Roles of GABAB, Muscarinic and Nicotinic Receptor Signaling in the Acquisition and Expression of Fructose and Fat-Conditioned Flavor Preferences and Acquisition of Quinine-Conditioned Flavor Avoidances in Rats

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ROLES OF GABA<sub>B</sub>, MUSCARINIC AND NICOTINIC RECEPTOR SIGNALING IN
THE ACQUISITION AND EXPRESSION OF FRUCTOSE AND FAT-
CONDITIONED FLAVOR PREFERENCES AND ACQUISITION OF QUININE-
CONDITIONED FLAVOR AVOIDANCES IN RATS.

by

FRANCIS M. ROTELLA

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This manuscript has been read and accepted for the Graduate Faculty in Psychology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

Roles of GABA\textsubscript{B}, Muscarinic and Nicotinic Receptor Signaling in the Acquisition and Expression of Fructose and Fat-Conditioned Flavor Preferences and Acquisition of Quinine-Conditioned Flavor Avoidances in Rats.

by

Francis M. Rotella

Advisor: Professor Richard J. Bodnar, Ph.D.

In addition to increased intake of sweet solutions by mammals, learning, particularly classically-conditioned “Pavlovian-like” learning, also plays an important role. An orosensory conditioned flavor preference (CFP) can be elicited by pairing one novel flavor (conditioned stimulus, CS+) with a fructose solution and a second novel flavor (CS-) with a saccharin solution. Rats will prefer the CS+ flavor in a subsequent 2-bottle choice test with both flavors mixed in saccharin. Previous pharmacological analyses revealed that systemic administration of dopamine (DA) D1 and D2 as well as NMDA, but not opioid, receptor antagonists eliminated the acquisition (learning) of fructose-CFP. Further, expression of an already-acquired fructose-CFP was significantly reduced by systemic DA D1 or D2, but not NMDA or opioid receptor antagonists. This dissertation research extended the pharmacological substrates of fructose-CFP by examining whether systemic administration of muscarinic (scopolamine: SCOP) and nicotinic (mecamylamine: MEC) cholinergic receptor antagonists, or a GABA\textsubscript{B} receptor agonist (baclofen: BAC) affected the learning and maintenance of fructose-CFP.
Whereas fructose-CFP acquisition was eliminated by SCOP, but not MEC or BAC, fructose-CFP expression was only marginally reduced by SCOP, MEC and BAC.

In addition to sugars, fats can also elicit CFP by pairing two novel flavors with different concentrations (e.g., 3.5% and 0.9%) of corn oil (CO). Previous studies indicated that acquisition of CO-CFP was eliminated by NMDA receptor antagonism, it was significantly reduced by DA D1 and D2, but not opioid receptor antagonists. Expression of CO-CFP was mildly reduced by DA D1, DA D2, NMDA or opioid receptor antagonists. In similar fashion, the effects of SCOP, MEC and BAC were evaluated upon acquisition and expression of CO-CFP. Interestingly, a similar pattern of results emerged for fat-CFP as was found for fructose-CFP. Thus, whereas CO-CFP acquisition was eliminated by SCOP, but not MEC or BAC, CO-CFP expression was significantly but marginally reduced by SCOP, MEC and BAC.

In addition to learned preferences, a conditioned flavor avoidance (CFA) can be produced by pairing a CS+ flavor with the bitter taste of quinine. The present studies evaluated whether fructose-CFP, CO-CFP and quinine-CFA share common neurochemical substrates by determining the systemic effects of DA D1 (SCH23390: SCH), DA D2 (raclopride: RAC), NMDA (MK-801), opioid (naltrexone: NTX), muscarinic (mAch: SCOP) or nicotinic (nAch: MEC) receptor antagonists as well as GABA_B (BAC) agonists on the acquisition of quinine-CFA. We first demonstrated that DA D1, NMDA and opioid, but not DA D2 receptor antagonism enhanced the CFA produced by the bitter taste of quinine, and then subsequently found that whereas MEC and BAC enhanced this avoidance, SCOP failed to alter quinine-CFA.
Therefore, this dissertation demonstrated the differential involvement of major neurotransmitter systems in two forms of preference-based and one form of avoidance-based learning. Accordingly, whereas the acquisition of sugar- and fat-preferences is primarily mediated by DA D1, DA D2, NMDA and mAch receptors, and their expression is primarily mediated by DA D1, DA D2, mAch and nAch receptors, the acquisition of quinine-avoidance is primarily mediated by DA D1, NMDA, opioid, nAch and GABA\textsubscript{B} receptors.
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I would like to thank my committee members for their assistance and patience throughout the preparation and review of this dissertation, Drs. Richard Bodnar, Carolyn Pytte, Robert Ranaldi, Jeff Beeler and Joshua Brumberg. I would especially like to thank my advisor and mentor Dr. Richard Bodnar for his dedicated support and guidance throughout not only this process, but in also contributing towards my personal growth. Additionally, I would like to thank all my graduate and undergraduate colleagues for their dedication and effort in helping this dissertation come to fruition. The research included in this dissertation would not have been possible without your collaborative efforts as well as your personal support, and for that I am especially grateful. Finally, I would like to thank my family and friends for continuing to support me throughout the years of working towards this dissertation. You all have my sincerest gratitude.
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<th>Full Form</th>
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<tr>
<td>3-APA</td>
<td>3-aminopropylphosphinic acid</td>
</tr>
<tr>
<td>α7-nAchR</td>
<td>Alpha 7 Nicotinic Acetylcholine Receptor</td>
</tr>
<tr>
<td>β2-nAchR</td>
<td>Beta 2 Nicotinic Acetylcholine Receptor</td>
</tr>
<tr>
<td>Ach</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>ADX71441</td>
<td>Selective Positive Allosteric Modulator of GABA&lt;sub&gt;B&lt;/sub&gt; Receptors</td>
</tr>
<tr>
<td>AM251</td>
<td>Cannabinoid Receptor Type 1 Inverse Agonist</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>AMY</td>
<td>Amygdala</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
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<tr>
<td>AP-5</td>
<td>(2R)-amino-5-phosphonopentanoate</td>
</tr>
<tr>
<td>BAC</td>
<td>Baclofen</td>
</tr>
<tr>
<td>CA1</td>
<td>Cornu Ammonis Region 1</td>
</tr>
<tr>
<td>CA3</td>
<td>Cornu Ammonis Region 3</td>
</tr>
<tr>
<td>CB1</td>
<td>Cannabinoid Receptor Type 1</td>
</tr>
<tr>
<td>CFA</td>
<td>Conditioned Flavor Avoidance</td>
</tr>
<tr>
<td>CFP</td>
<td>Conditioned Flavor Preference</td>
</tr>
<tr>
<td>CO</td>
<td>Corn Oil</td>
</tr>
<tr>
<td>CS</td>
<td>Conditioned Stimulus</td>
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<tr>
<td>CS+/FSQ</td>
<td>Flavored Fructose + Saccharin + Quinine Solution (Quinine-CFP)</td>
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<td>CS-/FS</td>
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<tr>
<td>CS+/3.5%CO</td>
<td>Flavored 3.5% Corn Oil and 0.3% Xanthan Gum Suspension (CO-CFP)</td>
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<td>CS-/0.9%CO</td>
<td>Flavored 0.9% Corn Oil and 0.3% Xanthan Gum Suspension (CO-CFP)</td>
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<td>CS+/Fs</td>
<td>Flavored Fructose + Saccharin Solution (Fructose-CFP)</td>
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<td>CS-/s</td>
<td>Flavored Saccharin Solution (Fructose-CFP)</td>
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<td>CS-</td>
<td>Negative Conditioned Stimulus</td>
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<td>CTA</td>
<td>Conditioned Taste Aversion</td>
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<td>D1</td>
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<td>D2</td>
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</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
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<tr>
<td>DAMGO</td>
<td>D-Ala2-NMe-Phe4-Glyol5-enkephalin</td>
</tr>
<tr>
<td>FS</td>
<td>Fructose + Saccharin (CS-: Quinine-CFA)</td>
</tr>
<tr>
<td>FSQ</td>
<td>Fructose + Saccharin + Quinine (CS+: Quinine-CFA)</td>
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<tr>
<td>GABA</td>
<td>γ-Aminobutyric acid</td>
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<td>GABA&lt;sub&gt;B&lt;/sub&gt;</td>
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<td>GABA&lt;sub&gt;A&lt;/sub&gt;</td>
<td>GABA-A Receptor</td>
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<tr>
<td>IG</td>
<td>Intragastric</td>
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<td>LiCl</td>
<td>Lithium Chloride</td>
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<tr>
<td>LMTD VEH</td>
<td>Limited Vehicle</td>
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<td>mAch</td>
<td>Muscarinic Acetylcholine Receptor</td>
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<td>MEC</td>
<td>Mecamylamine</td>
</tr>
<tr>
<td>mGluR5</td>
<td>Metabotropic Glutamate Receptor 5</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>------------------------------------</td>
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<tr>
<td>mOFC</td>
<td>Medial Orbital Frontal Cortex</td>
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<tr>
<td>mPFC</td>
<td>Medial Prefrontal Cortex</td>
</tr>
<tr>
<td>NAc</td>
<td>Nucleus Accumbens</td>
</tr>
<tr>
<td>nAch</td>
<td>Nicotinic Acetylcholine Receptor</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NS</td>
<td>Neutral Stimulus</td>
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<tr>
<td>NTX</td>
<td>Naltrexone</td>
</tr>
<tr>
<td>PPT/LDT</td>
<td>Pedunculopontine/Laterodorsal Tegmental Area</td>
</tr>
<tr>
<td>RAC</td>
<td>Raclopride</td>
</tr>
<tr>
<td>SCH</td>
<td>SCH23390</td>
</tr>
<tr>
<td>SCOP</td>
<td>Scopolamine</td>
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<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
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<td>US</td>
<td>Unconditioned Stimulus</td>
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<tr>
<td>VEH</td>
<td>Vehicle</td>
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<tr>
<td>VTA</td>
<td>Ventral Tegmental Area</td>
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Chapter 1: Introduction

Significance and Specific Aims:

Humans and animals learn to use orosensory cues (e.g., taste, odor, texture) to signal approach to energy-dense and nutritious substances (Capaldi, 1996; Sclafani, 1995), and avoidance to toxic substances (Dwyer, 2011; Fanselow and Birk, 1982; Freeman and Riley, 2009). Associative learning influences food-related preferences and avoidances, dissociating behavioral, neurochemical and neuroanatomical differences among four forms of food-related learning: conditioned flavor preferences (CFP) promoted by either the orosensory (flavor-flavor CFP (Sclafani and Ackroff, 1994)) or post-ingestive (flavor-nutrient CFP (Sclafani et al., 1999; Sclafani et al., 1999)) qualities of sugars and fats, conditioned taste-aversions (CTA), elicited by post-ingestive gastrointestinal malaise (Chambers and Bernstein, 1995; Garcia et al., 1974), or conditioned flavor avoidances (CFA) elicited by the negative orosensory qualities of quinine (flavor-flavor CFA (Dwyer, 2011).

One primary focus of this dissertation examines whether the pharmacological effects affecting orosensory-mediated flavor-flavor CFP produce similar patterns of effects upon flavor-flavor CFA in adult male Sprague-Dawley rats. The behavioral pharmacology mediating the acquisition and expression of sugar (fructose) and/or fat (corn oil) flavor-flavor CFP has been investigated using antagonists of dopamine (DA) DA D1 (SCH23390), DA D2 (raclopride), N-methyl-D-aspartate (NMDA: MK-801), or opioid (naltrexone: NTX) receptors (Baker et al., 2003; 2004; Dela Cruz et al., 2012a; 2012b; Golden and Houpt, 2007). The major forebrain targets (nucleus accumbens (NAc), amygdala (AMY) and medial prefrontal cortex (mPFC) of the meso-limbic and meso-cortical DA system originating in the ventral tegmental area (VTA) (Swanson, 1982) have been implicated in the mediation of the orosensory and post-ingestive
substrates of sugar-CFP (Amador et al., 2014; Bernal et al., 2008; 2009; 2010; Khaimova et al., 2004; Malkusz et al., 2012; 2014; 2015; Touzani et al., 2008; 2009a; 2009b; 2010b). However, the abilities of these receptor antagonists to alter the acquisition of quinine-CFA are unknown, and are examined in the present specific aims.

A second primary focus of this dissertation examines two additional neurotransmitter receptor systems in the acquisition and expression of fructose-CFP and fat-CFP and in the acquisition of quinine-CFA: the cholinergic muscarinic and nicotinic receptor systems and the GABA<sub>B</sub> receptor system. The cholinergic system, acting through muscarinic (mAch) and nicotinic (nAch) receptor signaling, has been implicated both directly and as a modulator of overlapping limbic and cortical DA circuits previously assessed in these flavor-flavor CFP studies (see review: Avena and Rada, 2012). The γ-Aminobutyric acid (GABA) system, and particularly its GABA<sub>B</sub> receptor, mediates both primary stimulatory (e.g., Ebenezer, 1995; Echo et al., 2002; Khaimova et al., 2004; Miner et al., 2010) and inhibitory (Buda-Levin et al., 2005; Wojnicki et al., 2006; Wong et al., 2009) behavioral effects upon food and palatable intake, and has also been shown to mediate responses within this same limbic DA circuit (Johnson and North, 1992). Therefore, additional specific aims examine the pharmacodynamic roles of GABA<sub>B</sub>, mAch and nAch receptors in mediating the associative learning processes conditioned by either primary appetitive stimuli (e.g., sugar or fat, CFP) or primary avoidant stimuli (e.g., quinine: CFA) in rats.

Specific Aim 1: Will muscarinic or nicotinic cholinergic receptor antagonism mediate the acquisition and expression of sugar (fructose)-CFP?

Previous pharmacological analyses have evaluated the neurochemical substrates of the acquisition (learning) and expression (maintenance) of the flavor-flavor component of sugar-
CFP. Systemic administration of either DA D1 (SCH23390) or D2 (raclopride) receptor antagonists eliminated both acquisition and expression of fructose-CFP in real-feeding, food-restricted rats and sucrose-CFP in sham-feeding, food-restricted rats (Baker et al., 2003; Hsiao and Smith, 1995; Yu et al., 2000a; 2000b). Subsequent studies revealed that systemic administration of the non-competitive NMDA receptor antagonist (MK-801) eliminated acquisition, but not expression of fructose-CFP (Golden and Houpt, 2007). In contrast, systemic administration of NTX, a general opioid receptor antagonist, reduced sweet intake, but failed to alter flavor-flavor-mediated sugar-CFP (Baker et al., 2004; Bernal et al., 2010; Yu et al., 1999). Therefore, DA D1, DA D2, and NMDA, but not opioid receptor signaling is required for the full learning (acquisition) and maintenance (expression) of fructose-CFP.

Evidence implicating cholinergic involvement in complex aspects of food intake appears to be due to activity in a limbic circuit (specifically the VTA and NAc) in which cholinergic signaling can act directly upon preferences and avoidances either in and of itself, or through interactions with brain DA (see review: Avena and Rada, 2012). Therefore, Specific Aim 1 investigates the role of muscarinic and nicotinic cholinergic receptor signaling in mediating the expression and acquisition of flavor preferences conditioned by fructose in rats by examining the ability of muscarinic (scopolamine: SCOP) and nicotinic (mecamylamine: MEC) receptor antagonists to block the expression and acquisition of fructose-CFP.

In addition to sugars, rodents are attracted to the flavor of fats (e.g., corn oil: CO) which may be partly mediated by taste receptors for fatty acids (Ackroff and Sclafani, 2007; 2009; Passilly-Degrace et al., 2009), as well as rewarding post-ingestive and orosensory properties, and can condition a flavor preference (Ackroff and Sclafani, 2009; Sclafani, 1999; 2004). The second Specific Aim addresses this corollary.
Specific Aim 2: Will muscarinic or nicotinic cholinergic receptor antagonism mediate the acquisition and expression of fat (corn oil)-CFP?

In contrast to fructose-CFP, systemic administration of DA D1 and D2 receptor antagonists produced marginal reductions in the expression of fat (CO)-CFP, and DA D2, but not DA D1 receptor antagonism hastened the extinction of fat-CFP acquisition (Dela Cruz et al., 2012a). Further, systemic administration of NMDA receptor antagonists significantly reduced the acquisition, but not the expression of fat-CFP. Finally, opioid receptor antagonism was ineffective in altering this fat preference similar to that observed for fructose-CFP (Dela Cruz et al., 2012b). Consequently, Specific Aim 2 addresses the potential involvement of muscarinic or nicotinic cholinergic receptor signaling in a parallel paradigm employed for the assessment of DA D1, DA D2, NMDA and opioid receptor signaling in the mediation of fat-CFP elicited by the ingestion of a flavor (e.g., cherry) paired with a higher (3.5%) CO concentration relative to a flavor (e.g., grape) paired with a lower (0.9%) CO concentration. This aim expands on the behavioral pharmacological relationships of the above neurochemical substrates beyond sugar preferences to that of fat preferences and thereby making a unifying statement about preferences in general. Thus, it is hypothesized that both subtypes of cholinergic receptor antagonists will influence the expression of fructose-CFP (Specific Aim 1) and fat-CFP (Specific Aim 2). However, only antagonism of the muscarinic subtype will modulate the acquisition of fructose- (Specific Aim 1) or fat- (Specific Aim 2) CFP.

The first two Specific Aims examine cholinergic receptor involvement in fructose- and fat-CFP. The next two Specific Aims evaluate GABA<sub>B</sub> receptor involvement.
Specific Aim 3: Does systemic pharmacological stimulation of GABA_B receptors influence the acquisition and expression of sugar (fructose)-CFP?

