second group (vehicle group, n = 8) received a vehicle injection (0.15 M NaCl, 1 ml/kg ip). The dose of MK-801 was selected based on efficacy as an NR antagonist in other learning paradigms, without inducing noticeable motoric side effects (Griesbach et al., 1998; Stuchlik et al., 2004; Weldon et al., 1997). Thirty minutes after the injections, rats were given 2-h access to either the CS+/F or CS-/S solution in a one-bottle conditioning session (see Table 5).

Following four days of conditioning, both groups received vehicle injections; 30 min later, all rats were given a 2-h, 2-bottle preference test with the CS+/S and CS-/S solutions. This was repeated 3 times so that all rats received a total of 6 CS+/F and 6 CS-/S conditioning days interspersed with 3 CS+/S vs. CS-/S 2-bottle preference test days.

2.5 Experiment 2: MK-801 and CFTP Expression

To examine the effects of MK-801 on the expression of preference and to determine if preference learning in the MK-801-treated rats was state-dependent, the rats (n= 16) from experiment 1 were administered MK-801 (100 µg in 1ml/kg ip) or vehicle (0.15 M NaCl, 1 ml/kg ip) 30-min prior to a 2-h, 2-bottle preference test of CS+/S vs. CS-/S. Rats were tested on 2 consecutive days. All rats received both MK-801 and vehicle injections, counterbalanced across days and groups.

2.5 Experiment 3: MK-801 and CTA

The rats (n=16) from experiment 1 and 2 were used to determine if treatment with MK-801 during CFTP conditioning had caused the acquisition of a CTA. Rats received no injections during this experiment, and were given ad libitum access to rodent chow. Rats were given 2-bottle preference tests (23 h/day) of Kool-Aid-flavored saccharin solutions vs. water for 4 consecutive days. All rats had two consecutive days of access to grape-flavored saccharin and two consecutive days of access to cherry-flavored saccharin (i.e. 2 days of CS+/S vs. water and 2 days of CS-/S vs. water.) The positions of the
solution bottle and the water bottle were alternated daily. Both intake and flavored-saccharin preference (flavored saccharin intake / total intake) were analyzed.

2.6 Experiment 4: DCS and CFTP Acquisition

Naive rats (n=23) were placed on food restriction and trained with 2-h maltose dextrin access as described above. Rats were divided into two groups. The rats in the first group (DCS group, n = 11) received a DCS injection (Sigma, St. Louis, MO, USA; 15mg in 1ml/kg ip) while rats in the second group (vehicle group, n = 12) group received a vehicle injection (0.15 M NaCl, 1 ml/kg ip). The dose of DCS was selected based on its efficacy at enhancing other forms of learning without adverse effects such as conditioned taste aversion (Andersen et al., 2002; Walker et al., 2002). Sixty minutes after the injections, rats were given 2-h access to either the CS+/F or CS-/S solution in a one-bottle conditioning session (see Table 5). Following four days of conditioning, both groups received vehicle injections; 60 min later, all rats were given a 2-h, 2-bottle preference test with the CS+/S vs. CS-/S solutions presented simultaneously. Intake was measured after 2 h. This was repeated four times so that all rats received a total of 8 CS+/F and 8 CS-/S conditioning days interspersed with 4 CS+/S vs. CS-/S 2-bottle test days.

To examine extinction, rats were then given 2-bottle preference tests (2 h/day) of CS+/S vs. CS-/S for 7 consecutive days without injections.

2.7 Experiment 5: DCS and CFTP Reversal Conditioning

DCS has been shown to accelerate extinction in other models of learning. Because CFTP is very resistant to extinction, the effects of DCS on reversal of CFTP were examined. Naive rats (n=18) were placed on food restriction and trained with 2-h maltose dextrin access as described above. A preference for the flavor paired with fructose was established in all rats as described above with 8 CS+/F and 8 CS-/S sessions. Three 2-h, 2-bottle preference tests with CS+/S vs. CS-/S were interspersed on days 9, 14, and 19 to measure the expression of the CFTP during acquisition. On the last 2-bottle preference test day (after 8 CS+/F and 8 CS-/S conditioning days), rats showed a CFTP of 0.8 ± 0.1 (expressed as the ratio of CS+/S over total 2-h intake).

After the CFTP was established, rats underwent reversal conditioning by reversing the pairing of fructose and CS solutions. Rats were divided into two groups. The rats in the first group (DCS group, n = 9) received a DCS injection (Sigma, St. Louis, MO, USA; 15mg in 1ml/kg ip) while rats in the second group (vehicle group, n = 9) received a vehicle injection (0.15 M NaCl, 1 ml/kg ip). Sixty minutes after the injections, rats were given 2-h access to either the rCS+/F (i.e., their old CS+ flavor now paired with 8% fructose) or rCS-/S (i.e., their old CS+ flavor now paired with 0.2% saccharin) in a one-bottle conditioning session. Following four days of conditioning, both groups received vehicle injections; 60 min later, all rats were given a 2-bottle preference test (2 h) with the rCS+/S and rCS-/S solutions. Intake was measured after 2 h. This was repeated four times so that all rats received a total of 8 rCS+/F and 8 rCS-/S conditioning days interspersed with 4 rCS+/S vs. rCS-/S 2-bottle test days.
3. Results

3.1 Experiment 1: MK-801 and CFTP Acquisition

To determine if NMDA neurotransmission is required for CFTP learning, food-restricted rats were injected with either vehicle (0.15 M NaCl, 1 ml/kg i.p., n= 8) or MK-801 (100 µg/kg, n = 8) 30 min before a daily 2-h conditioning session with either CS+/F or CS-/S. Although CS+/F intake was relatively stable across the 12 days of conditioning, there was a significant interaction of drug treatment and days [F(4,56) = 4.0, p < 0.01; see Figure 14A]. (Intakes were not recorded on days 3 and 7 due to technical errors.) Vehicle-treated rats drank significantly more CS+/F than MK-801-treated rats only on conditioning day 8. From day 2 to day 14, CS-/S intake increased significantly for both the vehicle and MK-801 groups, with a significant effect of days [F(4,56) = 28.7, p < 0.001] but not drug treatment (see Figure 14B).

The vehicle group acquired a CFTP after 8 conditioning sessions as shown by increased CS+ intake in the first 2-bottle preference test. Rats in the MK-801 group, however, did not acquire a CFTP after 12 conditioning sessions as shown by equal intake of the CS+/S and CS-/S in all three 2-bottle preference tests (see Figure 15A). Within the treatment groups, two-way ANOVAs on CS+/S vs. CS-/S intake across the three 2-bottle preference test days using test solution and test day as factors showed a significant interaction of test solution and days for the vehicle group (F[2,28]=14.39, p<0.05), but no significant effects for the MK-801 group. Thus, while vehicle-treated rats acquired a CFTP within 8 conditioning days, MK-801 treatment completely blocked CFTP acquisition.

When CS+/S and CS-/S intakes were compared between the MK-801 and vehicle groups on the final 2-bottle test day (see Figure 15B), there was a significant interaction of group and solution (F[1,28]= 25.02, p < 0.05). Post hoc comparisons showed that CS+/S intake was greater than CS-/S intake the vehicle group but not the MK-801 group; furthermore, the intake of CS+/S by the vehicle group was greater than CS+/S intake by the MK-801 group, and CS-/S intake by the vehicle group was less than CS-/S intake by the MK-801 group.

3.2 Experiment 2: MK-801 and CFTP Expression

The effect of MK-801 on CFTP expression was tested on the rats from experiment 1. MK-801 injected 30 min prior to a 2-bottle preference test did not alter CS+/S or CS+/S intake in either MK-801 or vehicle groups (see Figure 16). A two-way ANOVA with drug pretreatment and solution as factors showed no effect of drug pretreatment, but a significant effect of solution (F[1,28] = 28.18, p<0.001). Whether treated with MK-801 or vehicle immediately prior to the 2-bottle preference tests, there was no significant difference between CS+/S and CS-/S intakes in the MK-801 group. Conversely, the vehicle group drank significantly more CS+/S than CS-/S, regardless of pretreatment with MK-801 or vehicle immediately prior to the 2-bottle preference tests.

Thus CFTP learning in the MK-801 group was not dependent on MK-801 injection prior to expression testing (i.e. was not state-dependent learning). Furthermore,
MK-801 had no effect on expression of a previously acquired CFTP in drug-naïve rats, nor did it significantly affect overall intake. These results suggest a specific effect on CFTP acquisition of this dose of MK-801 (100 µg/kg); because only a single dose of MK801 was tested, however, we cannot completely rule out a role for NMDA receptors in intake or CFTP expression.

3.3 Experiment 3: MK-801 and CTA

Rats from Experiments 1 and 2 were given access to Kool-Aid/saccharin vs. water in 24-h, 2-bottle preference tests across 4 days (2 days of CS+/S vs. water and 2 days of CS-/S vs. water.) Kool-Aid/saccharin intake was higher than distilled water intake for both grape and cherry flavors in both the MK-801 group and vehicle group (see Figure 17). Two-way ANOVAs on Kool-Aid-saccharin preferences and intakes across the four 24-h, 2-bottle preference test days using group and solution (CS+/S and CS-/S) as factors showed no significant effects. (F[1,27] = 2.35, p = 0.13 for preference, F[1,27] = 2.55, p = 0.12 for intake). Although the vehicle group showed a CFTP for CS+/S when the CS+/S and CS-/S were presented simultaneously in Experiments 1 and 2, both Kool-Aid/saccharin solutions were highly preferred to water when presented individually.

Thus, MK-801-treated rats did not show a CTA to the Kool-Aid flavors. Therefore the absence of a CFTP for CS+/S in the MK-801 group cannot be ascribed to an opposing CTA induced by the drug treatment.

3.4 Experiment 4: DCS and CFTP Acquisition

To determine if the glycine-binding site of the NR contributes to CFTP learning, food-restricted rats were administered vehicle (0.15 M NaCl, 1 ml/kg i.p., n = 11) or DCS (15 mg/kg, i.p., n = 11) 60 minutes before a daily 2-h conditioning session with either CS+/F or CS-/S as in experiment 1. Across the 16 conditioning days, there was a significant increase in both CS+/F intake [main effect of days, F(7,147) = 14.27, p <0.001] and CS-/S intake [main effect of days, F(7,147) = 25.99, p <0.001]. There was no significant effect of drug treatment on either CS+/F or CS-/S intake during conditioning (see Figure 18).

DCS pretreatment during conditioning enhanced CFTP acquisition compared to vehicle-treated rats (see Figure 19). Both DCS and vehicle groups acquired a CFTP after 8-12 conditioning sessions as shown by increased CS+ intake in 2-bottle test sessions (see Figure 19A). Two-way ANOVAs on CS+ and CS- intake across the four 2-bottle test days during conditioning revealed a significant interaction of CS+/CS- treatment and days for both groups (F[3,63] = 23.8, p < 0.0001 for DCS group, F[3,63]=9.3, p < 0.0001 for vehicle group). Post hoc tests revealed that CS+ intake was higher than CS- intake for the DCS group on the second 2-bottle test (after 8 conditioning sessions) and for the vehicle group on the third 2-bottle test (after 12 conditioning sessions). This suggests that DCS-treated rats acquired the CFTP faster than the vehicle-treated rats.

When CS+/S and CS-/S intakes were compared between the DCS and vehicle groups on the final 2-bottle test day (see Figure 19B), there was a significant interaction of group and solution (F[1,40]= 5.62, p < 0.05). Post hoc comparisons showed that CS+/S
intake was greater than CS-/S intake in both groups, but the intake of CS+/S by the DCS group was greater than CS+/S intake by the vehicle group.

During unreinforced extinction testing with CS+/S and CS-/S in 2-bottle preference tests (2 h/day), both DCS-treated and vehicle-treated rats showed a higher intake of CS+ than CS- (See Figure 19C). Two-way ANOVAs on CS+ and CS- intake across the seven 2-bottle test days during extinction testing revealed a significant effect of solution but not days within each group (F[1,6]=62.15, p < 0.0001 for DCS group, F[1,6] = 23.32, p < 0.0001 for vehicle group), such that DCS-treated and vehicle-treated rats consumed more CS+ than CS- on all days with no apparent extinction. Thus a CFTP does not extinguish rapidly, if at all. A two-way ANOVA comparing CS+ intakes of DCS and vehicle groups across the seven 2-bottle test days revealed a significant effect of days (F[6,126] = 3.6, p < 0.002) but not groups, such that DCS-treated rats consumed more CS+ than vehicle-treated rats only in the first 2-bottle preference test. Thus there was a transient enhancement of CFTP by DCS that persisted briefly into extinction.

3.5 Experiment 5: DCS and CFTP Reversal Conditioning

CFTP learning is very resistant to extinction. Therefore, the effects of DCS on reversal conditioning, rather than extinction, were examined. Rats (n=18) were conditioned with CS+/F and CS+/S in daily 2-h conditioning sessions over 16 days as above.

Both DCS and vehicle groups acquired a CFTP after 16 conditioning sessions as shown by higher CS+ intake than CS- intake for both groups in a 2-bottle preference test prior to reverse conditioning. (t-test; p<0.001 for DCS, p<0.001 for vehicle; see Figure 28, test day 0).

Reversal conditioning abolished the CFTP in both DCS and vehicle groups after 16 conditioning sessions as shown by decreased CS+ and increased rCS+ intake in 2-bottle preference tests (see Figure 20, test days 1-4). Two-way ANOVAs on rCS+ and rCS- intake across the 2-bottle preference test days revealed a significant interaction of rCS+/rCS- treatment and days for both groups (F[4,64]=6.29, p < 0.0005 for DCS group, F[4,64] = 9.14, p < 0.0001 for vehicle group). Pretreatment with DCS prior to reversal conditioning did not reverse the CFTP any faster than the vehicle pretreatment. A two-way ANOVA comparing rCS- intakes in DCS and vehicle-treated groups across the five 2-bottle preference test days revealed a significant effect of test day (F[4,64] = 7.58, p<0.0001) but not drug pretreatment. Thus DCS did not potentiate reversal learning.

4. Discussion

This study examined the role of NR in CFTP learning using systemic injections of MK-801, a noncompetitive NR antagonist, and DCS, a NR glycine-site agonist. MK-801 or DCS was administered prior to 1-bottle conditioning trials with Kool-Aid flavors in solution with either highly preferred fructose (CS+/F) or the less preferred saccharin (CS-/S). MK-801 blocked CFTP acquisition, while DCS accelerated CFTP acquisition. Thus,
our results show that NR is necessary for CFTP learning and that the NR glycine-site contributes to CFTP learning.

As expected, rats reliably developed a preference for a flavor paired with 8% fructose over a flavor paired with 0.2% sodium saccharin (Baker et al., 2003; Baker et al., 2004; Sclafani and Ackroff, 1994). Although fructose has osmotic and caloric properties in addition to a preferred taste, the primary reinforcing effect of fructose is attributable to the NR glycine-site (Sclafani and Ackroff, 1994; Sclafani et al., 1993; Sclafani et al., 1999). Thus, gastric infusion of fructose is a weak US for conditioning a flavor preference (Ackroff et al., 2001; Sclafani et al., 1993; Sclafani et al., 1999), while sham-feeding of flavors in sweet solutions is sufficient to form flavor preferences (Myers and Sclafani, 2001a; Yu et al., 1999; Yu et al., 2000a, b).

When administered 30 min prior to conditioning trials, systemic injections of MK-801 blocked the acquisition of a CFTP. Thus NR neurotransmission is necessary for CFTP learning. Follow-up experiments ruled out several alternate explanations for these findings: MK-801 pretreatment did not induce a CTA; MK801 did not have an acute effect on ingestion that might have compromised CFTP learning; and no evidence was found for state-dependent learning.

It has been shown that lower doses of MK-801 (50 – 100 µg/kg ip) do not induce CTA, although a higher dose of MK-801 (200 µg/kg ip) paired with a novel taste can cause CTA acquisition (Bienkowski et al., 1998). The failure of MK-801-treated rats to increase CS+ intake during conditioning, therefore, might have been due to CTA acquisition rather than CFTP blockade. In order to verify that MK-801 did not reduce CS+/S intake by inducing a CTA in the MK-801-treated rats, the conditioned rats were given 23-h, 2-bottle tests of water vs. CS+/S or CS-/S for 4 consecutive days (see Figure 17). Both MK-801- and vehicle-treated rats showed a 90% or higher preference for the Kool-Aid flavor mixed with saccharin over water. Thus the reduced CS+/S intake seen in the MK-801 group was not due to the acquisition of a CTA.

MK-801 can also have acute behavioral effects that might have altered intake during conditioning days, and thus confounded CFTP learning. For example, MK-801 can induce ataxia and other locomotor effects that might reduce intake (Frantz and Van Hartesveldt, 1999). Conversely, MK-801 has been shown to increase the intake of highly palatable foods in fed rats and regular chow in fasted rats (Burns and Ritter, 1997) or at dark onset (Jahng and Houpt, 2001). After MK-801 pretreatment, however, we did not observe any ataxia, and all rats showed a very short latency to start drinking (< 1 min). Furthermore, the volume of CS+/F and CS-/S intakes during CFTP conditioning did not differ between MK-801- and vehicle-treated rats (data not shown).

The effects of MK-801 were specific to acquisition, because MK-801 pretreatment of rats with a previously acquired CFTP did not alter CFTP expression during 2-bottle preference tests (see Figure 16). Thus, while the NR is necessary for CFTP acquisition, it is not necessary for CFTP expression.

The same data indicate that MK-801 had no state-dependent effects on CFTP learning. MK-801 has been shown to support state-dependent learning in other models. For example, rats treated with MK-801 (100 µg / kg ip) during place-footshock aversion
conditioning appeared unable to express a place aversion when tested drug-free (Harrod et al., 2001). When tested immediately after MK-801 injection, however, the rats did express a place aversion. Our results contrast with the place aversion results: rats treated with MK-801 prior to CFTP acquisition did not express a CFTP during 2-bottle preference tests with or without MK-801 injection before the expression test.

The effect of MK-801 on CFTP learning are consistent with other reports that NR blockade impairs or inhibits olfactory learning in several paradigms. Systemic injections of MK-801 have impaired olfactory-water reward discrimination learning and its reversal (Griesbach et al. 1998), and olfactory-tactile preference learning in neonatal rats (Weldon et al., 1997). In addition, central administration of the competitive NR antagonist APV has identified critical sites for olfactory associations in different paradigms. APV infused into the prefrontal cortex (but not hippocampus) impaired memory of an odor-food reward association when infused in the prefrontal cortex (Tronel and Sara, 2003). APV infused into the basolateral amygdala disrupted learning in taste-potentiated odor-LiCl aversion (Ferry and Di Scala, 2000; Hatfield and Gallagher, 1995) and in odor-footshock fear conditioning (Walker et al., 2005).

In a second approach to exploring the role of NR, we found that systemic DCS enhanced CFTP learning. Our findings are in agreement with other studies in which DCS accelerated the rate of learning. For example, DCS has been shown in rats to enhance acquisition of spatial water-maze learning (Riekkinen and Riekkinen, 1997), inhibitory avoidance (Land and Riccio, 1999), and the extinction of fear-conditioning (Parnas et al., 2005) or fear-potentiated startle response (Walker et al., 2002). Relevant to gustatory learning, we have recently demonstrated that DCS potentiated acquisition of CTA (Houpt et al., 2005). DCS is also effective in the extinction of fear in phobic human subjects undergoing behavior modification (Ressler et al., 2004); phobic patients treated with DCS in combination with exposure therapy were shown to have greatly reduced symptoms of acrophobia that persisted for at least 3 months after treatment.