Studies of reward- or aversive-related learning revealed that regions within the mesolimbic DA system (e.g., VTA) are in turn modulated by and receive inputs from a number of other neurotransmitter systems including GABA using receptor activation rather than inhibition in influencing reward- and/or aversive-related learning (Johnson and North, 1992; Tan et al., 2012; van Zessen et al., 2012). Therefore, this specific aim addresses the specific effects of GABA_B receptor stimulation rather than inhibition.

There are two major receptor families for GABA: GABA_A and GABA_B. Systemic administration of the GABA_A receptor agonist, muscimol, interferes with memory consolidation processes in forms of inhibitory avoidance learning (Castellano and McGaugh, 1990) and conditioned taste aversions (DiSorbo et al., 2009). However, the timing of these effects suggests that the effects were due to retrograde amnesic effects (Salinas and McGaugh, 1995). In these studies, muscimol was most effective in interfering with learning when administered after the conditioning procedures (Castellano and McGaugh, 1990; DiSorbo et al., 2009). This approach is fundamentally different from the procedure employed in CFP studies. Further, the systemic effects of GABA_A agonism initially produce profound behavioral inhibitory effects associated with a cataleptic state followed by periods of hyperactivity (Vyazovskiy et al., 2007), which could potentially confound the learning effects of interest, similar to the reward-confounding behavioral effects of DA antagonists (Wise and Schwartz, 1981). The mechanism of action of such GABA_A agonists as muscimol occurs through stimulation of ionotropic GABA receptors, leading to a relatively quick hyperpolarization of the neuron and resulting in general depression of neuronal activity (see review: Johnston, 2014). Moreover, central muscimol is very
commonly used as a tool in producing generalized depression in addition to its GABA_A actions. On the other hand, GABA_B agonists such as baclofen act via stimulation of metabotropic GABA receptors, leading to a longer term cellular cascade of events resulting in a much slower depression and modulation of neuronal (particularly DA) activity (Kuba et al., 2000).

In addition, central stimulation of both subtypes of the GABA receptor (e.g., muscimol: GABA_A and baclofen: GABA_B) stimulate food intake in a receptor-specific manner (Miner et al., 2010). However, each seem to be expressed on different clusters of neurons (Sugita et al., 1992). Accordingly, GABA_A receptors are located mainly on non-DA-containing neurons (Churchill et al., 1992), while GABA_B receptors are located mainly on DA-containing neurons (Margretar-Mitrovic et al., 1999; Wirtshafter and Sheppard, 2001). Given the proposed interactions between GABA and DA in the modulation of CFP and CFA, this specific aim focuses on GABA_B signaling via systemic agonism of these receptors.

Another rationale for limiting our investigation to GABA_B receptors is their specific involvement in animal psychopathological models of food intake dysregulation (e.g., binge-eating: Berner et al., 2009; Broft et al., 2007; Buda-Levin et al., 2005; Corwin et al., 2012; de Beaurepatre et al., 2015; Wojnicki et al., 2006). However, the effects of peripheral GABA_B receptor activation (via baclofen: BAC) on food intake vary. In non-deprived rats, increases in food intake depend on time of consumption (Ebenezer and Patel, 2011), route of administration (Ebenezer and Prabhaker, 2007; Patel and Ebenezer, 2008a; 2008b), and acute tolerance (Bains and Ebenezer, 2013). In contrast, BAC can also decrease food intake in diabetic and diet-induced obese mice (Sato et al., 2007), as well as reduce intake of pure fat emulsions relative to chow under normal, limited-access and “binge-type” conditions in rats (Buda-Levin et al., 2005; Rao et al., 2008; Wang et al., 2011; Wojnicki et al., 2014; but see: Bains and Ebenezer, 2013).
and suppress intake of either a pure fat or a sugar-fat mixture (Avena et al., 2014; Berner et al., 2009; Corwin et al., 2009; Wojnicki et al., 2013; Wong et al., 2009).

Evidence implicating GABA<sub>B</sub> receptor involvement in complex aspects of food intake appears to be due to activity in a previously mentioned limbic circuit (specifically the VTA and NAc). Additional research suggests GABA<sub>B</sub> signaling mediates both medium spiny NAc GABA and VTA DA outputs, possibly interacting with DA-Ach activity here (Avena and Rada, 2012; Hoebel et al., 2007). Behavioral evidence also suggests the interaction of GABA<sub>B</sub> stimulation and DA-Ach involvement through baclofen’s ability to reduce nicotine self-administration in rats, in addition to being extended to glutamate (mGluR5) signaling (Markou et al. 2004). Therefore, Specific Aim 3 examines the roles of GABA<sub>B</sub> receptor signaling by determining whether the systemic GABA<sub>B</sub> (baclofen) receptor agonist will alter the acquisition and/or expression of fructose-CFP in an identical paradigm employed for the assessment of Specific Aim 1. As with the evaluation of the cholinergic system, the next Specific Aim examines GABA<sub>B</sub> receptor involvement in fat-CFP.

**Specific Aim 4: Does systemic pharmacological stimulation of GABA<sub>B</sub> receptors influence the acquisition and expression of fat (corn oil)-CFP?**

GABA<sub>B</sub> receptor stimulation has also been implicated in the mediation of fat intake. BAC administered into limbic and hypothalamic sites increased food intake (e.g., Arnt et al., 1979; Echo et al., 2002; Stratford and Kelley, 1997; Ward et al., 2000; Wirtshafter et al., 1993), and was reported to be mediated through GABA receptor interactions between the VTA and NAc (Miner et al., 2010). Systemic BAC increased fat intake under normal conditions (e.g., Bains and Ebenezer, 2013; Ebenezer and Patel, 2011), but suppressed fat intake under “binge-type” conditions (Avena et al., 2014; Berner et al., 2009; Buda-Levin et al., 2005; Corwin and
Wojnicki, 2009; Rao et al., 2008; Wojnicki et al., 2014; Wong et al., 2009). Furthermore, the reinforcing effects of rewards as well as cues associated with those rewards mediated by mesolimbic circuitry is modulated by \textit{GABA}\textsubscript{B} receptor stimulation in optogenetic studies of food intake (van Zessen et al., 2012). Indeed, given that many of the animal psychopathological studies were mimicking binge-eating with strong effects on diets containing fat, it would be expected that BAC effects should exert more powerful effects on acquisition and expression of fat-CFP if these learning processes are intimately related to the psychopathological model.

The first four Specific Aims relate to the second primary focus of the research. The first primary focus addresses the question as to whether CFA might utilize the same pharmacological systems and patterns of effects as CFP. Thus, the fifth and sixth Specific Aims examine this possibility.

**Specific Aim 5:** Can DA D1, DA D2, opioid and NMDA receptors, involved in mediating appetitive associative learning processes (e.g., sugar- and fat-CFP), similarly modulate underlying associative processes contributing to the behavioral effects of conditioned flavor avoidance (CFA) associative learning processes induced by primary avoidant stimuli (e.g., quinine, CFA)?

Flavor–taste avoidance learning occurs when an arbitrary flavor (CS, conditioned stimulus) is paired with a naturally non-preferred taste (US, unconditioned stimulus, e.g., bitter: quinine), and similar distinctions between flavor-flavor and flavor-nutrient processes with respect to avoidance and aversive conditioning have been assessed. Former studies elucidating the taste-specific (US: quinine) rather than post-ingestive (US: lithium chloride) effects of avoidance and aversive conditioning, respectively, have suggested that it is possible for rats to
learn to associate negative orosensory qualities (e.g., bitter) with that of neutral flavors (Fanselow and Birk, 1982; Dwyer, 2011).

Although there is much literature elucidating the pharmacology of flavor-toxin CTA elicited by the pairing of a neutral flavor (CS) with that of gastrointestinal malaise (US), flavor–taste CFA learning conditioned via non-visceral US’s (e.g., taste) has not been the subject of pharmacological analysis. Thus, Specific Aim 5 addresses this gap by examining the roles of those pharmacological substrates assessed previously for other forms of primarily taste-mediated learning (e.g., fructose- and fat-CFP), including DA D1, DA D2, NMDA and opioid receptor signaling in flavor avoidance conditioned by the bitter taste of quinine. One simple hypothesis is that pharmacological effects on CFP and CFA produce parallel effects. In this model, given that DA D1, DA D2 and NMDA, but not opioid receptor antagonism effectively block fructose-CFP acquisition (Baker et al., 2003; 2004; Golden and Houpt, 2007), it would be hypothesized that blockade of DA D1, DA D2 and NMDA, but not opioid receptors would systematically reduce the magnitude of quinine-CFA acquisition. An alternate hypothesis is that pharmacological antagonist effects would drive CFP and CFA in the same direction. In this model, given that DA D1, DA D2 and NMDA, but not opioid receptor antagonism effectively block fructose-CFP acquisition, it would be hypothesized that blockade of DA D1, DA D2 and NMDA, but not opioid receptors would systematically enhance the magnitude of quinine-CFA acquisition.

**Specific Aim 6:** Extending on the previous pharmacological assessments of fructose- and fat- CFP, will muscarinic or nicotinic cholinergic as well as GABAB receptors also mediate avoidance learning?

A. Cholinergic receptors have also been previously implicated in certain forms of CFA such as in lithium chloride-(LiCl) induced CFA (Coil et al., 1978; Ossenkopp and Giugno, 1990;
With respect to flavor-taste CFA, avoidant tastants such as quinine have been demonstrated to modulate Ach activity in itself (Ballester et al., 2005) and influence adrenal catecholamine release stimulated by Ach (Jang et al., 2001). Further, nicotine depressed quinine-evoked responses in the nucleus tractus solitarius that were blocked by MEC (Simons et al., 2006). Nicotine and quinine CFA generalized to each other in three mouse strains (Gyekis et al., 2012), with MEC blocking this ability in behavioral and electrophysiological assays (Oliveira-Maia et al., 2009).

These data appear to implicate cholinergic receptor signaling in flavor–taste CFA and therefore Specific Aim 6 addresses the pharmacological roles of muscarinic (SCOP) and nicotinic (MEC) receptor signaling in mediating the acquisition of quinine-induced CFA. Due to much behavioral evidence implicating muscarinic receptor signaling in the formation of learned associations conditioned by primary appetitive stimuli (Nisanov et al., 2016; Sharf et al., 2006; Sharf and Ranaldi, 2006), it is hypothesized that only manipulation of the muscarinic subtype will modulate the acquisition of quinine-CFA and that these effects will oppose those seen in fructose-CFP studies such that muscarinic cholinergic receptor blockade will enhance the magnitude of quinine-CFA.

B. Furthermore, given the inconsistent effects of GABA_B receptor agonism on various forms of food-related approach type learning (Bemer et al., 2009; Broft et al., 2007; Buda-Levin et al., 2005; Corwin et al., 2012; de Beaurepatre et al., 2015; Wojnicki et al., 2006), we assess the roles of GABA_B receptor signaling in food-related avoidance type learning, thereby shedding some light on these inconsistencies. As such, Specific Aim 6 also tests hypotheses as to the roles of GABA_B receptor signaling in the acquisition of quinine-CFA. Similar to the rationale noted in Specific Aims 3 and 5, if GABA_B receptor signaling is interacting with DA and/or muscarinic
Ach at integral limbic sites, it is hypothesized that agonism of GABA\textsubscript{B} receptors will modulate quinine-CFA in a manner opposite to that of DA and/or muscarinic receptor antagonism for fructose-CFP, thereby enhancing the magnitude of quinine-CFA.
Chapter 2: Background and Rationale

General Background:

To promote normal growth and development, humans require the intake of a multitude of substances including carbohydrates, fats, proteins, vitamins, minerals, fiber, and water. However, government data in 2015 indicated that the average diet was high in calories, added sugars, and saturated fats, but low in essential nutrients such as vitamin D, calcium, potassium, and fiber thereby posing health risks for a majority of the population (Dietary Guidelines Advisory Committee, 2015). This factor has contributed to increases in diet-related nutrition and metabolic disorders and diseases including obesity, type II diabetes, cardiovascular diseases and certain cancers (Dietary Guidelines Advisory Committee, 2015).

As an example, although obesity may be due partially to genetic factors (Choquet and Mayre, 2011), the presence of unlearned preferences and aversions (Menella and Beauchamp, 2005; Roitman et al., 2008; Sclafani, 2004), as well as the abundance of processed, energy dense and cost-efficient, yet nutrient-sparse foods (Dietary Guidelines Advisory Committee, 2015; Drenowski and Specter, 2004), another significant contributing factor to non-compliance with dietary guidelines and the development of diet-based nutrition and metabolic disorders is learning (Gibson and Brunstrom, 2007; Roitman et al., 2008; Scalfani, 2004; Yeomans, 2008). In addition to these factors, substantial animal research has shown that fats and sugars are both recognized as contributing to the palatability of foods, overeating, and diet-induced obesity through their inherent positive hedonic properties as well as learning processes associated with the preferences for fat- and sugar-rich foods (Sclafani, 1999). It has also been proposed that sugars, much like other substances of abuse such as alcohol, are able to elicit several components of addiction such as bingeing, withdrawal and craving in animal models (Avena et al., 2008a),
possibly contributing to the development of diet-induced obesity. Evidence supporting the inherent negative hedonic qualities and learning processes associated with the unpalatability and avoidance of bitter substances is far less conclusive (Dwyer, 2011; Fanselow and Birk, 1982). These preferences, and to a lesser extent, avoidances (Dwyer, 2011; Fanselow and Birk, 1982; Rotella et al., 2014; 2015), are based, in part, on learned associations between the various flavor elements in foods (e.g., flavor–flavor conditioning), and between flavor cues and post-ingestive consequences (e.g., flavor–nutrient/flavor-toxin conditioning).

Elucidating the neurochemical substrates of orosensory associative processes with an array of primary appetitive as well as aversive stimuli is critical to our understanding of dysregulations both in brain and bodily nutritional homeostatic mechanisms as well as conditioned goal-directed and consummatory behaviors associated with preventable nutritional and metabolic disorders. This research provides further insight as to alternate potential neurochemical substrates modulating traditional neurotransmitter systems (such as DA) in addition to mediating the activity of reward-related and/or limbic structures within the brain in and of themselves during orosensory associative processes. The series of Specific Aims were addressed by incorporating systemic pharmacological manipulations on our well-established flavor-flavor paradigms.

The remainder of the Background section is organized to cover the following topics: 1) conditioned flavor preferences (CFP) for sugars and fats and 2) pharmacological and neuroanatomical substrates of CFP. Based on these preliminary findings, the rationale for the studies of the dissertation research is then presented: 3) potential roles of cholinergic receptor systems in mediating CFP; 4) potential roles of GABA<sub>B</sub> receptors in mediating CFP; and 5)
conditioned flavor avoidances (CFA) and their potential pharmacology. This will be followed by Section 6 describing how these six specific aims are organized into the series of research studies.

1. Conditioned Flavor-Flavor Preferences (CFP):

1A. Sugar-CFP (Flavor-Flavor vs. Flavor-Nutrient Conditioning)

Rats use flavor cues (taste, odor, texture) to guide their selection of nutritious foods (Capaldi, 1996). Sugar-induced conditioned flavor preferences (CFP) occur when a novel flavor (CS+) is paired with a more-preferred sucrose (16%) or fructose (8%) and saccharin (0.2%) solution relative to a flavor (CS-) paired with a less-preferred saccharin (0.2%) solution. These sugar-CFPs are based on learned associations between food flavor elements (flavor-flavor conditioning) as well as between flavor and post-ingestive consequences (flavor-nutrient conditioning) (Sclafani, 1995). Flavor-flavor conditioning has been studied for sucrose in sham feeding rats (Yu et al., 1999; 2000a; 2000b), and for fructose in real-feeding rats (Baker al., 2003; 2004), given the inability of fructose to condition preferences after intragastric (IG) administration (Sclafani and Ackroff, 1994; Sclafani et al., 1993; 1999). In contrast, glucose is capable of producing CFP following oral and IG administration (Dela Cruz et al., 2014; Sclafani and Ackroff, 1994; Sclafani et al., 1993; 1999). Specific Aims 1 and 3 will expand our knowledge of the pharmacological substrates of sugar (e.g., fructose) flavor-flavor conditioning.

Conditioned flavor preferences (and subsequent description of conditioned flavor avoidance) is described throughout this dissertation as “Pavlovian-like” conditioning rather than true Pavlovian conditioning. For all studies, multiple UCSs were paired with a neutral stimulus (NS) as opposed to a single US and NS pairing in traditional Pavlovian conditioning studies. Thus, in sugar-CFP studies, the CS+ was operationally defined as the more preferred solution containing one NS (e.g. cherry Kool Aid) paired with two UCSs (fructose + saccharin: having
both orosensory and post-ingestive consequences) and the CS- was operationally defined as the less preferred solution containing a different NS (e.g. grape Kool Aid) paired with one UCS (saccharin: having only orosensory consequences). Thus, the “CS-” is not a negative stimulus, but rather a “less-preferred” stimulus. In fat-CFP studies, the CS+ was operationally defined as the more preferred solution containing one NS (e.g. cherry Kool Aid) paired with one UCS with a higher (3.5%) concentration of corn oil and the CS- was operationally defined as the less preferred solution containing a different NS (e.g. grape Kool Aid) paired with one UCS with a lower (0.9%) concentration of corn oil. Previous studies (Dela Cruz et al., 2012a, 2012b) show that rats consume less of the 0.9% concentration relative to the 3.5% concentration of corn oil. In quinine-CFA studies the CS+ was operationally defined as the less preferred solution containing one NS (e.g. cherry Kool Aid) paired with three UCSs (quinine + fructose + saccharin) and the CS- was operationally defined as the more preferred solution containing a different NS (e.g. grape Kool Aid) paired with two UCSs (fructose + saccharin). Therefore, we operationally define the CS+ as the more preferred solution and the CS- as the less preferred solution in CFP studies, while we operationally define the CS+ as the less preferred solution and CS- as the more preferred solution in CFA studies.