In this study, DCS-treated rats acquired a CFTP faster than the vehicle-treated rats. DCS also increased the magnitude of CFTP learning. The enhancement by DCS persisted transiently into extinction. Because the half-life of DCS is approximately 2 hours in rats (Baran et al., 1995), it is unlikely that the transient enhancement was due to any residual drug effect.

These results suggest that the NR glycine-binding site contributes to CFTP learning. Because exogenous DCS augments CFTP learning, endogenous glycine or D-serine may be a limiting factor in NR activation.

Unlike its enhancement of extinction in other models of learning (Ledgerwood et al., 2005; Richardson et al., 2004), DCS did not potentiate reversal learning in CFTP when the flavors paired with fructose and saccharin were reversed (i.e., the old CS+ was now paired with S and the old CS- was now paired with F). In both DCS- and vehicle-treated rats, intake of the previously established CS+ decreased and intake of the reversed CS+ increased in 2-bottle preference tests. There was no difference in intakes between the DCS and vehicle groups. It is important to note, however, that CFTP learning is very resistant to extinction even when the CS+/F contingency is removed (Sclafani and
Therefore, the increase in reversed CS+ intake may reflect an increased preference for the reversed CS+ without any change in the rat’s evaluation of the old CS+; eventually the flavors become isopala
table again. Thus, while the rats underwent reversal conditioning, it is not clear that reversal learning per se occurred. More detailed microstructural comparisons would be required to establish this.

We hypothesize that NMDA receptors are involved specifically in the associative processes underlying CFTP acquisition. Conceptually, CFTP learning is mediated by gustatory, olfactory, preference (reward), and associative processes. Although NR is abundant in olfactory and gustatory pathways, NR blockade does not appear to compromise olfactory processing of the CS flavor cues as measured behaviorally. For example, conditioned rats were able to express a CFTP in a 2-bottle preference test after MK-801 pretreatment, showing that they could discriminate the CS+/S flavor from the CS-/S flavor. These findings parallel the observation that intraamygdalar infusion of the NR antagonist APV did not attenuate expression of odor- and taste-guided aversions in rats with a previously acquired taste-potentiated odor aversion (Hatfield and Gallagher, 1995).

NR might be involved in reward processing during CFTP acquisition, but there is strong evidence that dopaminergic pathways are active during CFTP learning. The reward basis for preference and CFTP learning has been probed pharmacologically (Baker et al., 2003; Baker et al., 2004; Yu et al., 1999; Yu et al., 2000a, b). In real-feeding rats, both the dopamine D1 receptor antagonist SCH23390 and dopamine D2 receptor antagonist raclopride blocked the acquisition and attenuated the expression of CFTP conditioned with fructose-paired flavors (Baker et al., 2003). Thus, DA antagonists not only blocked acquisition of a CFTP, but also reduced intake of the preferred US and blocked the expression of a previously acquired CFTP. Therefore, DA neurotransmission is apparently related to the unconditioned rewarding properties of the gustatory US and the acquisition of conditioned rewarding properties by the flavor CS+. Endogenous opioids do not play a critical role. In the identical paradigm, the general opioid antagonist, naltrexone, reduced the intake of sweet solutions, but did not significantly attenuate the acquisition and expression of CFTP in either sham-feeding or real-feeding rats (Baker et al., 2004; Yu et al., 1999).

In conclusion, the dopamine D1 and D2 receptors (Baker et al., 2003; Baker et al., 2004; Yu et al., 1999; Yu et al., 2000a, b) and NR are necessary for the acquisition of CFTP. While dopamine D1 and D2 receptors are necessary for the expression of CFTP (Baker et al., 2003), NR is not. Furthermore, the glycine binding site of the NR may contribute to activation during CFTP acquisition. Thus, the NR may specifically mediate the associative processes that link the flavor of the CS+ with the rewarding properties of the gustatory US, allowing the CS+ to acquire rewarding properties and become preferred in expression tests.
Table 5. Experimental protocol for systemic drug injection prior to conditioning flavor-taste preferences. Four days of 2-h, 1-bottle presentations followed by a 2-bottle preference test. This cycle is repeated four times.

<table>
<thead>
<tr>
<th>Solutions</th>
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<tbody>
<tr>
<td><strong>Left side</strong></td>
<td><strong>Right side</strong></td>
<td></td>
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<tr>
<td><strong>Conditioning day 1</strong></td>
<td>Systemic injections of MK-801 (100µg/kg; 30 min prior to conditioning) or DCS (15 mg/kg; 60 min prior to conditioning) or vehicle (1 ml/kg)</td>
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<td></td>
<td>Cherry or grape Kool-Aid with 8% fructose</td>
<td>Empty bottle</td>
</tr>
<tr>
<td><strong>Conditioning day 2</strong></td>
<td>Systemic injections of MK-801 (100µg/kg; 30 min prior to conditioning) or DCS (15 mg/kg; 60 min prior to conditioning) or vehicle (1 ml/kg)</td>
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<tr>
<td></td>
<td>Empty bottle</td>
<td>Grape or cherry Kool-Aid with 0.2% saccharin</td>
</tr>
<tr>
<td><strong>Conditioning day 3</strong></td>
<td>Systemic injections of MK-801 (100µg/kg; 30 min prior to conditioning) or DCS (15 mg/kg; 60 min prior to conditioning) or vehicle (1 ml/kg)</td>
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<tr>
<td></td>
<td>Cherry or grape Kool-Aid with 8% fructose</td>
<td>Empty bottle</td>
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<tr>
<td><strong>Conditioning day 4</strong></td>
<td>Systemic injections of MK-801 (100µg/kg; 30 min prior to conditioning) or DCS (15 mg/kg; 60 min prior to conditioning) or vehicle (1 ml/kg)</td>
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<tr>
<td></td>
<td>Empty bottle</td>
<td>Grape or cherry Kool-Aid with 0.2% saccharin</td>
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<tr>
<td><strong>Preference test day 5</strong></td>
<td>Cherry or grape Kool-Aid with 0.2% saccharin</td>
<td>Grape or cherry Kool-Aid with 0.2% saccharin</td>
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Figure 14. Systemic injections of MK-801 did not effect CS intake during conditioning. Intake was measured after a 2-h, 1-bottle presentation. A. With the exception of conditioning day 8, there was no difference in CS+/F intake for rats treated with MK-801 during conditioning (squares) when compared to rats treated with vehicle (circles; black symbols). * p < 0.05 vs. MK-801. B. There was no difference in CS-/S intake for rats treated with MK-801 during conditioning (squares) when compared to rats treated with vehicle (circles; white symbols).
**Figure 15.** Systemic injections of MK-801 during conditioning blocked acquisition of CFTP. After every four conditioning days, CFTP expression was measured as intake during a 2-h, 2-bottle preference test. Rats received a saline injection prior to preference testing. A. Beginning with the second preference test after 8 conditioning days, rats treated with vehicle during conditioning (circles) consumed significantly more CS+/S (black symbols) than CS-/S (white symbols). Intake of CS+/S and CS-/S by the rats treated with MK-801 during conditioning (squares) were not different in any preference test. * p < 0.05 vs. own CS-. B. On the third preference test after 12 conditioning days, vehicle-treated rats consumed significantly more CS+/S (black bars) and significantly less CS-/S (white bars) than MK-801-treated rats. * p < 0.05 vs. own CS-; † p < 0.05 vs. MK-801 CS+; ° p < 0.05 vs. Mk-801 CS-. 

![Graph A](image1.png)  
![Graph B](image2.png)
Figure 16. Effects on intake of the acute administration of MK-801 in previously conditioned rats. Acute MK-801 did not affect the CFTP expression in a 2-h, 2-bottle preference test in rats treated with vehicle during conditioning (left side): CS+/S intake (black bars) was significantly greater than CS-/S intake (white bars) regardless of pretreatment. Furthermore, acute MK801 prior to the preference test did not reveal any state-dependent effects in rats treated with MK-801 during conditioning (right side): no difference was seen between CS+/S and CS-/S intake regardless of pretreatment. * p < 0.05 vs. own CS-.
Figure 17. Systemic injections of MK-801 during conditioning did not induce a CTA against the CS+ and CS- flavors. In 48-h, 2-bottle preference tests of Kool-Aid/saccharin solutions vs. water, all rats treated with either vehicle or MK-801 during conditioning showed significantly higher intake of both CS+/S (black bars) and CS-/S (white bars) compared to water (hatched bars). There was no difference in the preference for the CS+ and CS- solutions. * p < 0.05 vs. water.
Figure 18. Systemic injections of DCS did not effect CS intake during conditioning. Intake was measured after a 2-h, 1-bottle presentation. A. There was no difference in CS+/F intake for rats treated with DCS during conditioning (squares) when compared to rats treated with vehicle (circles; black symbols). B. There was no difference in CS-/S intake for rats treated with DCS during conditioning (squares) when compared to rats treated with vehicle (circles; white symbols).
Figure 19. Systemic injections of DCS during conditioning enhanced CFTP learning in rats. A. Rats treated with vehicle during conditioning showed significantly greater intake of CS+/S (black circles) than CS-/S (white circles) on the third preference test (after 12 conditioning days). Rats treated with DCS during conditioning showed significantly greater intake of CS+/S (black squares) than CS-/S (white squares) on the second preference test (after 8 conditioning days). Thus the DCS-treated rats learned the CFTP faster than vehicle-treated rats. B. On the fourth preference test after 16 conditioning days, all rats consumed significantly more CS+/S (black bars) than CS-/S (white bars); DCS-treated rats consumed significantly more CS+/S than vehicle-treated rats. C. Across seven consecutive daily 2-h, 2-bottle preference tests of CS+/S vs. CS-/S, all rats showed little or no sign of extinction. On the first test day, CS+/S intake was significantly greater in DCS-treated rats (black squares) than vehicle-treated rats (black circles). In both groups, CS+/S intake (black symbols) was significantly greater than CS-/S intake (white symbols) on all days. * p < 0.05 vs. own CS-; †p < 0.05 vs. vehicle/CS+.
Figure 20. Systemic administration of DCS did not potentiate reversal learning in rats. After acquiring a CFTP (as shown on test day 0), rats were reverse conditioned with alternate daily presentations of the previous CS+ flavor now mixed with saccharin (rCS-, black symbols) and the previous CS- now mixed with fructose (rCS+, white symbols). Preferences were assessed after every 4 reverse conditioning days (test days 5 – 20). In both groups, preference for the rCS-/S decreased while preference for the rCS+/S increased during reverse conditioning. Pretreatment during reverse conditioning with either vehicle (circles) or DCS (squares) had no effect on the magnitude or time course of reversal learning. * p < 0.05 vs. own rCS+.
CHAPTER 3

c-FOS IMMUNOLABELING IN SACCHARIN/ SACCHARIN CONDITIONED FLAVOR-TASTE PREFERENCES.

1. Introduction

Conditioned flavor-taste preference (CFTP) is a form of associative learning in which an animal comes to prefer a conditioned stimulus (CS; e.g., a Kool-Aid flavor), after it has been paired with an unconditioned stimulus (US; e.g., fructose or saccharin). Regions of the brain involved in the acquisition and developing expression of sweet taste and associated flavor preferences can be examined by studying the immunolabeling of c-Fos.

C-Fos immunohistochemistry has been used as a method for tracking neuronal changes in ingestive behavior (Houpt, 2000; Swank and Bernstein, 1994). This method was chosen to examine the associative learning of CFTP because it offers a number of advantages including quantifiable cellular resolution of neural activity, mapping of neuronal populations activated by a stimulus and differential patterns of c-Fos immunolabeling that can be interpreted using a vast database of literature.

C-Fos immunolabeling can be used to detect changes in brain activation that parallel the acquisition and expression of a new behavior through learning. For example, c-Fos has revealed brain regions that show altered responses before and after learning in CTA (Houpt et al., 1994; Koh and Bernstein, 2005; Swank et al., 1995), odor-fear conditioning (Funk and Amir, 2000), and odor discrimination tasks (Datiche et al., 2001). In order to use c-Fos as a tool in a learning paradigm, the model should meet several criteria. The CFTP meets all of these criteria.

1. A radical, long-term change in the behavioral response: As few as 12 conditioning days produce a robust preference for the flavor previously paired with fructose (CS+/S).

2. The behavior persists through extinction: This preference persists without US reinforcement for as many as 6 days.

3. Specific neural circuitry is differentially activated before and after the change in behavior: c-Fos has shown differential neural activity before and after the change in behavior in previous taste and olfactory studies. This suggests that c-Fos immunohistochemistry may reveal brain regions involved in CFTP learning.

4. Gene expression is required for the long-term change in behavior: The time course of the acquisition of a CFTP (days) suggests that gene expression is necessary for the
long-term change in behavior (i.e. for synaptic restructuring). (NB that in any particular model, expression of the c-Fos gene may or may not participate in long-term consolidation; in this proposal, however, c-Fos expression is used primarily as a marker of activation following synaptic restructuring, rather than the restructuring process per se.)

I examined two categories of brain regions for c-Fos induction after CFTP learning: 1) established gustatory and olfactory relays, and 2) potential sites of neural plasticity correlated specifically with associative conditioning.

1. Sensory Relays: As both taste and smell are integral components of flavor, I examined the major gustatory relays (e.g., nucleus of the solitary tract (NTS), parabrachial nucleus, and insular cortex) and major olfactory relays (e.g., olfactory bulb, accessory olfactory bulb, piriform cortex, orbitofrontal cortex, medial nucleus of the amygdala).

2. Sites of Plasticity: Plastic brain regions that mediate or are altered by olfactory taste conditioning can be identified by unique patterns of c-Fos expression in the brains of conditioned vs. unconditioned rats. To be correlated specifically with conditioning and not unconditioned sensory processing or experimental procedures, c-Fos induction in a brain region would have to meet the following criteria (Granger and Lynch, 1991; Houpt, 2000): 1) Unique patterns of c-Fos expression should be correlated with the conditioned and not the unconditioned response. 2) The c-Fos response to the CS+/S can overlap with the c-Fos response to the US (fructose and saccharin) or be in novel brain regions. 3) If the preference is reversed, thereby causing the behavior to change, c-Fos induction would no longer occur. 4) Any treatment that causes a change in the behavior will cause a proportional change in c-Fos expression. For example, increasing the number of conditioning trials in CFTP might lead to a stronger preference and a proportional increase in c-Fos expression.

Our laboratory and others have identified brain regions that meet these criteria in other models of taste or olfactory learning. For example, the intermediate NTS (Houpt, 2000; Swank et al., 1995) and amygdala (Navarro et al., 2000) are sites of plasticity because c-Fos was expressed in both regions after intraoral infusions of a tastant (CS+) previously paired with LiCl, but not in response to infusions of the tastant before conditioning or after non-contingent pairing with LiCl (CS-). The pattern of c-Fos after the taste CS+ alone overlapped with the pattern of c-Fos induced by the LiCl US alone (Houpt 2000).

Others have used c-Fos immunolabeling to identify plastic brain regions in odor discrimination and olfactory fear conditioning (Datiche et al., 2001; Funk and Amir 2000). In olfactory fear conditioning, Funk and Amir (2000) have reported that the response to the CS+ (in rats conditioned by pairing cedar oil vapor with footshock, then tested with the cedar oil vapor alone) is significantly different from the response to the
odor alone or the US (represented by a backwards conditioning group) in the accessory olfactory bulb, anterior olfactory nucleus, infralimbic cortex, orbital cortex, and the basolateral amygdala. Conversely, when examining olfactory discrimination, Datiche et al., (2001) found no difference in c-Fos induction in the piriform cortex after presentation of unconditioned odor, conditioned odor, the water reward US, or the test chamber alone. Thus the piriform cortex does not meet the criteria for a plastic brain region in olfactory discrimination, because c-Fos in the piriform cortex correlated primarily with exposure to a novel environment, rather than associative learning.

A qualitative difference would indicate that the neural circuit engaged by the CS+/S after learning is a new circuit formed by the associative learning. Overlap with US immunolabeling would indicate that the neural circuit engaged by the US before learning is also activated by the CS+ after learning. If distinctive patterns of immunolabeling are found between CS+ and CS-, follow-up experiments could determine if neural activation is correlated with aspects of acquisition (e.g. number of pairings or quality of US) or with aspects of expression (e.g. secondary to increased intake or orofacial responses.)

Alternatively, the response to the CS+ may be indistinguishable from the response to the CS-, i.e., both activate the same olfactory and gustatory pathways as unconditioned Kool-Aid administered to unconditioned rats. This negative result could be interpreted in three ways:

1. The CFTP and intraoral protocol is not sufficient to stimulate c-Fos expression. Possible modifications include increasing the number of training days or enhancing the contrast between taste US (e.g. condition with CS+/saccharin vs. CS-/water, rather than CS+/F vs. CS-/S).

2. The brain regions being examined do not express c-Fos when activated. Other potential markers of neural activity could then be examined. In particular, phoshoMAP kinase has been used to track gustatory (Swank and Sweatt, 2001) and olfactory responses (Zhang et al., 2003).

3. The brain regions being examined are not part of the CFTP associative neural circuit. Additional areas in the gustatory, olfactory and limbic pathways would be examined.

The role of potential associative brain regions identified by c-Fos expression could also be examined by site-specific pharmacological studies, electrolytic or excitotoxic lesions, or neurochemical profiling. Any of these studies would be significant, as the brain regions that mediate CFTP learning are unknown.

Our lab and others have used 8% fructose and 0.2% saccharin as the unconditioned stimuli in CFTP learning (Baker et al., 2003; Baker et al., 2004; Golden and Houpt, 2007). However, the use of fructose in ad libitum fed rats and in long-term conditioning has proved troublesome because of its potential postingestive effects. In order to eliminate the potential confounds of food restriction, restricted drinking sessions and potential postingestive effects, the present study examined the possibility of
conditioning a flavor preference using two different concentrations of sodium saccharin (Holman, 1975). One flavor (CS+) was paired in mixture with a concentrated 0.2% saccharin solution (US+) while a second flavor (CS-) was paired in mixture with a less-preferred dilute 0.05% saccharin solution (US-) on 2-bottle conditioning days (CS+/US+ vs. CS-/US-). These concentrations of saccharin were established as the optimal solutions in experiment 1. Acquisition was assessed with a 2-bottle preference test in which both flavors mixed with the dilute concentration of saccharin are presented simultaneously (CS+/US- vs. CS-/US-).

Once it was confirmed that a CFTP could be learned using this protocol, intraoral infusions were used to standardize the oral stimulation used to evoke c-Fos expression.

The present study also characterized the c-Fos response in both unconditioned rats and conditioned rats. Intraoral infusion intake and differential c-Fos immunolabeling patterns were analyzed. In unconditioned rats, we examined the c-Fos immunolabeling in response to intraoral infusions of deionized distilled water, 0.2% sodium saccharin (US+) and the CS+/US+ to establish a baseline of c-Fos activity. In conditioned rats, we examined the c-Fos immunolabeling in response to intraoral infusions of 0.05% sodium saccharin (US-), 0.2% sodium saccharin (US+), the CS-/US- and the CS+/US- in order to identify the brain regions involved in the associative learning of conditioned flavor-taste preferences. Brain regions examined included nuclei in the gustatory pathway (i.e., parabrachial nucleus, nucleus of the solitary tract and insular cortex), nuclei in the olfactory pathway (i.e., accessory olfactory bulb, anterior olfactory nucleus, central nucleus of the amygdala, anterior and posterior perirhinal cortex and perirhinal cortex) and nuclei thought to be involved in learning and cognition (i.e., medial and basolateral nuclei of the amygdala, dentate gyrus and orbitofrontal cortex) and the lateral habenula.