The primary purpose of the present series of studies was to analyze the pharmacology of CFP and CFA. Therefore, given the short-term (~2 h) effects of the drugs, it was imperative that we utilized a conditioning procedure in which rats were exposed to solutions for multiple sessions of short durations (~1h). It should be noted that for the development of fat and carbohydrate flavor preferences, paradigms using short-term (30min) or long-term (22h) training session lengths have been done (Lucas & Sclafani, 1999). They found that intake and/or preference could vary depending on the duration of the subsequent choice tests (either 30min or
22h), and that this influenced the extent to which carbohydrates were preferred over fats, and vice versa (Lucas & Sclafani, 1999). Thus, short-term choice tests do not always predict the long-term intakes and preferences for fats and carbohydrates, thereby limiting our pharmacological findings to only short-term tests.

1B. Fat-CFP

Rodents are also attracted to the flavor of fat (e.g., corn oil) which may be partly mediated by taste receptors for fatty acids (Ackroff and Sclafani, 2007; Ackroff and Sclafani, 2009; Passilly-Degrace et al., 2009), as well as rewarding post-ingestive and orosensory properties (Ackroff and Sclafani, 2009; Sclafani, 1999; Sclafani, 2004). Prior studies have shown that in addition to carbohydrates such as sugars, fats are also capable of eliciting robust and stable flavor-flavor and flavor-nutrient CFPs in rats (Dela Cruz et al., 2012a; 2012b; Lucas and Sclafani, 1989; Lucas and Sclafani, 1999). With respect to fat “Pavlovian-like” associative processes, similar distinctions between the capability of certain sugars (e.g., fructose vs. glucose) to condition flavor preferences via either orosensory or post-ingestive processes have been demonstrated utilizing varying methodologies, fats as unconditioned stimuli, and CS-US delay intervals (Ackroff and Sclafani, 2009). Accordingly, sham-feeding studies comparing nutritive and nonnutritive oil (corn oil and mineral oil, respectively) intake and preferences suggest that while both oils are capable of eliciting intake via their palatable orosensory properties, intake of nutritive oils are preferred during oil-oil choice testing (Mindell et al., 1990) and are preferred during separate oil emulsion-saccharin choice testing in food-restricted rats (Elizade and Sclafani, 1990). Accordingly, Specific Aims 2 and 4 expand our knowledge of the pharmacological substrates of fat (e.g., corn oil) flavor-flavor conditioning.
2. Pharmacological and Neuroanatomical Substrates of CFP:

2A. Acquisition and Expression of Fructose-CFP

The pharmacological substrates of conditioned flavor preferences (CFP) initially focused on brain DA and opioid systems (see reviews: Sclafani et al., 2011; Touzani et al., 2010a). Sweet taste activated mesolimbic and mesocortical DA circuits involved in the mediation of natural as well as drug rewards (e.g., Genn et al., 2004; Hajnal et al., 2003). DA receptor antagonism also suppressed the intake of sweet solutions in rats (Geary and Smith, 1985; Muscat and Willner, 1989; Xenakis and Sclafani, 1981).

As previously mentioned, flavor-flavor conditioning has been studied for sucrose in sham-feeding rats (Yu et al. 1999; 2000a; 2000b), and for fructose in real-feeding rats (Baker et al., 2003; 2004), given the inability of fructose to condition preferences after IG administration (Sclafani and Ackroff, 1994; Sclafani et al., 1993; 1999). Research was then aimed to assess the underlying pharmacological and neuroanatomical substrates moderating these distinct behavioral phenomena. Accordingly, systemic administration of DA D1 (SCH23390) or D2 (raclopride) receptor antagonists eliminated both acquisition and expression of fructose-CFP in real-feeding, food-restricted rats and sucrose-CFP in sham-feeding, food-restricted rats (Baker et al., 2003; Hsiao and Smith, 1995; Yu et al., 2000a; 2000b).

A critical role of the mesocorticolimbic DA system has been identified in reward processes and reward-related learning (Berridge and Robinson, 1998; Salamone and Correa, 2012; Smith, 2004; Wise, 2008). In this system, DA neurons located in the VTA project to cortical and limbic structures including the NAc, AMY and the mPFC (Swanson, 1982). Another brain DA system implicated in flavor learning (Caulliez et al., 1996) includes the A13 DA neurons located in the zona incerta that innervate the lateral hypothalamus (LH) (Wagner et al.,
Central DA receptor mediation of the acquisition of fructose-CFP is controlled by the AMY and mPFC (Bernal et al., 2009; Malkusz et al., 2012). Central DA receptor mediation of the expression of fructose-CFP is controlled by the NAc, AMY, medial orbital frontal cortex (mOFC) and LH (Amador et al., 2014; Bernal et al., 2008; Bernal et al., 2009; Malkusz et al., 2015).

In addition to DA, the roles of other neurotransmitter systems in mediating forms of CFP have been evaluated. For example, although it has been demonstrated that naloxone, a general opioid receptor antagonist, treatment during a flavor and glucose training session prevented rats from acquiring a preference for a glucose-paired flavor (Mehiel, 1996), subsequent studies demonstrated that systemic administration of NTX reduced sweet intake, but failed to alter acquisition or expression of flavor-flavor-mediated sugar-CFP induced by sucrose in sham-feeding rats or fructose in real-feeding rats (Baker et al., 2004; Yu et al., 1999). Central injections of NTX into the NAc, mPFC, AMY or LH also failed to affect fructose-CFP expression (Bernal et al., 2010; Malkusz et al., 2014). Systemic administration of NMDA receptor antagonists (MK-801) eliminated acquisition, but not expression of fructose-CFP (Golden and Houpt, 2007). Further, systemic administration of cannabinoid (CB1) receptor inverse agonists (AM251) reduced expression, but not acquisition of fructose-CFP (Miner et al., 2008). Therefore, DA D1, DA D2, NMDA and CB1, but not opioid receptor signaling is required for the full learning (acquisition) and maintenance (expression) of fructose-CFP, apparently in limbic sites associated with reward.

2B. Acquisition and Expression of Corn Oil-CFP

DA mediation of the rewarding effect of fat flavor is suggested by the findings that corn oil sham-feeding promotes NAc DA release (Liang et al., 2006), and DA D1 and D2 antagonists
suppress the sham feeding response to corn oil and real-feeding of fats in rats (Baker et al., 2001; Davis et al., 2006; Rao et al., 2008; Weatherford et al., 1988; 1990). DA D2, but not D1 antagonism also suppressed operant responding for corn oil in mice (Yoneda et al., 2007). In inbred mice, strain differences were observed in the ability of the D1 antagonist, SCH23390 to significantly reduce fat intake whereas the D2 antagonist, raclopride, had minimal effects on fat intake (Dym et al., 2010).

The opioid system has been implicated in the mediation of fat appetite and intake. In particular, there are many reports of opioid receptor antagonists suppressing fat intake in rats and mice (Cole et al., 1995; Dym et al., 2010; Glass et al., 2000; Higgs and Cooper, 1998; Islam and Bodnar, 1990; Marks-Kaufman et al., 1985; Naleid et al., 2007; Sahr et al., 2008). In addition, administration of the mu-selective opioid agonist, D-Ala2-NMe-Phe4-Glyol5- enkephalin (DAMGO) into the NAc stimulated high-fat intake in rats (Zhang et al., 1998). Place preferences conditioned by oral intake of corn oil or a high-fat snack food are also attenuated by NTX administration (Jarosz et al., 2006; Shide and Blass, 1991). Finally, it was demonstrated that the opioid system contributes to the acquisition for dietary fat but is not required for its maintenance and the reinforcement for fat intake and quantity consumed are differentially moderated by this system (Sakamoto, 2015).

Glutamate signaling has been shown to play a crucial role in learning and memory and the underlying synaptic plasticity (Rezvani, 2006). More specifically, glutamate receptor activation is required for food-related incentive learning. That is, glutamate antagonism within the AMY and NAc impaired appetitive instrumental learning (Hernandez et al., 2005; Kelley et al., 1997). Glutamate antagonism within the AMY also impaired both the acquisition and expression of conditioned taste avoidance (Yasoshima et al., 2000). Within the VTA, glutamate
antagonists impaired cue-sucrose learning and DA release in the NAc elicited by the sucrose-predictive cue (Stuber et al., 2008; Zellner et al., 2009; Zweifel et al., 2009). Interestingly, glutamate receptor antagonism by systemic administration of MK-801 blocked the acquisition of fructose-CFP (Golden and Houpt, 2007).

In contrast to the pronounced pharmacological effects observed on the acquisition and expression of fructose-CFP, the acquisition and expression of CO-CFP was only attenuated by DA D1 and D2 (Dela Cruz et al., 2012a), but not opioid receptor antagonism (Dela Cruz et al., 2012b), while the non-competitive NMDA receptor antagonist (MK-801) eliminated only the acquisition of CO-CFP (Dela Cruz et al., 2012b).

3. Potential Roles of Cholinergic Receptor Systems in Mediating CFP:

3A. Cholinergic Systems and Fructose-CFP (Specific Aim 1)

Ach has been implicated in food intake, particularly the “addictive” aspects of excessive sugar intake, by its interactions with brain DA systems (Avena and Rada 2012). Neuroanatomical interactions presumably occur through Ach inputs from the pedunculopontine and laterodorsal tegmental (PPT/LDT) nuclei to VTA DA cells (Holmstrand and Sesack, 2011; Maskos, 2008; Omelchenko and Sesack, 2005; Woolf et al., 1990) or through Ach or DA terminal innervation of Ach-containing interneurons in the NAc (Dautan et al., 2014; de Rover et al., 2002; Witten et al., 2010; Zhou et al., 2002). These interactions are integral in the formulation of central mechanisms involved in food reward (see reviews: Avena and Rada, 2012; Kelley et al., 2005; Laurent et al., 2014; Mark et al., 2011; McFadden et al., 2014; Nunes et al., 2013). Additionally, food intake increases Ach release in the AMY (Hajnal et al., 1998) and NAc (Avena et al., 2006; 2008a; 2008b; 2008c; Mark et al., 1992; Mark et al., 1995). Moreover, central studies of food and drug reward suggests that these brain DA-Ach systems interact to
modulate medium spiny GABAergic output within the NAc (Hoebel et al., 2007) as well as DAergic output within the VTA (Mark et al., 2011; Mifsud et al., 1989; Schilstrom et al., 1998; Schmidt et al., 2011). Additionally, muscarinic receptor antagonism with SCOP in the NAc reduced both deprivation-induced feeding (Pratt and Blackstone, 2009) and NAc DAMGO-induced feeding (Perry et al., 2009), and NAc sites at which SCOP suppressed feeding and DAMGO-induced feeding overlapped (Perry et al., 2014). DAMGO-induced increases in high-fat feeding were blocked by NTX and SCOP, but not by antagonists of DA, glutamate or nicotinic receptors (Will et al., 2006). VTA muscarinic receptor blockade also mediated cue-related responses to feeding such that SCOP administered into the VTA disrupted free-feeding and acquisition, but not the maintenance of food-related learning (Sharf and Ranaldi, 2006).

SCOP administered into the NAc core induced avoidance to flavor and spatial cues (Pratt et al., 2007). SCOP administered into the ventral hippocampus impaired memories for socially-transmitted food preferences (Carballo-Marquez et al., 2009). To determine cholinergic involvement in the development of sugar preferences, one of our preliminary studies (Rotella et al., 2015) demonstrated that SCOP, but not MEC completely blocked the acquisition of fructose-CFP. Also, both SCOP and MEC significantly reduced, but failed to block the expression of fructose-CFP.

3B. Cholinergic Systems and Corn Oil-CFP (Specific Aim 2)

Cholinergic receptor signaling have been implicated in the mediation of fat intake. Consumption of a high-fat diet for one week reduced acetylcholinesterase activity in the frontal cortex, hypothalamus and midbrain, as well as increased both β2-nAchR binding in the medial prefrontal cortex and substantia nigra, in addition to α7-nAchR binding in the lateral and ventromedial hypothalamus. MEC blocked the enhancements in exploratory and novelty-seeking
behaviors induced by high-fat consumption (Morganstern et al., 2012). Chronic nicotine reduced body weight in mice, particularly those maintained on a high-fat diet, an effect blocked by MEC co-treatment (Mangubat et al., 2012). Accumbal microinjections of SCOP markedly reduced fat intake elicited by accumbal administration of the mu-opioid receptor agonist, DAMGO, and also reduced food intake in food-deprived rats (Perry et al., 2009; Will et al., 2006). However, accumbal SCOP failed to affect fat intake itself (Will et al., 2006).

4. Potential Roles of the GABA_B System in Mediating CFP:

4A. GABA_B Systems and Fructose-CFP (Specific Aim 3)

Given the involvement of the DA or Ach systems in mediating fructose-CFP as well as other forms of NAc GABA-mediated food and drug related learning, another potential candidate neurotransmitter system which has not been evaluated in the modulation fructose-CFP is the GABAergic system. As previously mentioned in Specific Aim 3 we chose to limit our investigation to GABA_B receptors given evidence supporting the potential confounding behavioral, pharmacological and cognitive effects of systemic administration of GABA_A receptor agonists (e.g., muscimol) in similar learning paradigms (Salinas and McGaugh, 1995; Castellano and McGaugh, 1990; DiSorbo et al., 2009; Kuba et al., 2000; see review: Johnston, 2014), as well as research suggesting the possible lack of GABA_A involvement in the DA system (Miner et al., 2010; Sugita et al. 1992; Churchill et al., 1992; Margreta-Mitrovic et al., 1999; Wirtshafter and Sheppard, 2001) and evidence supporting the specific involvement of GABA_B signaling in animal psychopathological models of food intake dysregulation (e.g., binge-eating: Berner et al., 2009; Broft et al., 2007; Buda-Levin et al., 2005; Corwin et al., 2012; de Beaurepatre et al., 2015; Wojnicki et al., 2006).
Correspondingly, systemic administration of the GABA\textsubscript{B} agonist, baclofen, has been shown to influence the intake of both solid and liquid diets (Ebenezer, 1995), fats and sugars (Avena et al., 2014; Berner et al., 2009; Ebenezer and Pringle, 1992), and modulate short-term food intake (Patel and Ebenezer, 2010) as a function of texture (Wojnicki et al., 2013). Additionally, a systemic study comparing GABA\textsubscript{B} mediated alterations of food intake supported a primarily central role in baclofen’s effects, as 3-aminopropylphosphinic acid (3-APA), also a GABA\textsubscript{B} agonist but is impermeable to the blood-brain barrier, failed to alter food intake (Ebenezer and Patel, 2004). Moreover, central studies implicate GABA receptor involvement at limbic and hypothalamic structures in increasing food intake (Echo et al., 2002; Khaimova et al., 2004; Miner et al., 2010; Stratford and Kelley, 1997). As mentioned, however, it should be noted that BAC-induced modulations to food, sugar, and fat intake vary widely as a function of time of feeding (Ebenezer and Patel, 2011), route of administration (Bains and Ebenezer, 2013; Ebenezer and Prabhaker, 2007; Patel and Ebenezer, 2008a; 2008b), homeostatic state (Budnalevin et al., 2005; Rao et al., 2008; Sato et al., 2007; Wang et al., 2011; Wojnicki et al., 2014), and acute tolerance (Bains and Ebenezer, 2013) rendering it’s exact effects inconclusive. Given the evidence describing the mixed effects of BAC on intake per se, it is hypothesized that systemic GABA\textsubscript{B} agonist administration will alter the acquisition and expression of fructose-CFP.

4B. GABA\textsubscript{B} Systems and Corn Oil-CFP (Specific Aim 4)

GABA\textsubscript{B} receptor signaling has also been implicated in the mediation of fat intake. BAC administered into limbic and hypothalamic sites increased food intake (e.g., Arnt and Scheel-Kruger, 1979; Echo et al., 2002; Stratford and Kelley, 1997; Ward et al., 2000; Wirtshafter et al., 1993), and was reported to be mediated through GABA receptor interactions between the ventral
tegmental area and nucleus accumbens (Miner et al., 2010). Systemic BAC increased fat intake under normal conditions (e.g., Bains and Ebenezer, 2013; Ebenezer and Patel, 2011), but suppressed fat intake under “binge-type” conditions (Avena et al., 2014; Berner et al., 2009; Buda-Levin et al., 2005; Corwin and Wojnicki, 2009; Rao et al., 2008; Wojnicki et al., 2014; Wong et al., 2009). Additionally, the reinforcing effects of primary rewarding and conditioned stimuli mediated by mesolimbic circuitry was found to be modulated by GABA<sub>B</sub> receptor activation in optogenetic studies of food intake (van Zessen et al., 2012).

5. Conditioned Flavor-Flavor Avoidances (CFA):

5A. Quinine-CFA: Parallels of Fructose-CFP

Flavor–taste avoidance learning, occurs when an arbitrary flavor (CS) is paired with a naturally non-preferred taste (US, e.g., bitter, quinine). Fanselow and Birk (1982) originally reported that rats learned to avoid a flavor (e.g., almond) mixed into a quinine solution although their study was not a pure CFA because the animals had a second flavor (e.g., vanilla) mixed into a preferred saccharin solution. More recently, Dwyer (2011) trained rats to drink a CS+ flavor (e.g., cherry) added to a quinine solution and a CS− flavor (e.g., grape) added to water in separate sessions. In a subsequent two-bottle choice test, the rats avoided the CS+ when both CS flavors were presented in plain water.

Previous studies elicited quinine-CFA by employing a paradigm in which thirsty rats were trained to drink flavored water adulterated with quinine (Dwyer, 2011; Harris and Westbrook, 1998). Specific Aim 5 utilizes a different design to match that used in our flavor–taste preference conditioning studies in which hungry rats were trained with a flavored fructose and saccharin solution and a less preferred flavored saccharin solution (Baker et al., 2003). In this case, hungry rats are trained with two differently flavored fructose and saccharin (FS)
solutions with one adulterated with quinine. We examine a range of quinine concentrations to determine a concentration that is able to condition a flavor avoidance comparable in magnitude to the preference obtained in earlier fructose-CFP studies (Baker et al., 2003; 2004; Bernal et al., 2008; 2009; 2010; Golden and Houpt, 2007; Malkusz et al., 2012).

5B. Potential Pharmacological Substrates of Quinine-CFA (Specific Aim 5)

Conditioned flavor avoidances (CFA) can be induced by either ingested toxins that induce gastrointestinal distress (flavor–toxin learning; see review: Freeman and Riley, 2009) or by aversive tastes (flavor-taste learning; e.g., Dwyer, 2011; Fanselow and Birk, 1982).

Pharmacological analyses have examined DA D1, DA D2, NMDA and opioid antagonists in flavor-toxin CFA learning. DA D1, but not D2 antagonism disrupted the acquisition of a LiCl-induced CFA following systemic administration, and following central administration into the LH or NAc shell (Caulliez et al., 1996; Fenu et al., 2001; 2005; 2009). Blockade of NMDA, α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and metabotropic glutamate receptors in the AMY disrupted LiCl-induced CFA (Yasoshima et al., 2000). Naloxone enhanced taste aversions elicited by LiCl (Davis et al., 2009; Miceli et al., 1979; Smurthwaite et al., 1992). Given this as well as the differential effects of DA D1, DA D2, NMDA and opioid receptor signaling on flavor-flavor learning elicited by the palatability of fructose and corn oil, Specific Aim 5 assesses the potential role of these receptor candidates in modulating the acquisition and expression of flavor-flavor CFP elicited by the unpalatability of quinine.

5C. Cholinergic Systems and Quinine-CFA (Specific Aim 6A)

Cholinergic receptors have also been previously implicated in certain forms of CFA. In flavor-toxin CFA learning, SCOP attenuated LiCl-induced CFA without altering aversion to
quinine (Coil et al., 1978). SCOP and nicotine are capable of eliciting flavor-taste CFAs with the former abolished and the latter enhanced by lesions placed in the area postrema (Ossenkopp et al., 1986; Ossenkopp and Gugno, 1990). A possible role for cholinergic receptors in flavor-taste CFA is supported by the ability of quinine to inhibit Ach currents in alpha-9-alpha-10-containing nicotinic Ach receptors in Xenopus oocytes (Ballestero et al., 2005), and adrenal catecholamine secretion evoked by Ach stimulation of muscarinic and nicotinic receptors (Jang et al., 2001). In turn, nicotine depressed quinine-evoked responses in the nucleus tractus solitarius that were blocked by MEC (Simons et al., 2006). Nicotine and quinine CFA generalized to each other in three mouse strains (Gyekis et al., 2012), with MEC blocking this ability in behavioral and electrophysiological assays (Oliveira-Maia et al., 2009). Thus, these data appear to implicate cholinergic receptor signaling in flavor-taste CFA and as a result, Specific Aim 6A addresses the roles of muscarinic and nicotinic cholinergic receptor signaling in mediating quinine-induced CFA.

5D. GABA Systems and Quinine-CFA (Aim 6B)

A role for GABA_B receptor systems in conditioned aversions is supported by the ability of the GABA_B agonist, BAC, but not the GABA_B antagonist, saclofen, to suppress saccharin-induced drinking following pairing (Echo et al., 2002; Wilson et al., 2011). However, another study using a similar dose range found that systemic BAC failed to induce an aversion or affect ethanol-induced aversions (Chester and Cunningham, 1999). Further, although systemic BAC failed to alter operant responding to quinine-adulterated solutions (Petry and Heyman, 1997), it did enhance the discriminative abilities of D-amphetamine in a conditioned taste aversion procedure (Miranda et al., 2009). Accordingly, Specific Aim 6B tests the involvement of GABA_B receptor signaling in quinine-CFA.
6. Organization of the Studies:

The six specific aims of this dissertation research are systematically examined in four major documents that have successfully undergone peer review and have indeed been published. For ease of presentation, Chapters 3-6 present the four papers in the order that they were published. Thus, Dissertation Research Paper 1 (Chapter 3) addresses Specific Aim 5, Dissertation Research Paper 2 (Chapter 4) addresses Specific Aims 1 and 6A, Dissertation Research Paper 3 (Chapter 5) addresses Specific Aims 3 and 6B, and Dissertation Research Paper 4 (Chapter 6) addresses Specific Aims 2 and 4.

Dissertation Research Paper 1: The goals of Specific Aim 5 were to determine whether DA D1, DA D2, opioid and NMDA receptors, involved in mediating appetitive associative learning processes (e.g., sugar- and fat-CFP), would similarly moderate underlying associative processes contributing to the behavioral effects of CFA associative learning processes induced by primary aversive stimuli (e.g., quinine). Thus, we addressed the pharmacology of flavor-taste CFA by examining the roles of DA D1, DA D2, NMDA and opioid receptor signaling in flavor avoidance conditioned by the bitter taste of quinine. This paper, entitled “Role of NMDA, opioid and dopamine D1 and D2 receptor signaling in the acquisition of a quinine-conditioned flavor avoidance in rats”, was published in *Physiology and Behavior* (128, 133-140, 2014) (Chapter 3).

Dissertation Research Paper 2: The goals of Specific Aim 1 were to determine whether muscarinic or nicotinic cholinergic receptor antagonism mediates the acquisition and expression of sugar (fructose)-CFP. Further, one goal of Specific Aim 6 determines whether muscarinic or nicotinic receptor antagonism mediate quinine-CFA. Therefore, we evaluate the ability of SCOP and MEC for their effects upon the acquisition and expression of fructose-CFP (Specific Aim 1) and acquisition of quinine-CFA (Specific Aim 6A). This paper, entitled “Muscarinic and
nicotinic cholinergic receptor antagonists differentially mediate acquisition of fructose-conditioned flavor preference and quinine-conditioned flavor avoidance in rats”, was published in *Neurobiology of Learning and Memory* (123, 239-249, 2015) (Chapter 4).

**Dissertation Research Paper 3:** The goal of Specific Aim 3 was to determine whether systemic pharmacological stimulation of GABA\(_B\) receptors influence the acquisition and expression of sugar (fructose)-CFP. Further, a second goal of Specific Aim 6 determines whether stimulation of GABA\(_B\) receptors mediate quinine-CFA. Therefore, we evaluate the ability of BAC for its effects upon the acquisition and expression of fructose-CFP (Specific Aim 3) and acquisition of quinine-CFA (Specific Aim 6B). This paper, entitled “Baclofen differentially mediates fructose-conditioned flavor preference and quinine-conditioned flavor avoidance in rats”, was published in *European Journal of Pharmacology* (775, 15-21, 2016a) (Chapter 5).

**Dissertation Research Paper 4:** The goals of Specific Aim 2 were to determine whether muscarinic or nicotinic cholinergic receptor antagonism mediates the acquisition and expression of fat (corn oil)-CFP. Similarly, the goal of Specific Aim 4 determines whether stimulation of GABA\(_B\) receptors antagonism mediate the acquisition and expression of fat-CFP. Therefore, we evaluate the ability of SCOP and MEC (Specific Aim 2) as well as BAC (Specific Aim 4) for their effects upon the acquisition and expression of fat-CFP. This paper, entitled “Muscarinic, nicotinic and GABAergic receptor signaling differentially mediate fat-conditioned flavor preference in rats”, was published in *Pharmacology, Biochemistry, and Behavior* (150-151, 14-21, 2016b) (Chapter 6).
Chapter 3: Role of NMDA, opioid and dopamine D1 and D2 receptor signaling in the acquisition of a quinine-conditioned flavor avoidance in rats

1. Introduction:

Animals use flavor cues (taste, odor, texture) to guide their selection of nutritious foods and avoidance of toxic foods (or fluids) with learning shaping this selection (Capaldi, 1996; Sclafani, 1995). Four common types of food learning have been identified: CFP induced by the orosensory (flavor-taste learning; e.g., (Baker et al., 2004; Baker et al., 2003, Holman, 1975, Sclafani, 1995; Sclafani & Ackroff, 1994; Sclafani et al., 1993; Sclafani et al., 1999; Yu et al., 1999; 2000a; 2000b)) and/or the post-oral (flavor-nutrient learning; e.g., (Azzara et al., 2000; 2001; Touzani et al., 2008)) reinforcing properties of foods such as sugars, and CFA induced by either ingested toxins that induce gastrointestinal distress (flavor-toxin learning; see review: Freeman & Riley, 2009) or an aversive taste (flavor-taste learning; e.g., (Dwyer, 2011; Fanselow & Birk, 1982)). This paper is using the term, avoidance rather than aversion, as we did not measure taste reactivity following CFA and to allow consistency throughout the text. The fourth and least studied type of food learning, flavor-taste avoidance learning, occurs when an arbitrary flavor (CS) is paired with a naturally unpreferred taste (US, e.g., bitter quinine). Fanselow and Birk (1982) originally reported that rats learned to avoid a flavor (e.g., almond) mixed into a quinine solution although their study was not a pure CFA because the animals had a second flavor (e.g., vanilla) mixed into a preferred saccharin solution. More recently, Dwyer (2011) trained rats to drink a CS+ flavor (e.g., cherry) added to a quinine solution and a CS-flavor (e.g., grape) added to water in separate sessions. In a subsequent two-bottle choice test, the rats avoided the CS+ when both CS flavors were presented in plain water.
Numerous studies have investigated the neurochemical substrates of flavor-taste and flavor-nutrient CFP as well as flavor-toxin CFA using DA, NMDA and opioid receptor antagonists. In CFP studies, systemic treatment with DA D1 and D2 receptor antagonists attenuated the acquisition and expression of a flavor-taste CFP produced by the sweet taste of sucrose or fructose (Baker et al., 2003; Yu et al., 2000a; 2000b). In contrast, systemic DA D1 but not D2 antagonism blocked the acquisition, and to a lesser degree the expression of a flavor-nutrient CFP elicited by IG sucrose infusions (Azzara et al., 2001). Brain sites involved in DA modulation of flavor-taste and flavor-nutrient CFP by sugar include the NAc (Bernal et al., 2008; Touzani et al., 2008), AMY (Bernal et al., 2009; Touzani et al., 2009) and mPFC (Malkusz et al., 2012; Touzani et al., 2010). In flavor-toxin CFA studies, systemic DA D1, but not D2 antagonism disrupted the acquisition of a LiCl-induced CFA (Fenu et al., 2005; 2009). Central drug studies revealed that DA D1 receptor antagonists administered into either the LH (Caulliez et al., 1996) or shell of the NAc (Fenu et al., 2001) disrupted the acquisition of a LiCl-induced CFA.

In NMDA receptor signaling studies, the acquisition, but not the expression of flavor-taste mediated fructose-CFP was blocked by systemic treatment with the non-competitive NMDA antagonist, MK-801 (Golden & Houpt, 2007). Blockade of NMDA, AMPA and metabotropic glutamate receptors in the AMY disrupted LiCl-induced CFA (Yasoshima et al., 2000), and blockade of NMDA receptors in the AMY eliminated the acquisition of flavor-nutrient- CFP (Touzani et al., 2013). In contrast to DA and glutamate involvement, systemic or central administration of the general opioid antagonist, NTX had little or no effect on flavor preference conditioning by the taste or nutritive actions of sugar (Azzara et al., 2000; Baker et
al., 2004; Bernal et al., 2010; Yu et al., 1999). However, naloxone enhanced taste aversions elicited by LiCl (Davis et al., 2009; Miceli et al., 1979; Smurthwaite et al., 1992).

Flavor-taste CFA learning has not been the subject of pharmacological analysis, and the present study addressed this gap by examining the roles of DA D1, DA D2, NMDA and opioid receptor signaling in flavor avoidance conditioned by the bitter taste of quinine. In two prior studies, CFA was produced by training thirsty rats to drink flavored water adulterated with quinine (Dwyer, 2011; Harris & Westbrook, 1998). Here we used a different design to match that used in our flavor-taste preference conditioning studies in which hungry rats were trained with a flavored fructose + saccharin solution and a less preferred flavored saccharin solution (Baker et al., 2003). In this case, hungry rats were trained with two differently flavored fructose + saccharin (FS) solutions with one adulterated with quinine. The first experiment examined a range of quinine concentrations to determine a concentration that conditioned a flavor avoidance comparable in magnitude to the preference obtained in earlier fructose-CFP studies (Baker et al., 2004; 2003; Bernal et al., 2008; 2009; 2010; Golden & Houpt, 2007; Malkusz et al., 2012). The second experiment examined the systemic effects of DA D1 (SCH23390), D2 (raclopride), NMDA (MK-801) and opioid (naltrexone) receptor antagonists on the acquisition of the quinine-induced CFA. In these experiments, the avoidance of the quinine-paired CS+ flavor was evaluated in two-bottle tests with both flavored FS solutions presented without quinine. To determine if the high palatability of the FS solutions used in the choice tests may weaken the expression of the quinine conditioned avoidance, a third experiment was conducted in which the rats were given two-bottle tests using flavored saccharin solutions without fructose.
2. Methods:

2.1. Subjects: Male Sprague-Dawley rats (n=138, 250-275 g), obtained from Charles River Laboratories (Wilmington, MA), were housed individually in wire mesh cages and maintained on a 12:12 h light/dark cycle with chow (5001, PMI Nutrition International, Brentwood, MO) and water available ad libitum for the first week. All animals were then food-restricted to 85-90% of their body weight throughout behavioral testing. Food rations were provided 1 h after the end of daily training and testing sessions. The experimental protocols in the three experiments were approved by the Queens College Institutional Animal Care and Use Committee certifying that all subjects and procedures are in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

2.2. Test Solutions and Initial Training: The training solutions contained 8% fructose (Sigma Chemical Co., St. Louis, MO) and 0.2% sodium saccharin (Sigma Chemical Co.) with or without quinine (0.001-0.06%; Sigma Chemical Co.), each flavored with 0.05% unsweetened grape or cherry Kool-Aid (General Foods, White Plains, NY). Fructose rather than sucrose or glucose was used because, unlike these other sugars, fructose has minimal post-oral flavor conditioning effects (Sclafani & Ackroff, 1994; Sclafani et al., 1993; 1999). Half of the rats in each group had the cherry flavor added to the FS solution and the grape flavor added to the fructose + saccharin + quinine (FSQ) solution; the flavors were reversed for the remaining rats. In the two-bottle choice tests, the cherry and grape flavors were presented in either 8% fructose + 0.2% saccharin (Experiments 1 and 2) or 0.2% saccharin (Experiment 3) solutions. The flavored fructose + saccharin + quinine solution is referred to as the CS+/FSQ, and the flavored fructose + saccharin solution as the CS-/FS; and the same flavors used in the two-bottle tests are referred to as CS+ and CS-, respectively. All testing took place in the rat’s home cage during the mid-light
phase of the light:dark cycle. The food-restricted rats were initially trained to drink an unflavored 8% fructose and 0.2% saccharin solution from sipper tubes during five daily 1-h sessions. The sipper tube was mounted on the front of the cage held by a taut steel spring, and was positioned 3-6 cm above the cage floor.

2.3. Procedure: Rats were trained over eight one-bottle training sessions (1 h) to drink the CS-/FS solution on odd-numbered days, and the CS+/FSQ solution on even-numbered days. The eight training trials were divided into four pairs of sessions with a one-day break between each pair. In the first three training pairs, only one bottle was presented. In the fourth pair of training sessions (days 7 and 8), a second sipper tube containing water was also presented to acclimate the rats to the presence of two sipper tubes used during the choice tests; water intake was negligible in these training trials. The left-right position of the CS and water sipper tubes was counterbalanced over the two days. Training intakes were limited to 16 ml/session to correspond with the training procedure used in our prior fructose-CFP studies (Baker et al., 2003; 2004; Bernal et al., 2008; 2009; 2010; Malkusz et al., 2012). Following training, the rats were given six two-bottle choice test sessions (1 h) with unlimited access to the CS+ and CS- solutions. The position of the two bottles were left (L)-right (R)-R-L-L-R in half of the animals, and R-L-L-R-R-L in the remaining half. Solution intakes during the training and testing were measured by weighing (0.1 g) the bottles before and after the 1 h sessions.

2.4. Experiment 1: Quinine Concentration and CFA: Seven groups (n=6/7 each) of food-restricted rats, matched for unflavored fructose+ saccharin intakes, were tested in the conditioning procedure described above in which one of the following quinine concentrations was included in the CS+FSQ solution during one-bottle training: 0.001%, 0.002%, 0.004%,
0.008%, 0.012%, 0.016% and 0.030%. The animals then received six two-bottle tests with the CS+ and CS- flavors mixed in 8% fructose + 0.2% saccharin solutions.