2. Methods

2.1 Subjects

Male albino Sprague–Dawley rats (290–375 g, Charles River Laboratories, Wilmington, MA, USA) were housed individually in polycarbonate cages under a 12:12-h light-dark cycle with Purina rat chow and water available ad libitum. All testing took place in the rat's home cage during the first half of the lights-on phase. Food was available ad libitum for each experiment.

2.2 Intraoral Catheter Surgery

Following extinction testing in experiments 2, 3 and 4, chronic oral catheters were implanted. Under halothane anesthesia, rats were implanted with intraoral catheters made of PE-90 tubing that entered the mouth through the lateral cheek and were externalized on the dorsal surface between the scapulae, as described previously (Eckel and Ossenkopp, 1995). Intraoral catheters were flushed daily with water to maintain patency. Beginning on the day after surgery, rats received eight 2-bottle conditioning sessions (23 h/day) with the CS+/US+ and the CS-/US- presented simultaneously in order to maintain preferences. To habituate rats to intraoral infusions, four days after surgery
the rats were removed from their home cages and given intraoral infusions of deionized distilled water (dH₂O; 10 ml over 10 min) on three consecutive days. After the infusion, rats and any feces were weighed again as a measure of consumption, and rats were returned to their home cages.

For intraoral infusions, rats were weighed and placed in a glass aquarium subdivided into 4 individual compartments by Plexiglas sheets. Syringe pumps infused fluid into the mouth at a rate of 1 ml/min over 10 min. Rats were run in pairs in aquaria that were dedicated to a specific solution for the day (i.e., CS+ vs CS- or US+ vs US-) and were thoroughly cleaned between trials.

2.6 Experiment 1: Isopalatability

To determine the preference for varying concentrations of sodium saccharin, sodium saccharin solutions of 0.2%, 0.1%, 0.066%, 0.05%, 0.033% or 0.0% flavored with 0.05% cherry Kool-Aid were presented in random order in comparison to water in 23-h, 2-bottle preference tests. Cherry Kool-Aid was chosen as the flavor because it does not contain maltose dextrin as found in other Kool-Aid flavors.

Rats (n = 12) had access to each of the six saccharin concentrations for two consecutive days. Positions of the solution bottle and water bottle alternated daily. Thus rats received a total of six 2-bottle preference tests over a twelve day schedule. The sequence in which the saccharin concentrations were presented was determined for each rat using a random number generator.

2.7 Experiment 2: Acquisition and Expression of CFTP

To determine if a flavor-taste preference can be established without a caloric US, rats were conditioned using two different concentrations of sodium saccharin (see Table 6). It was our hypothesis that conditioning with saccharin as the sole US would streamline the experimental protocol by eliminating any potential postingestive effects, the need for food-restriction and allow for long-term presentation of the conditioning solutions. We also wished to test intraoral intake of conditioned solutions, so rats were implanted with intraoral catheters after CFTP learning.

The conditioning solutions were 0.2% sodium saccharin flavored with 0.05% unsweetened cherry Kool-Aid or 0.05% sodium saccharin flavored with 0.05% unsweetened grape Kool-Aid. The 0.2% saccharin-paired flavor is referred to as the CS+ and the 0.05% saccharin-paired flavor as the CS- because 0.2% saccharin is preferred to 0.05% saccharin and the 0.05% flavored saccharin solution is isopalatable to water (see results of experiment 1). In the 2-bottle preference tests and intraoral infusions, the cherry and grape flavors were each presented in a 0.05% saccharin solution; therefore increased intake of CS+ is a measure of a conditioned response acquired from the prior association of the flavor with the higher concentration of saccharin. CS+/US+ refers to the flavored high concentration saccharin solution used in conditioning, and CS+/US- refers to the same flavor in the lower concentration saccharin solution used during 2-bottle preference tests. The CS-/US- refers to the flavored low concentration saccharin solution used in conditioning and preference testing.
Rats (n = 6) received four 2-bottle conditioning sessions (23 h/day) with the CS+/US+ and the CS-/US- presented simultaneously. This pattern of four days of 2-bottle conditioning sessions was repeated twice prior to intraoral catheter surgery. Following each of the four day patterns were two days of 2-bottle preference tests. Positions of the flavored solution bottles were alternated daily (see Table 5).

To examine extinction, rats were then given the CS+/US+ and the CS-/US- in 2-bottle preference tests (23 h/day) for 4 consecutive days. Extinction testing occurred following the conditioning and 2-bottle preference sessions, but immediately prior to intraoral catheter surgery. The next day following surgery, rats received eight 2-bottle conditioning sessions (23 h/day) with the CS+/US+ and the CS-/US- presented simultaneously in order to maintain preferences.

Beginning four days after surgery, rats were habituated to intraoral infusions of dH2O on three days as described in the general methods. On day 4, half of the rats received infusions (10 ml/10 min) of the CS+/US- and the remaining rats received the CS-/US+; the flavors were reversed on the following day. Rats received infusions (10 ml/10 min) of a novel flavor, orange Kool-Aid, in a 0.05% saccharin solution on day 6. On day 7, half of the rats received infusions (10 ml/10 min) of the US+ (0.2% saccharin solution) and the remaining rats received the US- (0.05% saccharin solution); the solutions were reversed on the following day. After the infusion, rats and any feces were weighed as described in the general methods.

2.8 Experiment 3: Unconditioned Rats and c-Fos Immunolabeling

To determine the baseline c-Fos immunolabeling that is potentially evoked during CFTP learning, we examined fos expression in naive rats (n = 11) after intraoral infusions (10 ml/10 min) of dH2O, the US+ and the CS+/US+ solution.

Chronic oral catheters were implanted in 11 male albino SD rats. Rats were given ad libitum access to both food and water. To habituate rats to intraoral infusions, four days after surgery the rats were removed from their home cages and given intraoral infusions of dH2O (10 ml over 10 min) on three consecutive days. On day 4, rats received infusions (10 ml/10 min) of dH2O (n=3), 0.2% sodium saccharin solution (US+; n=4) or 0.2% sodium saccharin in a 0.05% cherry-flavored Kool-Aid solution (CS+/US+; n=4).

One hour after intraoral infusion, rats were overdosed with sodium pentobarbitol. When completely unresponsive, the rats were perfused transcardially, first with 100 ml of isotonic saline/0.5% sodium azide /1000 U heparin, and then with 400 ml phosphate-buffered 4% paraformaldehyde. The brains were removed, blocked, post-fixed for ~24 h and then transferred to 0.1 M phosphate buffer (PB) for storage at 4 °C. Individual blocks were transferred into 30% sucrose 72 h to 1 week prior to sectioning. Forty micron coronal sections were cut on a freezing, sliding microtome. Alternate sections were collected from the medulla at the level of the NTS (bregma –13.24 mm to –14.08 mm) and the pons at the level of the PBN (bregma –9.16 mm to –9.68 mm). Alternate sections were collected from the forebrain through the hypothalamus and amygdala (bregma –0.92 mm to –3.3 mm). Olfactory bulbs were cut in half and one half was used to collect alternate coronal sections (bregma 5.2 mm to 2.7 mm) while the other half was
used to collect the first four or five sagittal sections (lateral 1.4 mm to 1.9 mm). Coordinates were based on Paxinos and Watson’s atlas [Paxinos, 1986]. Sections were immediately processed for c-Fos-like immunoreactivity.

Free-floating tissue sections were washed for 10 min in 0.1 M phosphate-buffered saline (PBS) and then incubated for 30 min in 0.2% Triton/1% bovine serum albumin (BSA)/PBS. After two washes in PBS/BSA for 10 min each, sections were incubated overnight with a rabbit anti-c-Fos antiserum (Ab-5, Oncogene Research) at a dilution of 1:20,000. After two 10 min washes in PBS/BSA, sections were then incubated for 1 h with a biotinylated goat anti-rabbit antibody (Vector Laboratories) at a dilution of 1:200. Antibody complexes were amplified using the Elite Vectastain ABC kit (Vector Laboratories), and visualized by a 5-min reaction in 0.05% 3,3-diaminobenzidine tetrahydrochloride (DAB). Sections were stored in 0.1 M PB until mounted onto gelatin-coated glass slides and coverslipped using Permount.

Cells expressing darkly-positive, nuclear c-Fos immunolabeling were quantified using the MindsEye software program. Regions were digitally-captured at 4x magnification. For the PIRIa, PIRIp and LHB, the counting was restricted to the area delineated by a hand-drawn outline. Outlining was not necessary for the AOB, AON, BLA, CeA, DG, MeA (ventral and dorsal), MeP (ventral and dorsal), PRH, VLO/LO or GUS CTX because these regions mostly filled the counting frame or had little c-FLI outside the area of interest. Unilateral cell counts were collected for a single section of AOB, AON, PIRIa and VLO/LO and multiplied by two to reflect a bilateral cell count. The sum of bilateral cell counts were collected for a single section of the BLA, CeA, MeA, MeP, DG, GUS CTX, PIRIp and PRH for each rat. Bilateral cell counts were averaged 6 sections of the LHB for each rat. The individual mean counts for each region were then averaged across rats within experimental groups.

2.9 Experiment 4: CFTP and c-Fos Immunolabeling

The neural circuitry of the associative learning underlying CFTP is largely unknown. To determine what brain regions might be involved in CFTP expression, I examined c-Fos immunolabeling in rats (n = 22) that have learned a CFTP using two different concentrations of sodium saccharin and are experiencing intraoral infusions of US+, US-, CS+/US+ and CS-/US- solutions (see Table 7).