2.5. Experiment 2: Pharmacological Effects on Acquisition of Quinine-CFA: Five groups of food-restricted rats received systemic injections of a) vehicle (VEH group, n=13; 1 ml 0.9% normal saline/kg body weight, ip), b) MK-801 (MK801 group, n=11; 100 ug/kg, ip, Sigma Chemical Co.), c) SCH23390 (SCH group, n=12; 200 nmol/kg, ip, Sigma Chemical Co.), d) raclopride (RAC group, n=11; 200 nmol/kg, ip, Sigma Chemical Co.) or naltrexone (NTX group, n=12; 1 mg/kg, ip, Sigma Chemical Co.) 30 min prior to each of eight one-bottle training sessions with the CS-/FS solution on odd-numbered days and CS+/FSQ (0.030% concentration) on even-numbered days. The doses of MK-801, SCH, RAC and NTX were chosen based on their use in prior studies investigating the acquisition of fructose-CFP (Baker et al., 2003; 2004; Golden & Houpt, 2007). To assess whether drug effects were functionally equivalent to increasing the quinine concentration, a sixth group of rats (VEH.06% group, n=12) received vehicle injections followed 30 min later with the CS-/FS solution or a CS+/FSQ solution containing 0.06% quinine. Following training, all groups received six two-bottle sessions with the CS+ and CS- flavors mixed in 8% fructose + 0.2% saccharin solutions.

2.6. Experiment 3: To assess whether the highly palatable 8% fructose + 0.2% saccharin solution used in two-bottle testing in Experiments 1 and 2 influenced the expression of the quinine conditioned flavor avoidance, two groups of rats received either vehicle or SCH (200 nmol/kg) 30 min prior to each of eight one-bottle training sessions with the CS-/FS and CS+/FSQ (0.030% concentration). However, in the six two-bottle choice sessions, the CS+ and CS- flavors were presented in 0.2% saccharin solutions.
2.7. Data analysis: In each experiment, training intakes were averaged over the four CS+/FSQ and four CS-/FS sessions and were analyzed with a two-way randomized-blocks ANOVA (CS conditions x Groups). Intakes during the preference tests were averaged over sessions 1-2, 3-4, and 5-6 (referred to as Tests 1, 2, and 3) to control for side position effects. A three-way randomized-blocks ANOVA compared the CS intakes of the concentration groups in Experiment 1 (Group x CS x Test), and the CS intakes of the drug groups in Experiments 2 and 3 (Group x CS x Test). Separate two-way ANOVAs evaluated total CS intakes and percent CS+/s intakes of the different groups in the three experiments. When main or interaction effects were found, Bonferroni corrected comparisons (p<0.05) detected significant effects.

3. Results:

3.1. Quinine Concentration and CFA: During one-bottle training, the mean intake of the CS-/FS solution significantly exceeded that of the CS+/FSQ solution (14.7 vs. 11.9 g/1 h, F(1,36)= 111.82, p<0.0001), and significant differences were also observed across the seven quinine concentrations (F(6,36)= 15.99, p<0.0001) and for the interaction between CS and concentration (F(6,36)= 22.63, p<0.0001). As indicated in Figure 1A, one-bottle training intakes of the CS-/FS and CS+/FSQ solutions failed to differ at quinine concentrations 0.001%, 0.002%, 0.004% and 0.008%. CS+/FSQ intakes were significantly lower than CS-/FS intakes with the 0.012%, 0.016% and 0.03% quinine concentrations (Figure 1A).

In the two-bottle choice tests, overall, CS+ intakes (13.9 g) failed to differ from CS-intakes (14.5 g). However, significant differences were observed among tests (F(2,72)= 5.68, p<0.018) and for the interaction between quinine concentration and tests (F(12,72)= 6.16, p<0.047). Figure 1B depicts CS- and CS+ intakes in Test 1 across the seven training quinine concentrations. Whereas the six lower quinine concentrations elicited similar CS- and CS+
Figure 1. Elicitation of Quinine-conditioned flavor avoidance (CFA) is dependent on quinine concentration. Panel A: One-bottle training intakes (mean +SEM, g/60 min) of CS+/FSQ and CS-/FS solutions in separate groups of animals given CS+/FSQ solutions containing 0.001%, 0.002%, 0.004%, 0.008%, 0.012%, 0.016% or 0.030% quinine. Panel B. Two-bottle choice Test 1 intakes (mean +SEM, g/60 min) of the CS+ flavor and CS- flavor presented in FS solutions. The percentages of CS+ intake over total intake are denoted above each pair of values. Significant differences are denoted between CS+/FSQ and CS-/FS intake are denoted (*).
intakes, rats trained with the highest (0.03%) quinine concentration displayed significantly lower CS+ intake than CS- intake, indicative of an avoidance of the flavor associated with the FSQ solution. This effect was transitory in that this group and all other groups failed to display any differences in CS+ and CS- intakes in Tests 2 and 3 (data not shown). Evaluation of the percent CS+ intakes failed to reveal any differences among concentrations, tests or their interaction. Thus, the 29% CS+ percent intake of the 0.03% group was lower, but not significantly so, than those (48-54%) of the other six quinine groups.

3.2. Pharmacological effects on Quinine-CFA: In the one-bottle training sessions, overall, CS-/FS intake significantly exceeded CS+/FSQ intake (12.7 vs. 2.0 g/1 h, F(1,60)= 1303.21, p<0.0001), there were significant group differences (F(5,60)= 13.11, p<0.0001), and there was a significant interaction between groups and CS (F(5,60)= 3.31, p=0.011). CS-/FS intake for all six groups was significantly higher than CS+/FSQ intake (Figure 2), demonstrating the ability of quinine at concentrations of 0.03% and 0.06% (VEH.06% group) to reduce intake. CS-/FS intake was significantly lower in the SCH and NTX groups relative to VEH group; the MK-801, RAC and VEH groups did not differ significantly in their CS-/FS intakes (Figure 2). CS+/FSQ intake was significantly lower in MK801, SCH and NTX groups relative to the VEH group; the RAC and VEH groups did not differ in CS+/FSQ intakes (Figure 2). The CS+/FSQ intake of the VEH.06% group was significantly lower than that of the VEH group trained with 0.03% quinine (Figure 2).

In the two-bottle choice tests, there were significant differences in the overall CS+ (8.8 g) and CS- (15.5 g) intakes (F(1,19)= 359.90, p<0.0001), as well as for the interactions between groups and tests (F(10,120)= 5.12, p<0.043) and tests and CS (F(2,120)= 3.71, p<0.039). Within-group comparisons revealed that VEH rats consumed more CS- than CS+ only during Test 1.
Figure 2. (Experiment 2): One-bottle training intakes (mean ±SEM, g/60 min) of CS+/FSQ and CS-/FS solutions during training sessions 30 min following systemic administration of vehicle (VEH group), MK-801 (MK801 group), SCH23390 (SCH group), raclopride (RAC group) or naltrexone (NTX group). These groups were trained with a CS+/FSQ solution containing 0.03% quinine whereas the VEH.06% group was trained with a CS+/FSQ solution containing 0.06% quinine. Significant differences are denoted between CS+/FSQ and CS-/FS intake are denoted (*) as are any drug effect relative to VEH (+).
(Figure 3A), consistent with the results of Experiment 1. In contrast, the MK801, SCH and NTX groups consumed more CS- than CS+ in all three tests (Figure 3B, C, E), indicating that the NMDA, DA D1 and opioid antagonists prolonged the CFA effects of quinine. In contrast, the RAC group, like the VEH group, consumed more CS- than CS+ in Test 1 only (Figure 3D). The three drug groups (SCH, MK801, NTX) that displayed a persistent CS+ avoidance (Figure 3B,C,E), were the groups that also showed significantly reduced CS+/FSQ intake during training relative to the VEH group (Figure 2). In contrast, the RAC group was similar to the VEH group in its training and test intakes. The persistent quinine-induced CFA in rats trained with MK-801, SCH and NTX was unexpected, and therefore, an additional vehicle control group was added to determine whether the effects of these drugs were behaviorally and functionally equivalent to increasing the concentration of quinine during training. This explanation appeared to be plausible in that the VEH.06% group trained with a 0.06% quinine solution in the CS+/FSQ consumed significantly more CS- than CS+ in Test 1 and 2 although not in Test 3 (Figure 3F).

Analysis of the percent CS+ intake data revealed significant differences across tests (F(2,120)= 4.13, p<0.029), but not among groups or for the interaction between groups and tests. Trends in the data indicate that the avoidance in all five groups were comparable (30-37%) for the first test pair in animals exposed to the 0.03% quinine solution, and were lower (23%) for the first test pair in animals exposed to the 0.06% quinine solution (Figure 3). However, whereas vehicle-trained (43-50%) and RAC-trained (40-46%) rats displayed increasing indifference to the two flavors in the second and third test pairs, rats trained with MK-801 (32-33%), SCH (35-36%) and NTX (29-39%) displayed persistent quinine-induced CFA during the second and third test pairs (Figure 3). Finally, vehicle-treated rats exposed to 0.06% quinine showed a steady erosion of the avoidance in the second (34%) and third (40%) test pairs (Figure 3).
Figure 3. (Experiment 2): Two-bottle choice test intakes (mean ±SEM, g/60 min) of the CS+ flavor and CS- flavor presented in fructose+saccharin solutions in Tests 1-3 in groups trained with vehicle (A), MK-801 (B), SCH23390 (C), raclopride (D), naltrexone (E) or VEH and a CS+/FSQ solution containing 0.06% quinine (F). The percentages of CS+/FSQ intake over total intake are denoted above each pair of values. Significant differences are denoted between CS+/FSQ and CS-/FS intake are denoted (*).
3.3. Quinine-Induced CFA expressed with Saccharin Solutions: To test the possibility that the transitory nature of quinine-induced CFA in the VEH group was due to “masking” effects of the very palatable FS solution used in two-bottle testing, this experiment employed an identical paradigm in vehicle-trained rats except that the CS+ and CS- flavors were mixed in a 0.2% saccharin solution during the two-bottle test. A SCH group was similarly trained and tested to determine if the testing with CS flavors in 0.2% saccharin solutions influenced the results obtained with vehicle and SCH23390 injections. In the one-bottle training sessions, overall, CS-/FS intake significantly exceeded CS+/FSQ intake (10.6 vs. 2.5 g/1 h, F(1,9)= 769.41, p<0.0001), the two groups significantly differed from each other (F(1,9)= 286.26, p<0.0001), and there was a significant interaction between groups and CS (F(1,9)= 6.31, p=0.033). CS-/FS intake in both groups was significantly higher than CS+/FSQ intake (Figure 4A). SCH-trained rats displayed significantly less CS-/FS intake relative to vehicle-trained rats; CS+/FSQ intakes failed to differ between groups (Figure 4A).

In the two-bottle choice tests, there were significant differences in the overall CS+ (6.3 g) and CS- (13.9 g) intakes (F(1,9)= 19.03, p<0.002), as well as for the interaction between tests and CS (F(2,180)= 3.69, p<0.045) but not between groups or for the interaction between groups and tests. Within-group comparisons revealed that VEH rats consumed significantly more CS- than CS+ only in Test 1(Figure 4B), demonstrating an identical avoidance pattern to that observed with rats tested with the CS+ and CS- flavors presented in FS solutions in Experiments 1 and 2. In contrast, the SCH rats consumed significantly more CS- than CS+ in all three Tests (Figure 4C) as observed in Experiment 2. Analysis of the percent CS+ intake data revealed significant effects across tests (F(2,18)= 4.02, p<0.036), but not between groups and for the interaction between groups and tests. Trends in the data indicate that the avoidances in the
Figure 4. (Experiment 3. Panel A): One-bottle training intakes (mean +SEM, g/60 min) of CS+/FSQ and CS-/FS solutions during training sessions 30 min following systemic administration of vehicle (VEH group) or SCH23390 (SCH group). Two-bottle choice test intakes (mean +SEM, g/60 min) of the CS+ flavor and CS- flavor presented in 0.2% saccharin solutions in Tests 1-3 in VEH group (B) and SCH group (C). The percentages of CS+/FSQ intake over total intake are denoted above each pair of values. Significant differences are denoted between CS+/FSQ and CS-/FS intake are denoted (*) as are any drug effect relative to VEH (+).
vehicle (26%) and SCH (19%) groups were comparable for the first test pair (Figure 4). However, whereas vehicle-trained rats (40-49%) displayed increasing indifference to the two flavors in the second and third test pairs, rats trained with SCH displayed persistent quinine-induced CFA during the second (28%) and third (25%) test pairs (Figure 4).

4. Discussion:

The present study examined the roles of DA, NMDA and opioid receptor signaling in the acquisition of a flavor-taste CFA induced by the bitter taste of quinine added to a flavored fructose+saccharin solution. This was of interest given that the acquisition of a flavor-taste CFP induced by the sweet taste of fructose is blocked by systemic or central administration of DA D1, DA D2, NMDA, but not opioid receptor antagonists (Baker et al., 2003; 2004; Bernal et al., 2008; 2009; 2010; Golden & Houpt, 2007; Malkusz et al., 2012).

The results of Experiment 1 demonstrated that a CFA could be produced by adulterating a fructose+saccharin solution with quinine depending upon the concentration of the bitterant. At very low concentrations (0.001-0.008%), the added quinine failed to reduce CS+/FSQ training intake relative to the CS-FS, and did not produce significant differences in CS+ and CS- intakes in the two-bottle choice tests. Although adulteration with 0.012% or 0.016% quinine significantly depressed CS+/FSQ training intake, it was insufficient to produce a CFA in the two-bottle tests. However, when the quinine concentration was raised to 0.03%, CS+/FSQ intake was significantly reduced relative to CS-/FS intake in one-bottle training trials, and a quinine CFA was observed in two-bottle Test 1 but not in Tests 2 and 3. The transitory nature of the 0.3% quinine-induced CFA was also observed in the VEH groups of Experiments 2 and 3 that were tested with CS flavors presented in fructose+saccharin and saccharin-only solutions, respectively. The data from Experiment 3 indicates that the transitory quinine CFA observed in
the first two experiments was not due to the use of palatable fructose+saccharin solutions in the choice tests. Instead, the quinine CFA was found to be related to the concentration of the bitter adulterant. The VEH.06% rats trained with a CS+FSQ containing 0.06% quinine consumed less solution during training and displayed a more persistent CS+ avoidance (Tests 1 and 2) than did the VEH rats with the CS+FSQ containing 0.03% quinine (Test 1 only), indicating that the persistence of the quinine CFA could be increased by increasing quinine concentration.

Our observation of a quinine-induced CFA in hungry rats trained with fructose-saccharin solutions extends a prior report of quinine-induced CFAs in thirsty rats trained with flavored water using the same grape and cherry CS flavors (Dwyer, 2011). Note that this and prior studies evaluated quinine-induced CFA in only one or two two-bottle choice sessions (Dwyer, 2011; Fanselow & Birk, 1982; Harris & Westbrook; 1998) unlike the six test sessions of the present study, and thus provide no data on the persistence of the CFA in thirsty rats. In studies of fructose-CFP, the conditioned preference persisted over 6 to 10 two-bottle test sessions which indicates that at least some forms of flavor-taste learning are resistant to extinction (Baker et al., 2003; 2004; Bernal et al., 2008; 2009; 2010; Malkusz et al., 2012).

The prior sugar-conditioned flavor-taste preference studies revealed that flavor conditioning was dependent of upon intact DA D1 and D2 (Baker et al., 2003; Yu et al., 2000a; 2000b) and NMDA (Golden & Houpt, 2007), but not opioid (Baker et al., 2004; Yu et al., 1999) receptor signaling. That is, systemic administration of SCH23390, raclopride and MK-801, but not naltrexone, during flavor training reduced or eliminated the learning of the sweet taste based CS+ preference. This is in marked contrast to the present findings that the same doses of SCH23390, MK-801, and naltrexone, but not raclopride, enhanced the quinine-CFA as indicated by the more persistent CS+ avoidance displayed by the SCH, MK801 and NTX groups compared
to the VEH group. The effects of SCH23390, MK-801 and naltrexone on the quinine-based CFA were behaviorally similar to that produced by increasing the quinine concentration (from 0.03 to 0.06%) in the CS+FSQ training solution. That is, the rats trained with the 0.06% quinine adulterated CS+FSQ solution underconsumed the solution to the same degree as the SCH, MK801, and NTX groups trained with the 0.03% quinine-adulterated CS+FSQ solution, and they showed a more persistent CS+ avoidance (Tests 1 and 2) approaching that of the drug groups. Thus it is possible that SCH233890, MK-801, and naltrexone injections enhanced the quinine-CFA by increasing the aversiveness of the FSQ training solution, although the mere observation of behavioral equivalence of the antagonist effects on the one hand, and the increased concentration effects on the other does not specify that identical mechanisms of action are involved. Further studies are needed to address this issue.