The conditioning solutions were 0.2% or 0.05% sodium saccharin flavored with 0.05% unsweetened grape or cherry Kool-Aid. Half of the rats received the cherry-flavored 0.2% saccharin solution and grape-flavored 0.05% saccharin solution; the flavors were reversed for the remaining rats. The rest of the rationale and nomenclature for the conditioning solutions is identical to experiment 2.

Rats received eight 2-bottle conditioning sessions (23 h/day) immediately prior to intraoral catheter surgery with the CS+/US+ and the CS-/US- presented simultaneously. Following the initial conditioning days, chronic oral catheters were implanted. Following surgery, rats received three 2-bottle conditioning sessions (23 h/day) with the CS+/US+ and the CS-/US- presented simultaneously in order maintain preferences. Immediately prior to the start of the intraoral infusion training with distilled water, a single 23-h, 2-
bottle preference test was performed to confirm that a preference for the CS+ flavor had been conditioned. Positions of the solution bottles were alternated daily.

To habituate rats to intraoral infusions, four days after surgery the rats were removed from their home cages and given intraoral infusions of dH2O (10 ml over 10 min) on three consecutive days. On day 4, rats received intraoral infusions of either the CS+/US- (n=6), CS-/US- (n=6), US+ (n=5) or the US- (n=5). Rats were transcardially perfused 1 h later. Tissue was collected, processed and analyzed for c-Fos-like immunoreactivity as described above in experiment 3.

Statistical Analysis
Bottle intakes were measured by weighing the bottles before and after each session to the nearest 0.1 g. Behavioral data was analyzed with two-way repeated ANOVA, using the Newman–Keuls test for post hoc comparisons (Statistica, StatSoft, Tulsa, OK). Intraoral intake data were analyzed with 1-way ANOVA, using the Newman–Keuls test for post hoc comparisons. c-Fos data was analyzed within brain regions with 1-way ANOVA, using the Newman–Keuls test for post hoc comparisons. Data are presented as mean intake ± standard error of the mean.

3. Results

3.1 Experiment 1: Isopalatability
The preference expressed for a cherry flavored sodium saccharin solution was dependent on the concentration of saccharin (Figure 21). Two-way ANOVAs showed that there was a significant interaction of solution and concentration for intake \([F(5, 100)=12.1, p<0.05]\). Rats drank significantly more dH2O and generally avoided the unsweetened Kool-Aid solution. There was no significant difference in total intake between the cherry Kool-Aid flavored 0.033%, 0.05% and 0.066% concentrations of saccharin solution and dH2O. Rats drank significantly more of the cherry Kool-Aid flavored 0.1% and 0.2% concentrations of saccharin solution in comparison to dH2O. Therefore, Kool-Aid in 0.05% saccharin was chosen as a neutral flavor-taste pairing (CS-/US-) and Kool-Aid in 0.2% saccharin was chosen as a preferred flavor-taste pairing (CS+/US+).

3.2 Experiment 2: Acquisition and Expression of CFTP
Total intake for CS+/US+ was significantly greater in comparison to CS-/US- throughout seven days of 23-h, 2-bottle conditioning (Figure 22). An eighth day of conditioning data was lost due to technical error. Two-way ANOVA across the eight days of conditioning with CS+/US+ and CS-/US- revealed a significant effect of solution \([F(1,5)=25.00, p=0.004]\) but not days with no interaction for intake (Figure 22). Intake for CS+/US+ was significantly greater from the first day of conditioning.

Total intake for CS+/US- was significantly greater in comparison to CS-/US- throughout eight days of 23-h, 2-bottle preference testing (Figure 23). Two-way ANOVA across the 2-bottle preference tests with CS+/US- and CS-/US- revealed a significant
effect of solution \[F(1,5)=35.04, p=0.002\] and days \[F(7,35)=3.9, p=0.003\] but no interaction for intake (Figure 23). CS+/US- total intake was significantly higher during the first two preference tests (after four days of conditioning) and remained significantly higher in the second two preference tests (after an additional four days of conditioning). CS+/US- total intake continued to be significantly higher through four consecutive extinction test days.

There was no difference in intraoral intake between the US+, US-, CS+/US- and CS-/US- solutions, although all solutions had significantly higher intraoral intake than \(\text{dH}_2\text{O}\) (Figure 24). One-way ANOVA across the 5 intraoral infusion groups revealed a significant effect of solution \([F(5,30)=8.16, p<0.05]\).

3.3 Experiment 3: Unconditioned Rats and c-Fos Immunolabeling

There was no difference in intraoral intake between the \(\text{dH}_2\text{O}\), US+ and CS+/US+ solutions (Figure 25). One-way ANOVA across the 3 intraoral infusion groups revealed no significant effect of solution.

In unconditioned rats, c-Fos immunolabeling was found to be extensive in olfactory pathway areas in the brain (i.e., anterior olfactory nucleus, anterior and posterior piriform cortex)(Table 1). There was no c-Fos immunolabeling in hindbrain regions associated with ingestive behavior (i.e., parabrachial nucleus, nucleus of the solitary tract). c-Fos immunolabeling in the basolateral complex of nuclei in the amygdala was significantly greater for the \(\text{dH}_2\text{O}\) group than for the other two groups (Figure 26). One-way ANOVA showed a significant interaction of group for activated c-Fos cells per section \([F(2,8)=11.30, p < 0.05]\). Although there was no significant interaction \([F(2,8)=4.27, p = 0.055]\), c-Fos immunolabeling in the dorsal region of the posterior medial nucleus of the amygdala was significantly greater for the \(\text{dH}_2\text{O}\) group than for the CS+/US+ group \((p < 0.05)\) and was approaching significance in comparison to the US+ group \((p = 0.07)\). c-Fos immunolabeling in response to intraoral infusions of \(\text{dH}_2\text{O}\) was greater in regions of the amygdala known to be associated with emotion and fear.

3.4 Experiment 4: CFTP and c-Fos Immunolabeling

Total intake for CS+/US+ was significantly greater in comparison to CS-/US- throughout eleven days of 23-h, 2-bottle conditioning (Figure 27). Two-way ANOVA across the eleven days of 23-h, 2-bottle conditioning with CS+/US+ and CS-/US- showed a significant effect of solution \([F(1,26)=231.40, p=0.00000]\) and days \([F(10,260)=14.25, p=0.00000]\) with a significant interaction \([F(10,260)=2.53, p=0.006]\) for intake. Extending the results in experiment 1, intake for CS+/US+ was significantly greater from the first day of conditioning.

Total intake for CS+/US- was significantly greater in comparison to CS-/US- for the one day of 23 h 2-bottle preference testing (Figure 28). One-way ANOVA for the 2-bottle preference test revealed a significant interaction of solution and days for intake \([F(1,52)=238.46, p<0.05]\). CS+/US- total intake was significantly higher during the single preference test (after eleven days of conditioning).
There was no difference in intraoral intake between the CS+/US-, CS-/US-, US- and US+ solutions (Figure 29). One-way ANOVA across the four intraoral infusion groups revealed no significant effect of solution.

c-Fos immunolabeling was extensive in established olfactory, gustatory and learning and cognition neural tracts (Table 9). There was no c-Fos immunolabeling in hindbrain regions associated with ingestive behavior (i.e., parabrachial nucleus, nucleus of the solitary tract). For most of the sites examined, there was no significant difference between groups in c-Fos immunolabeling. However, in the lateral habenula there was a noticeable difference between the CS+/US- and other groups (Figure 30; for an example of c-Fos immunolabeling in the lateral habenula see figure 31). The c-Fos immunohistochemistry was run in two separate groups and there was a difference in intensity between the two immunohistochemistry runs. Once the counts were normalized to the average for each run, the difference between the CS+/US- and other groups approached significance (p < 0.07). A t-test analyzing the difference in c-Fos immunolabeling between the CS+/US- and CS-/US- groups was significant (p < 0.05). A t-test analyzing the difference in c-Fos immunolabeling between the CS+/US- (conditioned rats) and CS+/US+ (unconditioned rats) groups was also significant (p < 0.05).

4. Discussion

This study establishes a method of conditioning a flavor-taste preference in free-feeding rats using long-term exposure to two different concentrations of saccharin as the unconditioned stimuli. After determining what concentrations of saccharin mixed in Kool-Aid were less preferred, isoprefereed and more preferred in comparison to dH$_2$O, rats were conditioned with 0.2% sodium saccharin paired with one Kool-Aid flavor and 0.05% sodium saccharin paired with an alternate Kool-Aid flavor. Rats consumed significantly more of the flavor previously paired with the higher concentration of saccharin when both flavors are mixed in the lower concentration of saccharin and presented in 23-h, 2-bottle tests. Thus, rats can learn a CFTP based solely on the orosensory stimuli of varying concentrations of saccharin solution mixed in Kool-Aid flavors. Examination of differential patterns of c-Fos immunolabeling in olfactory, gustatory and learning and cognition brain regions in unconditioned and conditioned rats revealed an increase in c-Fos activity in the lateral habenula receiving intraoral infusions of CS+/US- after conditioning. This suggests that the lateral habenula may play a role in the expression of a CFTP.

In confirmation of prior work (Holman, 1975), rats could learn a CFTP when trained with flavored sodium saccharin solutions of two different concentrations. Rats trained with flavored 0.2% and 0.05% sodium saccharin solutions had a greater intake of the flavor paired with the higher concentration in 2-bottle preference tests when both flavors were presented in the lower concentration of saccharin. This higher intake is attributed to the rats associating the CS+ flavor with the 0.2% concentration of saccharin during conditioning. This finding is encouraging in that it shows that a flavor preference
can be conditioned in long-term testing with non-caloric solutions in free feeding rats, based solely on gustatory stimulation.

Similar to flavor-taste preferences conditioned with 8% fructose and 0.2% saccharin (Golden and Houpt, 2007; Sclafani and Ackroff, 1994), flavor-taste preferences conditioned with 0.2% and 0.05% saccharin were strongly resistant to extinction. In contrast to flavor-taste preferences conditioned with fructose, saccharin/saccharin CFTP requires even fewer pairings. This result can be attributed to the long-term conditioning (i.e., 23 h) presentations. Whether or not saccharin/saccharin conditioning would work with short-term presentations has yet to be explored.

The lack of significant differences in intraoral intake of neutral or palatable solutions confirms that rats experienced comparable stimulation. Thus, any differential c-Fos immunolabeling pattern is not due to differences in intake volume.

The significant increase in c-Fos immunolabeling in the BLA in response to intraoral infusion of dH2O may indicate that even after three sessions of intraoral dH2O infusion, water infusion is somehow stressful. In experiment 2, intraoral intake of dH2O was significantly less for water in comparison to all other solutions, including a novel flavor that the conditioned rats had not previously experienced. In experiment 3, intraoral intake of the US+ and the CS+/US+ for unconditioned rats was equal to the intake of dH2O. These findings suggest that dH2O infused directly into the oral cavity may induce a stress response even in experienced rats. Alternately, exposure to flavor may lead to less c-Fos immunolabeling or may be inhibiting c-Fos activity.

The lateral habenula had a noticeably higher amount of c-Fos immunolabeling in the CS+/US- in comparison to the other groups in the conditioned rats and a significant increase in comparison to the CS+/US+ group in the unconditioned rats. Although this difference was not significantly different, there was a trend towards significance. This lack of significance may be rectified with the addition of more rats. A significant finding would be in agreement with findings in other studies of olfactory learning (Roullet et al., 2005; Tronel and Sara, 2002). Tronel and Sara (2002) trained rats to identify the placement of a food reward in one of three odor impregnated sponges by associating the reward’s location consistently with the same odor. An unpaired control group received the reward prior to entering the training apparatus where it was exposed to the same environment as the trained group. 24 hr later the trained group experienced a single reinforced retrieval trial and the unpaired group was exposed to the reinforcement and the environment in the training apparatus for the same amount of time as the trained group. c-Fos immunolabeling was significantly greater in the habenula of the trained rats after the retrieval test in comparison to the unpaired rats or home cage controls (Tronel and Sara, 2002). These results are similar to our findings in which the conditioned rats receiving intraoral infusion of the CS+/US- had increased c-Fos activity in the lateral habenula in comparison to the other groups.

Roullet and colleagues (2005) trained mildly water-deprived rats to follow an odor (limonene) in a four-arm radial arm maze to a water reward. Following another odor (geraniol) was not rewarded. Ten days later trained rats were given 20 reinforced trials in the maze to measure brain activity after memory retrieval. Three control groups consisted
of rats that received the water reward after finishing the maze but were not exposed to the odors, rats that were exposed to the odors and allowed to roam the maze without reward and rats that never left the home cage. After the retrieval test, c-Fos immunolabeling was significantly greater in the lateral habenula of the trained rats and the rats that received a reward just for completing the maze in comparison to the other two control groups (Roullet et al., 2005). Again, the results they report of the trained group were similar to our results, however we did not have a control group that matches the their reward/no odor group.

The role of the lateral habenula in reward is largely unknown. However, anatomical studies have shown that the lateral habenula receives input from the nucleus accumbens and the internal segment of the globus pallidus. The lateral habenula also has afferent projections to dopamine neurons in the substantia nigra pars compacta and the ventral tegmental area and to serotonin neurons in the dorsal raphe (Kimura et al., 2007).

The present results show that rats can be conditioned to learn a flavor-taste preference with two unconditioned stimuli that have no postingestive effects and that the effects of this learning are long lasting. The question of the role of the lateral habenula in stimulus-reward association has been raised in the recent past (Tronel and Sara, 2002) and again more recently (Ji and Shepard, 2007; Matsumoto and Hikosaka, 2007). Further study is needed to examine the role of the lateral habenula in reward pathways and associative learning.
**Table 6.** Experimental protocol for saccharin/saccharin conditioned flavor-taste preferences and intraoral infusions. Four days of 23-h, 2-bottle presentations followed by a 2-bottle preference test. This cycle is repeated twice, followed by four days of extinction testing. Rats are then implanted with intraoral catheters and after a period of rest are given intraoral infusions.

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Left side</th>
<th>Right side</th>
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<tbody>
<tr>
<td><strong>Cond. days 1-4</strong></td>
<td>CS+/US+</td>
<td>CS-/US-</td>
</tr>
<tr>
<td></td>
<td>(bottle position alternated daily)</td>
<td></td>
</tr>
<tr>
<td><strong>Pref. days 5-6</strong></td>
<td>CS+/US-</td>
<td>CS-/US-</td>
</tr>
<tr>
<td><strong>Extinct. Days 9-12</strong></td>
<td>CS+/US-</td>
<td>CS-/US-</td>
</tr>
<tr>
<td></td>
<td>(bottle position alternated daily)</td>
<td></td>
</tr>
<tr>
<td><strong>Surgery Day</strong></td>
<td>Intraoral catheter surgery</td>
<td></td>
</tr>
<tr>
<td><strong>Rest Days 1-4</strong></td>
<td>Conditioning day solutions</td>
<td></td>
</tr>
<tr>
<td><strong>Habituation Days 1-3</strong></td>
<td>Intraoral infusions of dH2O</td>
<td></td>
</tr>
<tr>
<td><strong>Infusion Day 1</strong></td>
<td>CS+/US- or CS-/US- (counterbalanced)</td>
<td></td>
</tr>
<tr>
<td><strong>Infusion Day 2</strong></td>
<td>CS+/US- or CS-/US- (counterbalanced)</td>
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</tr>
<tr>
<td><strong>Infusion Day 3</strong></td>
<td>Novel Kool-Aid flavor</td>
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<tr>
<td><strong>Infusion Day 4</strong></td>
<td>US+ or US- (counterbalanced)</td>
<td></td>
</tr>
<tr>
<td><strong>Infusion Day 5</strong></td>
<td>US+ or US- (counterbalanced)</td>
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</tbody>
</table>

CS+/-= grape or cherry kool-aid; US- = 0.05% saccharin; US+ = 0.2% saccharin.

All intraoral infusions were 10 ml/10min.
Table 7. Experimental protocol for saccharin/saccharin conditioned flavor-taste preferences, intraoral infusions and c-Fos immunoreactivity. Four days of 23-h, 2-bottle presentations are followed by a 2-bottle preference test. This cycle is repeated twice, followed by four days of extinction testing. Rats are then implanted with intraoral catheters and after a period of rest are given intraoral infusions. Specific brain regions are then probed with c-Fos immunohistochemistry.

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Left side</th>
<th>Right side</th>
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</thead>
<tbody>
<tr>
<td>Cond. days 1-8</td>
<td>CS+/US+</td>
<td>CS-/US-</td>
</tr>
<tr>
<td></td>
<td>(bottle position alternated daily)</td>
<td></td>
</tr>
<tr>
<td>Surgery Day</td>
<td>Intraoral catheter surgery</td>
<td></td>
</tr>
<tr>
<td>Rest Days 1-4</td>
<td>Conditioning day solutions</td>
<td></td>
</tr>
<tr>
<td>Pref. test day</td>
<td>CS+/US-</td>
<td>CS-/US-</td>
</tr>
<tr>
<td>Habituation Days 1-3</td>
<td>Intraoral infusions of dH₂O</td>
<td></td>
</tr>
<tr>
<td>Infusion Day</td>
<td>CS+/US- (n=6), CS-/US- (n=6), US+ (n=5) or US- (n=5)</td>
<td></td>
</tr>
<tr>
<td>1 hr later</td>
<td>Rats transcardially perfused, tissue collected</td>
<td></td>
</tr>
<tr>
<td></td>
<td>c-Fos immunohistochemistry</td>
<td></td>
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</tbody>
</table>

CS+/- = grape or cherry kool-aid; US- = 0.05% saccharin; US+ = 0.2% saccharin. All intraoral infusions were 10 ml/10min.
Figure 21. Flavored saccharin concentrations in direct comparison to dH$_2$O (white bars). In 23h, 2-bottle tests, rats prefer a 0.1% or a 0.2% saccharin solution, are indifferent to a 0.033%, 0.05% or a 0.066% saccharin solution and avoid a 0.0% saccharin solution mixed in 0.05% Kool-Aid (black bars) in comparison to dH$_2$O.
During CFTP acquisition, total intake was significantly greater for the CS+/US+ solution in comparison to the CS-/US- solution across seven days of conditioning. Total intake was measured after a 23-h, 2-bottle presentation. Total intake for the CS+/US+ (black circles) was significantly greater than the intake for the CS-/US- (white circles) from the first day of conditioning on. * p < 0.05 vs. CS-/US-.
Figure 23. During CFTP expression testing, total intake was significantly greater for the CS+/US- solution in comparison to the CS-/US- solution. Total intake was measured in two 23-h, 2-bottle expression tests after every four days of 23h, 2-bottle conditioning days. Total intake for the CS+/US- (black circles) was significantly greater than the intake for the CS-/US- (white circles) for the four days of expression testing and continued on through four consecutive days of extinction testing. * p < 0.05 vs. CS-/US-.
Figure 24. There was no difference in total intraoral intake in conditioned rats. There was no difference in total intraoral intake of 0.2% saccharin (US+), 0.05% saccharin (US-), Kool-Aid flavor previously paired with 0.2% saccharin now in 0.05% saccharin (CS+/US-), Kool-Aid flavored 0.05% saccharin (CS-/US-) and a novel Kool-Aid flavor mixed in a US- solution (novel). However, all of the 0.05% saccharin sweetened solution groups had intakes significantly greater than the dH2O group. Total intraoral intake was measured after a 10ml/10min intraoral infusion.* p < 0.05 vs. dH2O.
Figure 25. There was no difference in total intraoral intake in unconditioned rats. There was no difference in the total intraoral intake of deionized distilled water (dH₂O), 0.2% saccharin (US+) and 0.05% cherry Kool-Aid mixed in 0.2% saccharin (CS+/US+). Total intraoral intake was measured after a 10ml/10min intraoral infusion.
Table 8. Mean cell counts of cells displaying c-Fos immunolabeling in brain regions of unconditioned rats. c-Fos immunolabeling was significantly greater in the basolateral complex of the amygdala of unconditioned rats for deionized distilled water (dH$_2$O) in comparison to 0.2% saccharin (US+) and 0.05% cherry Kool-Aid mixed in 0.2% saccharin (CS+/US+). There was no difference in immunolabeling in any other brain region.