4.1 DA D1 and D2 Antagonist Effects: In CFP studies, systemic DA D1 and D2 antagonists reduce the acquisition of flavor-taste sugar preferences (Baker et al., 2003; Hsiao & Smith, 1995; Yu et al., 2000a; 2000b), whereas only DA D1 antagonists reduce the acquisition of flavor-nutrient sugar preferences (Azzara et al., 2001). These DA antagonist effects involve central sites of action, notably the NAc, AMY and mPFC (Bernal et al., 2008; 2009; Malkusz et al., 2012; Touzani et al., 2008; 2009a; 2010). One interpretation of how DA antagonists block sugar-based CFPs is that the drugs reduce the reward value of the sweet taste or post-oral actions of sugars. An extension of this hypothesis would be that DA antagonists also reduce the negative reward value of aversive tastes or post-oral aversive states. In fact, DA D1 receptor antagonists administered into either the LH (Caulliez et al., 1996) or shell of the NAc (Fenu et al., 2001) disrupted the acquisition of a flavor-toxin CFA induced by LiCl in a manner similar to reductions in the acquisition of flavor-nutrient CFP elicited by IG glucose infusions (Touzani et
The selective effects of SCH23390, but not raclopride injections on quinine-induced CFA is similar to reports that only SCH23390 altered flavor-toxin avoidance produced by LiCl injections (Fenu et al., 2005; 2009). However, whereas SCH23390 blocked the development of the LiCl-CFA, the drug enhanced the quinine-CFA in the present study. The quinine and LiCl conditioning procedures differed in several important respects which complicate comparisons between the different drug effects. Note, in particular, that in one experiment, SCH23390 significantly attenuated a LiCl-induced CFA when injected 5 min after the CS training sessions but not when injected 30 min prior to the CS training sessions as in the present study (Fenu et al., 2001). Thus, to determine if SCH23390, or other drugs, differentially influence the learning of a bitter taste (quinine) or toxic drug (LiCl) CFA, it is essential to use similar training paradigms (drug dose, injection timing, CS flavor, etc.).

Conceivably, DA D1 antagonism may enhance quinine-conditioned flavor avoidance because it selectively increases the aversiveness of bitter adulterants. However, there is little evidence concerning the impact of systemic SCH23390 on quinine avoidance; indeed, one study reported that SCH microinfusions into the ventral pallidum suppressed saccharin, but not quinine intake (Shimura et al., 2006). In Experiments 2 and 3, SCH23390 reduced the intake of both the FS and FSQ solutions so the drug effect on quinine avoidance per se cannot be differentiated. The failure of raclopride injections to alter CS+FSQ training intake or CS+ preference, relative to the vehicle treatment, is consistent with one report that this DA D2 antagonist did not alter quinine solution intake in rats (Phillips et al., 1991).

4.2 NMDA Antagonist Effects: In a CFP study, systemic NMDA receptor antagonism eliminated the acquisition, but not expression of a flavor-taste fructose preference (Golden & Houpt, 2007). Blockade of NMDA, AMPA and metabotropic glutamate receptors in the AMY,
but not the LH or parabrachial nucleus, also disrupted a flavor-LiCl CFA (Tucci et al., 1998; Vales et al.; 2006; Yasoshima et al., 2000), whereas blockade of NMDA receptors in the AMY eliminated the acquisition of flavor-nutrient-CFP (Touzani et al., 2013). Yet the present study demonstrated that systemic treatment with MK-801 significantly prolonged the flavor-taste CFA induced by quinine. During training, MK-801-treated rats displayed significant reductions in CS+FSQ, but not CS-FS intake as compared to VEH-treated rats, suggesting that it enhanced the avoidance of the quinine adulterated solution. Yet, a previous study (Vardigan et al., 2010) reported that systemic MK-801 did not reduce quinine solution intake in thirsty rats. Golden and Houpt (2007) hypothesized that NMDA receptor signaling mediates the learning process by which a flavor CS is associated with the reward value of a gustatory US. Thus, it is not clear why systemic NMDA receptor antagonism with MK-801 blocks a fructose-CFP (Golden & Houpt, 2007), but enhances a quinine-CFA. CFA is induced by MK-801 as well as other NMDA antagonists (Fowler et al., 2011; Jackson & Sanger, 1989; Traverso et al., 2003; 2012; Turgeon et al., 2000), and it enhances ethanol-induced CFA (Blenkowski et al., 1998).

4.3 Opioid Antagonist Effects: In CFP studies, neither systemic nor central (NAc) opioid receptor antagonism altered the acquisition or expression of flavor-taste or flavor-nutrient sugar preferences (Azzara et al., 2000; Baker et al., 2004; Bernal et al., 2010; Yu et al., 1999). Yet the present study demonstrated that NTX significantly prolonged the flavor-taste CFA induced by quinine. This effect may be related to the finding that opioid antagonism increased quinine aversion in rats (Cagniard & Murphy, 2013; Le Magnen et al., 1980; Siviy & Reid, 1983), although this is not a consistent result (Ferraro et al., 2002; Goodwin et al., 2001; Levine et al., 1982; Parker et al., 1992). Opioid receptor antagonism with naloxone is also reported to enhance taste aversions elicited by LiCl (Davis et al., 2009; Micelli et al., 1979; Smurthwaite et al.,
NTX as well as delta-opioid agonists and antagonists produce conditioned flavor avoidance by themselves (Hutchinson et al., 2000; Kautz et al., 1989; Parker & Rennie, 1992). Yet, opioid antagonism blocks morphine-induced taste aversions (Fox et al., 2006; Stevenson et al., 1992).

4.4 Do antagonists prolong flavor-taste quinine CFA by eliciting CFA themselves: The ability of NTX and MK-801 to induce a CFA when paired with unflavored saccharin or sucrose raises the question as to whether aversive drug effects contributed to the enhanced quinine-CFA observed in the present study. However, Golden and Houpt (2007) did not find any evidence that MK-801 at the dose used in the present study induces a conditioned flavor avoidance. To our knowledge, there are no reports that systemic SCH23390 conditions sweet taste avoidance; one study found that systemic SCH failed to produce a saccharin avoidance (Fenu et al., 2001). It is worth noting the features of the conditioning paradigm used in the present study that differ from the typical drug-induced CFA procedures. In particular, in the present study, the antagonist drugs were administered before the presentation of the CS flavor, whereas in standard CFA studies the drug is administered after the CS flavor is consumed. Nevertheless, it is possible that taste avoidance can occur in such a "backward" conditioning preparation if the drug onset is slow enough to functionally occur simultaneous with or after the presentation of the CS flavor. A second more important difference between the present paradigm and the typical CFA procedure is that the antagonist drugs were given prior to both the CS- and the CS+ solutions during training sessions so that both flavors were associated with the aversive effects, if any, of the drugs. In the typical drug CFA procedure, only CS+ is paired with the drug and the CS- is paired with a vehicle treatment. The critical importance of this design feature is indicated by early studies of opioid antagonist effects on sugar-conditioned flavor preferences. In particular, one
study (Mehiel, 1996) reported that naloxone blocked a sugar CFP when the drug was administered prior to CS+ training sessions only, whereas other studies reported that naltrexone did not block a sugar CFP when the drug was administered prior to both CS+ and CS- training sessions (Azzara et al., 2000; Yu et al., 1999). Thus, it is not certain that drug-induced aversive effects contributed to the prolonged quinine-CFA observed in the present study, but this issue certainly requires further analysis.

In summary, whereas DA D1, DA D2 and NMDA, but not opioid receptor antagonism blocks the acquisition of sweet taste-based CFP, DA D1, NMDA and opioid, but not DA D2 receptor antagonism enhances a bitter taste-based CFA.
Chapter 4: Muscarinic and nicotinic cholinergic receptor antagonists differentially mediate acquisition of fructose-conditioned flavor preference and quinine-conditioned flavor avoidance in rats.

1. Introduction:

Sugar-CFPs are based on learned associations between food flavor elements (flavor-flavor conditioning) as well as between flavor and post-ingestive consequences (flavor-nutrient conditioning) (Sclafani, 1995). Flavor-flavor conditioning has been studied for sucrose in sham-feeding rats (Yu et al., 1999, 2000a, 2000b), and for fructose in real-feeding rats (Baker al., 2003, 2004) given the inability of fructose to condition preferences after IG administration (Sclafani and Ackroff, 1994; Sclafani et al., 1993, 1999). In contrast, glucose is capable of producing CFP following oral and IG administration (Dela Cruz et al., 2014; Sclafani and Ackroff, 1994; Sclafani et al., 1993, 1999). Previous pharmacological analyses have evaluated the neurochemical substrates of the acquisition (learning) and expression (maintenance) of the flavor-flavor component of sugar-CFP. Systemic administration of either DA D1 (SCH23390) or D2 (raclopride) receptor antagonists eliminated both acquisition and expression of fructose-CFP in real-feeding, food-restricted rats and sucrose-CFP in sham-feeding, food-restricted rats (Baker et al., 2003; Hsiao and Smith, 1995; Yu et al., 2000a, 2000b). Central DA receptor mediation of the acquisition and expression of fructose-CFP is differentially controlled by the NAc, AMY, mPFC, mOFC, and LH (Amador et al., 2014; Bernal et al., 2008, 2009; Malkusz et al., 2012, 2015). Systemic administration of NMDA receptor antagonists (MK-801) eliminated the acquisition, but not the expression of fructose-CFP (Golden and Houpt, 2007). However, systemic administration of cannabinoid (CB1) receptor inverse agonists (AM251) reduced the expression, but not the acquisition of fructose-CFP (Miner et al., 2008). In contrast, systemic and
NAc administration of NTX reduced sweet intake, but failed to alter flavor-flavor-mediated sugar-CFP (Baker et al., 2004; Bernal et al., 2010; Yu et al., 1999). Therefore, DA D1, DA D2, NMDA and CB1, but not opioid receptor signaling is required for the full learning (acquisition) and maintenance (expression) of fructose-CFP, apparently in limbic sites associated with reward.

CFA can be induced by either ingested toxins that induce gastrointestinal distress (flavor–toxin learning; see review: Freeman and Riley, 2009) or by an aversive taste (flavor-taste learning; e.g., Dwyer, 2011 Fanselow and Birk, 1982). Pharmacological analyses have examined DA D1, DA D2, NMDA and opioid antagonists in flavor-toxin CFA learning. DA D1, but not D2 antagonism disrupted the acquisition of a LiCl-induced CFA following systemic administration, and following central administration into the LH or NAc shell (Caulliez et al., 1996; Fenu et al., 2001, 2005, 2009). Blockade of NMDA, AMPA and metabotropic glutamate receptors in the amygdala disrupted LiCl-induced CFA (Yasoshima et al., 2000). Naloxone enhanced taste aversions elicited by LiCl (Davis et al., 2009; Miceli et al., 1979; Smurthwaite et al., 1992). Our laboratory (Rotella et al., 2014) previously examined the pharmacological substrates of flavor-taste CFA learning using a design to match that used in our flavor–flavor CFP studies. In this case, food-restricted rats were trained with two differently flavored fructose + saccharin (FS) solutions with one adulterated with quinine (0.03%: FSQ). In contrast to the greater persistence of fructose-CFP over a week or more of testing (Baker et al., 2003, 2004), quinine (0.03%)-CFA typically lasts for one pair of sessions. However, the persistence of quinine-CFA was significantly enhanced by systemic administration of DA D1, NMDA and opioid, but not DA D2 receptor antagonists administered during training (Rotella et al., 2014). Thus, whereas DA D1, DA D2 and NMDA, but not opioid receptor antagonism blocks the
acquisition of sweet taste-based CFP, DA D1, NMDA and opioid, but not DA D2 receptor antagonism enhanced the duration of a bitter taste-based CFA.

Avena and Rada (2012) have implicated acetylcholine (Ach) in the mediation of food intake, particularly the “addictive” aspects of excessive sugar intake, by its interactions with brain DA systems. One Ach-DA neuroanatomical interaction presumably occurs through Ach inputs from the PPT/LDT nuclei to identified DA cells in the VTA (Holmstrand and Sesack, 2011; Maskos, 2008; Omelchenko and Sesack, 2005; Woolf et al., 1990). The second Ach-DA interaction presumably occurs through DA terminal innervation of Ach-containing interneurons in the NAc (de Rover et al., 2002; Witten et al., 2010; Zhou et al., 2002), although cholinergic PPT/LDT innervation is found there as well (Dautan et al., 2014). NAc cholinergic-DA interactions act through local DA D2 receptors (Alcantara et al., 2003), mediate accumbal DA release that also involves glutamate signaling (Cachope et al., 2012; Chuhma et al., 2014; Threlfell and Cragg, 2011), and provide feedback control of VTA DA release (Rahman and McBride, 2002). These interactions are integral in the formulation of central mechanisms involved in food reward (see reviews: Avena and Rada, 2012; Kelley et al., 2005; Laurent et al., 2014; Mark et al., 2011; McFadden et al., 2014; Nunes et al., 2013), and suggest that cholinergic receptor mechanisms may also play a role in acquisition and expression of fructose-CFP mediated by systemic (Baker et al., 2003; Yu et al., 2000a, 2000b) and accumbal (Bernal et al., 2008; Malkusz et al., 2012) DA. NAc cholinergic interneurons play a role in regulation of body weight and metabolism (Hajnal et al., 2000). Food intake increases Ach release in the AMY (Hajnal et al., 1998) and the NAc (Mark et al., 1992, 1995). Sugar intake under bingeing conditions potently increases NAc Ach release that is mediated by deprivation, sham intake and weight of the animals (Avena et al., 2006, 2008a, 2008b, 2008c). Further, VTA Ach and NAc
DA are concomitantly released by the orexigenic peptide, ghrelin (Jerlhag et al., 2012), and activity of dorsomedial hypothalamic cholinergic neurons increases following overnight food deprivation (Groessl et al., 2013). Although food intake was significantly reduced by chronic nicotine (Dandekar et al., 2011), the nicotinic cholinergic receptor antagonist, MEC suppressed ghrelin-induced food intake (Dickson et al., 2010), and chronic 18-methoxycoronaridine reduced long-term sucrose intake (Taraschenko et al., 2011). Pilocarpine, a muscarinic cholinergic receptor agonist, administered into the NAc core increased chow intake (Nunes et al., 2013). Muscarinic receptor antagonism with SCOP in the NAc reduced both deprivation-induced feeding (Pratt and Blackstone, 2009) and NAc DAMGO-induced feeding (Perry et al., 2009), and NAc sites at which SCOP suppressed feeding and DAMGO induced feeding overlapped (Perry et al., 2014). DAMGO-induced increases in high-fat feeding were blocked by NTX and SCOP, but not by antagonists of DA, glutamate or nicotinic receptors (Will et al., 2006). Muscarinic receptor blockade also mediated cue-related responses to feeding such that SCOP administered into the VTA disrupted food-related learning (Sharf and Ranaldi, 2006). SCOP administered into the NAc core induced avoidance to flavor and spatial cues (Pratt et al., 2007). SCOP administered into the ventral hippocampus impaired memories for socially-transmitted food preferences (Carballo-Marquez et al., 2009). Thus, these data appear to implicate the cholinergic receptor system in a limbic circuit that mediates not only food intake per se, but is also involved in the development of preferences.

Cholinergic receptors have also been previously implicated in certain forms of CFA. In flavor-toxin CFA learning, SCOP attenuated LiCl-induced CFA without altering aversion to quinine (Coil et al., 1978). SCOP and nicotine are capable of eliciting flavor-taste CFAs with the former abolished and the latter enhanced by lesions placed in the area postrema (Ossenkopp et
A possible role for cholinergic receptors in flavor-taste CFA is supported by the ability of quinine to inhibit Ach currents in alpha-9-alpha-10-containing nicotinic Ach receptors in Xenopus oocytes (Ballestero et al., 2005), and adrenal catecholamine secretion evoked by Ach stimulation of muscarinic and nicotinic receptors (Jang et al., 2001). In turn, nicotine depressed quinine-evoked responses in the nucleus tractus solitarius that were blocked by MEC (Simons et al., 2006). Nicotine and quinine CFA generalized to each other in three mouse strains (Gyekis et al., 2012), with MEC blocking this ability in behavioral and electrophysiological assays (Oliveira-Maia et al., 2009). Thus, these data appear to implicate cholinergic receptor signaling in flavor-taste CFA.

Much of the evidence implicating cholinergic involvement in complex aspects of food intake appears to be due to activity in a limbic circuit (specifically the VTA and NAc) in which cholinergic signaling can act directly upon preferences and avoidances either in and of itself, or through interactions with brain DA. Therefore, the present study investigated the role of muscarinic and nicotinic cholinergic receptor signaling in mediating the expression and acquisition of flavor preferences conditioned by fructose in rats. Our previous evaluation of DA receptor involvement in fructose-CFP initially examined systemic receptor-selective antagonist effects (Baker et al., 2003; Yu et al., 2000a, 2000b) followed by antagonist administration into central candidate limbic sites (Amador et al., 2014; Bernal et al., 2008, 2009; Malkusz et al., 2012). Hence, the present study initially employed a systemic approach in examining the dose-dependent effects of muscarinic (SCOP) or nicotinic (MEC) cholinergic receptor antagonists upon the expression and acquisition of fructose-CFP. A parallel study then examined whether systemic SCOP or MEC altered the acquisition of quinine-CFA in a fructose-saccharin solution.
2. Methods:

2.1 Subjects: Male Sprague-Dawley rats (n=124, 250-275 g), obtained from Charles River Laboratories (Wilmington, MA), were housed individually in wire mesh cages, maintained on a 12:12 h light/dark cycle (lights on: 7 AM, lights off: 7 PM) at a constant ambient temperature of 22°C with chow (5001, PMI Nutrition International, Brentwood, MO) and water available ad libitum for the first week. All animals were then food-restricted to 85-90% of their body weight throughout behavioral testing to insure short-latency responses to presentation of the training and test solutions. Food rations were provided 1 h after the end of daily training and testing sessions. The experimental protocols were approved by the Queens College Institutional Animal Care and Use Committee (Protocol 69) certifying that all subjects and procedures are in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

2.2 Fructose-CFP Initial Training and Test Solutions: During initial training in the fructose-CFP paradigm, rats were trained to drink an unflavored 0.2% sodium saccharin (Sigma Chemical Co., St. Louis, MO) solution during five daily 1-h sessions to guarantee sampling as previously described (Baker et al., 2003, 2004); this initial unflavored training solution was the same concentrations as the flavored CS- solution used in the subsequent conditioning paradigms. The sipper tube was mounted on the front of the cage held by a taut steel spring, and was positioned 3-6 cm above the cage floor. Solution measurement (0.1 ml gradations and accuracy) was insured by using a retrofitted testing sipper tube that has been previously validated (Baker et al., 2003, 2004; Rotella et al., 2014; Yu et al., 1999, 2000a, 2000b). This training procedure was repeated daily until all rats approached the sipper tubes with short (< 1 min) latency, typically within three days. The limited food rations were given 1 h after each training session.
The two training solutions in the *fructose-CFP expression and acquisition* studies were a 8% fructose + 0.2% saccharin solution and a saccharin (0.2%) solution, each flavored with 0.05% unsweetened grape or cherry Kool-Aid (General Foods, White Plains, NY). The 8% fructose + 0.2% saccharin-paired flavor is referred to as the CS+/Fs, and the 0.2% saccharin-paired flavor as the CS-/s (Baker et al., 2003, 2004; Yu et al., 1999, 2000a, 2000b). Half of the rats in each drug paradigm had the cherry flavor added to the CS+/Fs solution and the grape flavor added to the CS-/s solution; flavors were reversed for the remaining rats. In all two-bottle preference choice tests for fructose-CFP, the cherry and grape flavors were presented in 0.2% saccharin solutions (CS+, CS-). All training and testing in both paradigms took place in the rat’s home cage during the mid-light phase (~11 AM- 4 PM) of the light:dark cycle.

2.3 Muscarinic and Nicotinic Cholinergic Receptor Antagonists and Fructose-CFP Expression: Thirty-one rats were given ten daily 1-bottle training sessions (0.5 h/day) with 16 ml of the CS+/Fs solution presented on odd-numbered days, and 16 ml of the CS-/s solution presented on even-numbered days. On days 9 and 10, the rats had access to a second sipper tube containing water. This familiarized the rats to the presence of two sipper tubes used during the choice tests; water intake was negligible in these training trials. The left-right position of the CS and water sipper tubes was counterbalanced over the two days. The order of presentation of the CS+/Fs first followed by subsequent presentation of the CS-/s solution during training was identical to that used in our and other previous studies examining the pharmacological substrates of fructose-CFP in real-feeding rats (Baker et al., 2004; Golden & Houpt, 2007) and sucrose-CFP in sham-feeding rats (Yu et al., 2000a, 2000b). Following training, the rats were given eight 2-bottle choice test sessions (0.5 h/day) with unlimited (~45 ml) access to the CS+ and CS-flavors mixed in 0.2% saccharin solutions. Solution intakes during training and testing were
measured by weighing (0.1 g) the bottles before and after the 1 h sessions. The animals were limited to eight 2-bottle sessions because previous research (Baker et al., 2003, 2004; Yu et al., 2000a, 2000b) demonstrated that the magnitude of the preference did not change during this testing interval. Therefore, each animal received vehicle, and three doses of either the muscarinic or nicotinic cholinergic receptor antagonist. All 31 rats initially received a pair of vehicle injections that were used to match the animals into muscarinic and nicotinic groups as well as to match them as a function of receiving three of the five doses. Following vehicle treatment, the first group of fifteen rats received pairs of three doses of SCOP injections at 0.1 (n=7), 1 (n=7), 2.5 (n=8), 5 (n=8) and 10 (n=15) mg/kg 30 min prior to the two-bottle choice test. Following vehicle treatment, the second group of sixteen rats received pairs of three doses of MEC injections at 1 (n=7), 2 (n=7), 4 (n=16), 6 (n=9) and 8 (n=9) mg/kg 30 min prior to the two-bottle choice test. The 30-min interval for systemic administration of the muscarinic and nicotinic cholinergic receptor antagonists prior to the experimental condition in this and the other two paradigms was based on this commonly-used interval in many other systemic studies. Thus, all groups of rats were tested in two consecutive daily sessions at vehicle and three drug doses with the left–right position of the CS+ and CS- solutions counterbalanced across sessions to control for position effects. Half of the rats in each group were tested with an ascending drug dose order, and the remaining rats were tested with a descending drug dose order.

2.4 Muscarinic and Nicotinic Cholinergic Receptor Antagonists and Fructose-CFP Acquisition: Six groups of rats, matched for their intakes of the unflavored 0.2% saccharin solution prior to training, were given ten 1-bottle training sessions (1 h/day) with 16 ml of the CS+/Fs solution presented on odd-numbered days, and 16 ml of the CS-/s solution presented on even-numbered days. The first group (VEH) of eight rats received daily vehicle injections 30
min prior to each training session. The second (SCOP 1) and third (SCOP 2.5) groups received daily injections of SCOP at doses of 1 (n=8) and 2.5 (n=8) mg/kg respectively 30 min prior to each training session. The fourth (MEC 4) and fifth (MEC 6) groups received daily injections of MEC at doses of 4 (n=8) and 6 (n=7) mg/kg respectively 30 min prior to each training session. The sixth (n=6) group received daily injections of vehicle 30 min prior to each training session, but were limited in CS+ and CS- intakes to the reduced levels observed during training in the drug groups (LMTD VEH). Following training, all groups were given six daily 2-bottle choice sessions (1 h/day) with unlimited (~45 ml) access to the CS+ and CS- flavors mixed in 0.2% saccharin solutions; no drugs were administered prior to these sessions. The positions of the CS+ and CS- solutions were counterbalanced across sessions.

2.5 Quinine-CFA Initial Training and Test Solutions: During initial training in the quinine-CFA paradigm, rats were trained to drink an unflavored 8% fructose (Sigma Chemical Co.) and 0.2% sodium saccharin solution as previously described (Rotella et al., 2014); this initial unflavored training solution was the same concentrations as the flavored CS- solution used in the subsequent conditioning paradigms. Initial training was otherwise identical to the fructose-CFP paradigm. The two training solutions in the quinine-CFA acquisition study were 8% fructose + 0.2% saccharin with or without quinine (0.03%: Sigma Chemical Co.). Each solution was flavored with 0.05% unsweetened grape or cherry Kool-Aid (Rotella et al., 2014). Half of the rats in each group had the cherry flavor added to the fructose + saccharin solution and the grape flavor added to the fructose + saccharin + quinine solution; the flavors were reversed for the remaining rats. In the two-bottle choice tests, the cherry and grape flavors were presented in 8% fructose + 0.2% saccharin solutions. The flavored fructose + saccharin + quinine solution is referred to as the CS+/FSQ, and the flavored fructose + saccharin solution as the CS-/FS; and the
same flavors used in the two-bottle tests are referred to as CS+ and CS-, respectively (Rotella et al., 2014). All training and testing in this paradigm took place in the rat’s home cage during the mid-light phase (~11 AM- 4 PM) of the light:dark cycle.

2.6 Muscarinic and Nicotinic Cholinergic Receptor Antagonists and Quinine-CFA Acquisition: Rats were trained over eight one-bottle training sessions (1 h) to drink 16 ml of the CS-/FS solution on odd-numbered days, and 16 ml of the CS+/FSQ solution on even-numbered days. The CS-/FS solution was presented first to minimize potential potent neophobic effects of the fructose-saccharin solution adulterated by quinine (CS+/FSQ), and encourage intake as performed previously (Rotella et al., 2014). The eight training trials were divided into four pairs of sessions with a one-day break between each pair (Rotella et al., 2014). In the first three training pairs, only one bottle was presented. In the fourth pair of training sessions (days 7 and 8), a second sipper tube containing water was also presented to acclimate the rats to the presence of two sipper tubes used during the choice tests; water intake was negligible in these training trials. The left-right position of the CS and water sipper tubes was counterbalanced over the two days. The first group (VEH) of eighteen rats received daily vehicle injections 30 min prior to each training session. The second (SCOP 1) and third (SCOP 2.5) groups received daily injections of SCOP at doses of 1 (n=7) and 2.5 (n=8) mg/kg respectively 30 min prior to each training session. The fourth (MEC 4) and fifth (MEC 6) groups received daily injections of MEC at doses of 4 (n=8) and 6 (n=7) mg/kg respectively 30 min prior to each training session. Because the two SCOP and the two MEC groups displayed comparable CS-/F and CS+/FSQ intakes during training, an additional limited vehicle group was not employed in this study. Following training, all groups received six two-bottle sessions with the CS+ and CS- flavors mixed in 8% fructose + 0.2% saccharin (FS) solutions (unlimited ~45 ml) access). The position
of the two bottles were left (L)-right (R)-R-L-L-R in half of the animals, and R-L-L-R-R-L in the remaining half. Solution intakes during the training and testing were measured by weighing (0.1 g) the bottles before and after the 1 h sessions.

2.7 Statistics: In fructose-CFP expression studies, training intakes were averaged over the five CS+/Fs and five CS-/s sessions and evaluated by a t-test. Intakes during the two-bottle preference tests were averaged over the two sessions at each dose and evaluated with two-way repeated-measures analyses of variance (ANOVA, CS condition vs. Dose) for each group. Separate ANOVAs evaluated percent CS+/g intakes and total intake as a function of dose for the three groups. In fructose-CFP acquisition studies, training intakes were averaged over the five CS+/Fs and CS-/s sessions and were analyzed with a two-way randomized-blocks ANOVA (CS conditions x Groups). Intakes during the two-bottle preference tests were averaged over sessions 1-2, 3-4, and 5-6 (referred to as Tests 1, 2, and 3) to control for side position effects. A three-way randomized-blocks ANOVA compared the CS intakes of the six groups (Group x CS x Test). A separate two-way ANOVA evaluated percent CS+ intakes and total intakes of the six groups across the three tests. In quinine-CFA acquisition studies, training intakes were averaged over the four CS+/FSQ and CS-/FS sessions and were analyzed with a two-way randomized-blocks ANOVA (CS conditions x Groups). Intakes during the preference tests were averaged over sessions 1-2, 3-4, and 5-6 (referred to as Tests 1, 2, and 3) to control for side position effects. A three-way randomized-blocks ANOVA compared the CS intakes of the five groups (Group x CS x Test). A separate two-way ANOVA evaluated percent CS+ intakes and total intakes of the five groups across the three tests. When main or interaction effects were observed in any ANOVA, Bonferroni corrected comparisons (p<0.05) detected significant effects. Drug-induced changes in acquisition or expression preferences were operationally defined as a significant change in
percent CS+ intakes relative to vehicle, and/or a failure to observe significant differences between CS+ and CS- intakes in the two-bottle preference tests.

3. Results:

3.1 Muscarinic and Nicotinic Cholinergic Receptor Antagonists and Fructose-CFP Expression: During 1-bottle training, CS+/Fs intake (12.6 g (SEM: ±0.3)) significantly exceeded (t(30)= 3.77, p<0.0005) CS-/s intake (11.1 g (SEM: ±0.4)). In the 2-bottle preference tests conducted following SCOP treatment, overall, CS+ (6.6 g) intakes significantly exceeded CS- (1.4 g) intakes (F(1,14)= 102.13, p<0.0001), and intakes significantly differed as functions of SCOP dose (F(5,70)= 65.29, p<0.0001) and for the interaction between conditions and doses (F(5,70)= 42.25, p<0.0001). CS+ intake was significantly higher than corresponding CS- intake following vehicle and all SCOP doses (Figure 5A, *). All SCOP doses significantly reduced CS+, but not CS- intake relative to vehicle (Figure 5A, +). Percent CS+ preferences significantly differed (F(5,54)= 5.16, p<0.0006) as a function of SCOP dose with the three highest (2.5 (65%), 5 (65%), 10 (68%) mg/kg) doses significantly reducing percent CS+ preferences relative to vehicle (90%) (Figure 5A, +). Although significant fructose-CFP preferences were noted across all SCOP doses after learning of the preference had taken place, these data indicate a dose-dependent ability of SCOP to significantly reduce, but not eliminate the magnitude of the expression of CS+ preferences at the three highest doses.

In the 2-bottle preference tests conducted following MEC treatment, overall, CS+ (7.1 g) intakes significantly exceeded CS- (1.2 g) intakes (F(1,15)= 570.01, p<0.0001), and intakes significantly differed as functions of MEC doses (F(5,75)= 197.16, p<0.0001) and for the interaction between conditions and doses (F(5,75)= 104.40, p<0.0001). CS+ intake was significantly higher than CS- intake following vehicle and all MEC doses (Figure 5B, *). MEC
Figure 5. (Cholinergic receptor antagonists and fructose-CFP expression): Intakes (mean ±SEM, g/30 min) of CS+ and CS- solutions in two-bottle preference tests in animals pretreated (30 min) with systemic scopolamine (Panel A) or mecamylamine (Panel B). Significant differences are denoted between CS+ and CS- intake within an injection condition (*) as well as drug-induced effects upon CS+ and CS- intake relative to corresponding vehicle values (+). The percentages of CS+ intake over total intake are indicated above each pair of values with significant effects denoted (+).
doses between 2 and 8 mg/kg, but not 1 mg/kg significantly reduced CS+, but not CS- intake relative to vehicle (Figure 5B, +). Percent CS+ preferences significantly differed (F(5,58)= 7.75, p<0.0001) as a function of MEC dose with the three highest (4 (68%), 6 (67%), 8 (73%) mg/kg) doses significantly reducing percent CS+ preferences relative to vehicle (89%) (Figure 5B. +). Although significant fructose-CFP preferences were noted across all MEC doses after learning of the preference had taken place, these data indicate a dose-dependent ability of MEC to significantly reduce, but not eliminate the magnitude of the expression of CS+ preferences at the three highest doses.

3.2 Muscarinic and Nicotinic Cholinergic Receptor Antagonists and Fructose-CFP Acquisition: During 1-bottle training, overall CS+/Fs intake (6.0 g) significantly (F(1,7)= 26.58, p<0.0013) exceeded CS-/s intake (4.1 g), and there were significant differences among groups (F(5,35)= 21.54, p<0.0001) and for the interaction between groups and conditions (F(5,35)= 3.02, p<0.023). CS+/Fs and CS-/s intakes of the SCOP 1, SCOP 2.5, MEC 4 and MEC 6 groups were significantly lower than corresponding training intakes of the VEH group (Figure 6, +). However, training intakes of all drug groups failed to differ from corresponding LMTD VEH group intakes, except for lower CS-/s intake in the SCOP 2.5 group (Figure 6).

Following training, the rats were given three pairs of two-bottle preference tests without drug treatment. Significant differences were observed among groups (F(5,35)= 3.42, p<0.013), among tests (F(2,14)= 4.44, p<0.032), between CS+ and CS- conditions (F(1,7)= 57.24, p=0.0001), and for the interactions between groups and tests (F(10,70)= 9.59, p<0.017), groups and conditions (F(5,35)= 5.99, p<0.0004), and among groups, tests and times (F(10,70)= 2.59, p<0.01), but not for the interaction between tests and conditions (F(2,14)= 1.36, ns). Within-group comparisons revealed that CS+ intake was significantly greater than CS- intake in
**Figure 6.** (Cholinergic receptor antagonists and fructose-CFP acquisition training): Intakes (mean ±SEM, g/60 min) of CS+/Fs and CS-/s solutions during one-bottle training sessions in animals receiving vehicle (VEH), scopolamine at doses of 1 (SCOP 1) or 2.5 (SCOP 2.5) mg/kg, mecamylamine at doses of 4 (MEC 4) or 6 (MEC 6) mg/kg, or vehicle, but limited to drug-induced intakes (LMTD VEH) 30 min prior to each training session. Significant differences are denoted in drug-induced effects upon CS+/Fs and CS-/s intake relative to corresponding VEH intake (+).
Tests 1-3 in the VEH (Figure 7A, *), MEC 4 (Figure 7D, *), MEC 6 (Figure 7E, *) and LMTD VEH (Figure 7F, *) groups. CS+ and CS- intakes failed to differ in any of the tests in the SCOP 1 (Figure 7B) and SCOP 2.5 (Figure 7C) groups. The SCOP 1 group displayed significantly lower CS+ intake in all three tests and significantly higher CS- intake in the last two tests relative to VEH (Figure 7B, +), whereas the SCOP 2.5 group displayed significantly lower CS+, but not CS- intake in the first two tests relative to VEH (Figure 7C, +). Comparisons with the LMTD VEH group revealed significantly lower CS+ intake across all three tests for the SCOP 1 and SCOP 2.5 groups, and significantly higher CS- intakes in the last two tests in the SCOP 1 group. Significant differences in the percent CS+ intake were observed among groups (F(5,35)= 6.48, p<0.0002) and for the interaction between groups and tests (F(10,70)= 2.98, p<0.035), but not among tests (F(2,14)= 0.15, ns). The percent CS+ intakes were stable across the three tests in the VEH (85-92%, Figure 7A), MEC 4 (82-83%, Figure 7D), MEC 6 (67-75%, Figure 7E) and LMTD VEH (74-88%, Figure 7F) groups. In contrast, the SCOP 1 (40-54%, Figure 7B) and the SCOP 2.5 (45-58%, Figure 7C) groups displayed significantly lower percent CS+ preferences across all three tests relative to either the VEH or LMTD VEH groups. Thus, these data indicate that SCOP, but not MEC, administered during training eliminated the subsequent acquisition of fructose-CFP.