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>dH2O</th>
<th>US+</th>
<th>CS+/US+</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeA (V &amp; D)</td>
<td>171 ± 93</td>
<td>124 ± 9</td>
<td>114 ± 17</td>
</tr>
<tr>
<td>MeP (V)</td>
<td>190 ± 42</td>
<td>110 ± 32</td>
<td>103 ± 18</td>
</tr>
<tr>
<td>MeP (D)</td>
<td>142 ± 46</td>
<td>57 ± 12</td>
<td>58 ± 5</td>
</tr>
<tr>
<td>CeA</td>
<td>105 ± 16</td>
<td>55 ± 14</td>
<td>56 ± 8</td>
</tr>
<tr>
<td>BLA</td>
<td>147 ± 10</td>
<td>57 ± 18</td>
<td>71 ± 8</td>
</tr>
<tr>
<td>AOB</td>
<td>51 ± 23</td>
<td>85 ± 21</td>
<td>49 ± 13</td>
</tr>
<tr>
<td>AON</td>
<td>419 ± 87</td>
<td>769 ± 318</td>
<td>459 ± 47</td>
</tr>
<tr>
<td>DG</td>
<td>51 ± 10</td>
<td>53 ± 10</td>
<td>49 ± 6</td>
</tr>
<tr>
<td>GUS CTX</td>
<td>86 ± 14</td>
<td>92 ± 14</td>
<td>70 ± 8</td>
</tr>
<tr>
<td>LHB</td>
<td>19 ± 2</td>
<td>16 ± 3</td>
<td>33 ± 7</td>
</tr>
<tr>
<td>PIRIa</td>
<td>239 ± 46</td>
<td>181 ± 62</td>
<td>168 ± 10</td>
</tr>
<tr>
<td>PIRIp</td>
<td>123 ± 50</td>
<td>113 ± 41</td>
<td>127 ± 26</td>
</tr>
<tr>
<td>PRH</td>
<td>89 ± 5</td>
<td>57 ± 9</td>
<td>62 ± 9</td>
</tr>
<tr>
<td>VLO/LO</td>
<td>348 ± 111</td>
<td>335 ± 41</td>
<td>292 ± 99</td>
</tr>
</tbody>
</table>
Figure 26. c-Fos immunolabeling was significantly greater in the basolateral complex of the amygdala of unconditioned rats for deionized distilled water (dH₂O) in comparison to 0.2% saccharin (US+) and 0.05% cherry Kool-Aid mixed in 0.2% saccharin (CS+/US+). * p < 0.05 vs. water.
Figure 27. During CFTP acquisition, total intake was significantly greater for the CS+/US+ solution in comparison to the CS-/US- solution across eleven days of conditioning. Total intake was measured after a 23-h, 2-bottle presentation. Total intake for the CS+/US+ (black circles) was significantly greater than the intake for the CS-/US- (white circles) from the first day of conditioning on. * p < 0.05 vs. CS-/US-; † p < 0.05 vs. first day of the same solution.
**Figure 28.** During CFTP expression testing, total intake was significantly greater for the CS+/US- solution in comparison to the CS-/US- solution. Total intake was measured in one 23-h, 2-bottle expression test after eleven days of 23h, 2-bottle conditioning days. Total intake for the CS+/US- (black circles) was significantly greater than the intake for the CS-/US- (white circles) for the single day of expression testing. * p < 0.05 vs. CS-/US-. 
Figure 29. There was no difference in the total intraoral intake of 0.05% saccharin (US-), 0.2% saccharin (US+), Kool-Aid flavored 0.05% saccharin (CS-/US-) and Kool-Aid flavor previously paired with 0.2% saccharin now in 0.05% saccharin (CS+/US-). Total intraoral intake was measured after a 10ml/10min intraoral infusion.
Table 9. Mean cell counts of cells displaying c-Fos immunolabeling in brain regions of conditioned rats. c-Fos immunolabeling approached greater significance in the lateral habenula of conditioned rats in a Kool-Aid flavor previously paired with 0.2% saccharin now in 0.05% saccharin (CS+/US-) in comparison to 0.05% saccharin (US-), 0.2% saccharin (US+) and Kool-Aid flavored 0.05% saccharin (CS-/US-). T-test showed a significant difference between the CS+/US- and CS-/US- (p < 0.05). There was no difference in immunolabeling in any other brain region.

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>US-</th>
<th>US+</th>
<th>CS-/US-</th>
<th>CS+/US-</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeA (V &amp; D)</td>
<td>188 ± 81</td>
<td>169 ± 50</td>
<td>155 ± 36</td>
<td>191 ± 64</td>
</tr>
<tr>
<td>MeP (V)</td>
<td>146 ± 52</td>
<td>116 ± 38</td>
<td>153 ± 43</td>
<td>155 ± 52</td>
</tr>
<tr>
<td>MeP (D)</td>
<td>161 ± 87</td>
<td>103 ± 38</td>
<td>102 ± 50</td>
<td>122 ± 38</td>
</tr>
<tr>
<td>CeA</td>
<td>184 ± 97</td>
<td>171 ± 52</td>
<td>198 ± 62</td>
<td>168 ± 50</td>
</tr>
<tr>
<td>BLA</td>
<td>160 ± 91</td>
<td>128 ± 53</td>
<td>146 ± 44</td>
<td>143 ± 49</td>
</tr>
<tr>
<td>AOB</td>
<td>209 ± 45</td>
<td>198 ± 36</td>
<td>217 ± 67</td>
<td>183 ± 49</td>
</tr>
<tr>
<td>AON</td>
<td>476 ± 217</td>
<td>585 ± 213</td>
<td>425 ± 169</td>
<td>419 ± 105</td>
</tr>
<tr>
<td>DG</td>
<td>73 ± 42</td>
<td>57 ± 19</td>
<td>60 ± 14</td>
<td>62 ± 24</td>
</tr>
<tr>
<td>GUS CTX</td>
<td>240 ± 87</td>
<td>360 ± 171</td>
<td>376 ± 88</td>
<td>338 ± 89</td>
</tr>
<tr>
<td>LHB</td>
<td>50 ± 26</td>
<td>43 ± 18</td>
<td>44 ± 14</td>
<td>121 ± 47</td>
</tr>
<tr>
<td>PIRla</td>
<td>281 ± 124</td>
<td>170 ± 116</td>
<td>243 ± 113</td>
<td>153 ± 57</td>
</tr>
<tr>
<td>PIRlp</td>
<td>163 ± 63</td>
<td>146 ± 43</td>
<td>138 ± 34</td>
<td>149 ± 36</td>
</tr>
<tr>
<td>PRH</td>
<td>240 ± 131</td>
<td>151 ± 60</td>
<td>139 ± 43</td>
<td>122 ± 42</td>
</tr>
<tr>
<td>VLO/LO</td>
<td>348 ± 186</td>
<td>236 ± 164</td>
<td>314 ± 157</td>
<td>194 ± 82</td>
</tr>
</tbody>
</table>
Figure 30. c-Fos immunolabeling of the lateral habenula in conditioned rats approached significance in a Kool-Aid flavor previously paired with 0.2% saccharin now in 0.05% saccharin (CS+/US-) in comparison to 0.05% saccharin (US-), 0.2% saccharin (US+) and Kool-Aid flavored 0.05% saccharin (CS-/US-). T-test showed a significant difference between the CS+/US- and CS-/US- (p < 0.05).
Figure 31. An example of c-Fos immunolabeling in the lateral habenula of rats after receiving intraoral infusions of either CS-/US- or CS+/US-.
FUTURE AIMS

There are three specific studies I would do to follow up the experiments discussed in this dissertation. First, I would like to further examine the role of NMDA receptors in CFTP by giving site-specific injections of NMDA agonists and antagonists in brain regions that have been implicated in associative learning. This study would help determine the location of the associative process in CFTP learning.

Second, NMDA agonists and antagonists could be used in an attempt to manipulate c-Fos immunolabeling as seen in CFTP learning. Pharmacological manipulation of NMDA receptors may change the differential pattern of c-Fos immunolabeling in either unconditioned or conditioned rats. The results of this experiment could determine what critical sites are involved in the acquisition or expression of a CFTP.

Third, the role of the lateral habenula in reward is largely unknown. Although it has been suggested that the lateral habenula may play a role in predicting the value of a reward, it would be beneficial to phenotype the cells that express c-Fos immunolabeling in the lateral habenula during CFTP acquisition and expression.
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ABSTRACTS AND POSTER PRESENTATIONS

Golden G.J., Houpt T.A. Intraoral intake and c-Fos induction in rats with conditioned flavor taste preferences. Society for the Study of Ingestive Behavior, 14th annual meeting, July 24-29, 2007
Golden G.J., Houpt T.A. D-cycloserine potentiates conditioned flavor-taste preference learning, but not reversal learning. Society for Neuroscience, 35th annual meeting, November 12-16, 2005


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