3.3 Muscarinic and Nicotinic Cholinergic Receptor Antagonists and Quinine-CFA Acquisition:

In the one-bottle training sessions, overall, CS-/FS intake significantly exceeded CS+/FSQ intake (8.4 vs. 1.7 g/1 h, F(1,17)= 528.14, p<0.0001), there were significant group differences (F(4,68)= 69.44, p<0.0001), and there was a significant interaction between groups and CS (F(4,68)= 18.77, p=0.0001). CS-/FS intake for all five groups was significantly higher than CS+/FSQ intake (Figure 8A, *), replicating the previously-demonstrated (Rotella et al., 2014) ability of
Figure 7. (Cholinergic receptor antagonists and fructose-CFP acquisition testing): Intakes (mean +SEM, g/30 min) of CS+ and CS- solutions in three pairs of two-bottle preference tests (unlimited (~45 ml) access, Tests 1, 2, 3) in animals that received VEH (Panel A), SCOP 1 (Panel B), SCOP 2.5 (Panel C), MEC 4 (Panel D), MEC 6 (Panel E) or LMTD VEH (Panel F) during training. Significant differences are denoted between CS+ and CS- intake within an injection condition (*) as well as drug-induced effects upon CS+ and CS- intake relative to corresponding vehicle values (+). The percentages of CS+ intake over total intake are denoted above each pair of values with significant effects denoted relative to VEH (+) and LMTD VEH (#).
Figure 8. (Cholinergic receptor antagonists and quinine-CFA acquisition training and testing. Panel A): One-bottle training intakes (mean +SEM, g/60 min) of CS+/FSQ and CS-/FS solutions during training sessions in animals receiving systemic administration of VEH, scopolamine at doses of 1 (SCOP 1) or 2.5 (SCOP 2.5) mg/kg, or mecamylamine at doses of 4 (MEC 4) or 6 (MEC 6) mg/kg 30 min prior to each training session. Animals were given 16 ml of solutions during training as indicated in the Panel A y-axis. Significant differences are denoted between CS+/FSQ and CS-/FS intake are denoted (*) as are any drug effect relative to VEH (+). Two-bottle choice test intakes (mean +SEM, g/60 min, unlimited (~45 ml) access) of the CS+ and CS-flavors presented in fructose+saccharin solutions in Tests 1-3 in groups trained with VEH (Panel B), SCOP 1 (Panel C), SCOP 2.5 (Panel D), MEC 4 (Panel E) or MEC 6 (Panel F). Significant differences are denoted between CS+/FSQ and CS-/FS intakes within an injection condition (*) as well as drug-induced effects upon CS+ and CS- intake relative to corresponding VEH values (+). The percentages of CS+/FSQ intake over total intake are denoted above each pair of values. Significant differences are denoted between percent CS+/FSQ intakes relative to VEH (+).
quinine at a 0.03% concentration to reduce intake. CS-/FS and CS+/FSQ intakes were significantly lower in the SCOP 1, SCOP 2.5, MEC 4 and MEC 6 groups relative to the corresponding VEH group (Figure 8A, +). The SCOP and MEC groups failed to differ from each other in CS-/FS and CS+/FSQ intakes.

In the two-bottle choice tests, there were significant differences in the overall CS+ (6.4 g) and CS- (15.4 g) intakes (F(1,17)= 252.39, p<0.0001), as well as significant differences among groups (F(4,68)= 11.26, p<0.0001), across tests (F(2,34)= 114.71, p<0.0001), and for the interactions between groups and tests (F(8,136)= 7.63, p<0.013), between groups and conditions (F(4,68)= 10.07, p<0.0001) and among groups, tests and conditions (F(8,136)= 5.23, p<0.0001), but not between tests and conditions (F(2,34)= 0.08, ns). Within-group comparisons revealed that the VEH group consumed significantly more CS- than CS+ only during Test 1 (Figure 8B, *), consistent with the limited duration of quinine-CFA observed previously (Rotella et al., 2014). CS- intake was significantly greater than CS+ intake during Tests 1 and 3 in the SCOP 1 group (Figure 8C, *), and during test 2 in the SCOP 2.5 group (Figure 8D, *). CS- intake was significantly greater than CS+ intake across all three tests in the MEC 4 (Figure 8E, *) and MEC 6 (Figure 8F, *) groups. CS- intake was significantly lower in Test 1 of the SCOP 2.5 group relative to corresponding VEH (Figure 8D, +). CS+ intake was significantly lower in Test 2 of the MEC 4 group (Figure 8E, +) and in Tests 1 and 2 of the MEC 6 group (Figure 8F, +).

Analysis of the percent CS+ intake data revealed significant differences among groups (F(4,68)= 11.43, p<0.0001), across tests (F(2,34)= 10.74, p<0.0002), and for the interaction between groups and tests (F(8,136)= 6.27, p<0.0001). The SCOP 1 group displayed significantly greater quinine-CFA during Test 1 relative to vehicle (17.0% vs. 33.9%; Figure 8C); all other SCOP effects failed to differ from the VEH group. In contrast, MEC treatment during training
significantly enhanced the magnitude of quinine-CFA across all three tests in the MEC 4 (Figure 8E) and MEC 6 (Figure 8F) relative to the VEH group. Thus, these data indicate that MEC, but not SCOP, administered during training significantly enhanced and prolonged the subsequent acquisition of quinine-CFA.

4. Discussion:

These experiments indicate that systemic pretreatment with muscarinic and nicotinic receptor antagonists differentially altered the expression and acquisition of dructose-CFP as well as the acquisition of quinine-CFA.

4.1 Cholinergic Receptor Antagonism and Fructose-CFP Expression: Expression of fructose-CFP, defined by significant preferences for the CS+ flavor over the CS- flavor, failed to be affected by a wide dose range of the muscarinic antagonist, SCOP or the nicotinic antagonist, MEC. However, the magnitude of fructose-CFP expression, defined by changes in percent CS+ intake, was significantly reduced by high doses of SCOP (2.5-10 mg/kg: 65-68%) and MEC (4-8 mg/kg: 67-73%) relative to vehicle (89-90%). These reductions were accompanied by reductions in total, and particularly CS+, intake. These intake reductions of saccharin by systemic SCOP and MEC correspond to previous reductions of sweet intake following systemic nicotinic receptor antagonism (Taraschenko et al., 2011) and chow intake following systemic of muscarinic and nicotinic receptor antagonism (Dandekar et al., 2011; Dickson et al., 2010; Perry et al., 2009; Pratt and Blackstone, 2009; Will et al., 2006). The very limited inhibition of systemic muscarinic or nicotinic cholinergic receptor antagonism of expression of fructose-CFP stands in contrast to previously-observed systemic pharmacological effects. Thus, systemic DA D1 (SCH23390) or D2 (raclopride) receptor antagonists dose-dependently eliminated the expression of both fructose-CFP in real-feeding rats and sucrose-CFP in sham-feeding rats.
(Baker et al., 2003; Hsiao and Smith, 1995; Yu et al., 2000a, 2000b). Whereas a CB1 receptor inverse agonist (AM-251) reduced expression of fructose-CFP (Miner et al., 2008), neither NMDA (MK-801) nor opioid (naltrexone) receptor antagonism affected expression of fructose-CFP (Baker et al., 2004; Bernal et al., 2010; Golden and Houpt, 2007). Thus, muscarinic or nicotinic cholinergic receptor antagonism plays a minimal role in the maintenance of an already-acquired CFP for fructose.

4.2 Cholinergic Receptor Antagonism and Fructose-CFP Acquisition: Acquisition of fructose-CFP was eliminated by systemic muscarinic (SCOP), but not nicotinic (MEC) cholinergic receptor antagonists administered during training. This was demonstrated by the failure of two-bottle CS+ and CS- intakes to differ in rats receiving SCOP during training. The magnitude of fructose-CFP observed in vehicle-trained (85-92%) rats was eliminated in SCOP-trained rats receiving 1 (40-54%) and 2.5 (45-58%) mg/kg doses, effects indicative of indifference. Because these SCOP doses significantly reduced CS+/Fs and CS-/s intakes during training, it is possible that this loss of preference could be alternatively due to specific primary actions of muscarinic cholinergic receptor antagonism or to secondary actions of reducing the opportunities to learn the preference due to reduced intake per se. The addition of the LMTD VEH group receiving vehicle injections, but limited in comparable CS+ and CS- intakes to those of the drug groups controlled for this second possibility, and displayed comparable, significant fructose-CFP preferences relative to vehicle-trained rats. These data strongly suggest that the inability of SCOP-trained rats to display preferences was thus due to its cholinergic receptor antagonism. Further, the SCOP doses (1-2.5 mg/kg) capable of eliminating fructose-CFP acquisition were incapable of affecting fructose-CFP expression. In contrast, MEC-trained rats displayed significant preferences with CS+ intake significantly higher than CS- intake, and
comparable percent CS+ intake preferences following the 4 (82-83%) and 6 (67-75%) mg/kg
doses relative to vehicle- (85-92%) and LMTD VEH- (74-88%) trained groups. This failure of
systemic nicotinic receptor antagonism to affect fructose-CFP acquisition occurred despite
MEC’s significant reductions in CS+/Fs and CS-/s intakes during training to levels observed for
SCOP. Hence, systemic muscarinic (SCOP), but not nicotinic (MEC) receptor antagonism
administered during training eliminated fructose-CFP acquisition. These data extend the
circumstances under which muscarinic receptor blockade mediated other cue-related feeding
responses, including disruptions in food-related learning following VTA administration (Sharf
and Ranaldi, 2006), avoidance to flavor and spatial cues following NAc core administration
(Pratt et al., 2007), and memories for socially-transmitted food preferences following ventral
hippocampal administration (Carballo-Marquez et al., 2009). These effects are also consistent
with the ability of food, and especially sugars, to increase Ach release in the AMY and the NAc
(Avena et al., 2006, 2008a, 2008b, 2008c; Hajnal et al., 1998; Mark et al., 1992, 1995). The
systemic SCOP-induced elimination of fructose-CFP acquisition is similar in magnitude to the
abilities of systemic DA D1, DA D2 and NMDA receptor antagonists to eliminate fructose-CFP
acquisition in real-feeding rats (Baker et al., 2003; Golden and Houpt, 2007; Hsiao and Smith,
1995) and sucrose-CFP acquisition in sham-feeding rats (Yu et al., 2000a, 2000b).

4.3 Cholinergic Receptor Antagonism and Quinine-CFA Acquisition: Systemic
administration of nicotinic (MEC), but not muscarinic (SCOP) cholinergic receptor antagonism
significantly enhanced and prolonged the acquisition of quinine-CFA. Vehicle-trained rats
displayed a significant, transitory quinine-CFA as demonstrated by a significant aversion
observed after the first (34%), but not second (48%) or third (47%) pairs of tests. Systemic
nicotinic cholinergic antagonism significantly increased the magnitude and duration of quinine-
CFA as indicated by rats trained with the 4 (18-24%) and 6 (11-13%) mg/kg MEC doses. In contrast, the SCOP 1 group displayed a significant aversion after the first test pair (17%), but failed to differ from vehicle-trained rats thereafter, whereas the SCOP 2.5 group failed to display quinine-CFA after any preference test. It should be noted that both systemic SCOP and MEC significantly reduced CS-/FS intake and CS+/FSQ intake during training relative to vehicle. This raises the possibility that the greater subsequent avoidance responses could be due to non-specific malaise brought about by pairing the antagonist with the flavored solutions during training. Both LiCl-induced conditioned taste aversions and attenuation of neophobic responses were blocked by central SCOP pretreatment into the insular cortex (Ferreira et al., 2002; Gutierrez et al., 2003a, 2003b; Naor and Dudai, 1996) and NAc shell (Ramirez-Lugo et al., 2006), but not when SCOP was administered after the presentation of the new taste. Although nicotinic receptor antagonists have not been evaluated in this paradigm, galantamine, an acetylcholinesterase inhibitor and positive allosteric modulator of nicotinic Ach receptors, reduced nicotine seeking without producing malaise (Hopkins et al., 2012). These data suggest that systemic nicotinic, but not muscarinic receptor antagonism, enhances and prolongs quinine-CTA by acting on its specific cholinergic signaling mechanism, and not through non-specific malaise-induced effects. Further studies altering the timing of cholinergic antagonist injections are necessary to completely rule out participation by this non-specific effect. These effects extend our previous (Rotella et al., 2014) findings demonstrating that the persistence of quinine-CFA was significantly enhanced by systemic administration of DA D1, NMDA and opioid, but not DA D2 receptor antagonists administered prior to training. Thus, systemic nicotinic, DA D1, NMDA and opioid, but not muscarinic, or DA D2 receptor antagonists administered during training enhanced and prolonged the acquisition of quinine-CFA. The inability of SCOP to affect
quinine-CFA acquisition is consistent with its previously-described inability to alter quinine aversion while attenuating LiCl-induced CFA (Coil et al., 1978). Although systemic SCOP elicited a flavor-taste CFA (Ossenkopp et al., 1986), the present data indicate that SCOP- and quinine-CFA fail to synergize. The ability of MEC to enhance and prolong quinine-CFA is consistent with previous observations that nicotine depressed quinine-evoked responses in the nucleus tractus solitarius that were blocked by MEC (Simons et al., 2006), and CFAs induced by nicotine and quinine generalized to each other in mice (Gyekis et al., 2012) that were blocked by MEC (Oliveira-Maia et al., 2009). However, the ability of MEC to enhance and prolong quinine-CFA appears specific to this type of “inhibitory” learning. Recent studies demonstrated that chronic co-treatment with systemic MEC prevented the occurrence of depressive-like behavior elicited by chronic restraint stress as measured by the forced swim test, sucrose preference and body weight control (Aboul-Fotouh, 2015). Moreover, whereas nicotine facilitated a version of “inhibitory” learning called negative occasion setting, systemic MEC co-treatment extended the number of training sessions to elicit this behavior (Meyer et al., 2015).
Chapter 5: Baclofen differentially mediates fructose-conditioned flavor preference and quinine-conditioned flavor avoidance in rats.

1. Introduction:

GABA or its agonists administered into limbic and hypothalamic sites increase food intake (e.g., Arnt et al., 1979; Echo et al., 2002; Grandison and Guidotti, 1977; Soderpalm and Berridge, 2000; Stratford and Kelley, 1997; Ward et al., 2000; Wirtshafter et al., 1993). Feeding elicited by the GABA_B agonist, BAC is mediated through GABA receptor interactions between the VTA and NAc (Miner et al., 2010). Indeed, brain dopamine and cholinergic systems modulate medium spiny NAc GABA output and VTA dopamine output (see reviews: Avena & Rada, 2012; Hoebel et al., 2007). Although peripheral BAC increased rodent chow and fat intake under specific dose regimens and intake conditions (e.g., Bains and Ebenezer, 2013; Ebenezer and Patel, 2011), it decreased food intake in diabetic and diet-induced obese mice (Sato et al., 2007). Peripheral BAC also selectively reduced fat intake under normal, limited-access and “binge-type” conditions (; as well as intakes of pure fat or a sugar-fat mixture (Avena et al., 2014; Berner et al., 2009; Buda-Levin et al., 2005; Corwin et al., 2009; Rao et al., 2008; Wojnicki et al., 2006; Wong et al., 2009 but see Covelo et al., 2014). Given the complex effects of GABA_B receptor signaling on feeding, the present study investigated whether systemic BAC mediated expression and acquisition of fructose-CFP as well as quinine-CFA in rats.

2. Methods:

2.1 Subjects: Male Sprague-Dawley rats (n=74, 250-275 g), obtained from Charles River Laboratories (Wilmington, MA), were housed individually in wire mesh cages, maintained on a 12:12 h light/dark cycle (lights on: 7 AM, lights off: 7 PM) at a constant ambient temperature of 22°C with chow (5001, PMI Nutrition International, Brentwood, MO) and water available ad
libitum for the first week. All animals were then food-restricted to 85-90% of their body weight throughout behavioral testing to insure short-latency responses to presentation of the training and test solutions. Food rations were provided 1 h after the end of daily training and testing sessions. The experimental protocols were approved by the Queens College Institutional Animal Care and Use Committee certifying that all subjects and procedures are in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

2.2 Fructose-CFP Initial Training and Test Solutions: During initial training in the fructose-CFP paradigm, rats were trained to drink an unflavored 0.2% sodium saccharin (Sigma Chemical Co., St. Louis, MO) solution during five daily 1-h sessions to guarantee sampling as previously described (Baker et al., 2003, 2004); this initial unflavored training solution was the same concentration as the flavored CS- solutions used in the subsequent conditioning paradigms. The sipper tube was mounted on the front of the cage held by a taut steel spring, and was positioned 3-6 cm above the cage floor. Solution measurement (0.1 ml gradations and accuracy) was insured by using a retrofitted testing sipper tube that has been previously validated (Baker et al., 2003, 2004; Rotella et al., 2014, 2015; Yu et al., 1999, 2000a). This training procedure was repeated daily until all rats approached the sipper tubes with short (< 1 min) latency, typically within three days. The limited food rations were given 1 h after each training session.

The two training solutions in the fructose-CFP expression and acquisition studies were an 8% fructose + 0.2% saccharin solution and a saccharin (0.2%) solution, each flavored with 0.05% unsweetened grape or cherry Kool-Aid (General Foods, White Plains, NY). The 8% fructose + 0.2% saccharin-paired flavor is referred to as the CS+/Fs, and the 0.2% saccharin-paired flavor as the CS-/s. Half of the rats in each drug paradigm had the cherry flavor added to the CS+/Fs solution and the grape flavor added to the CS-/s solution; flavors were reversed for
the remaining rats. In all two-bottle preference choice tests for fructose-CFP, the cherry and grape flavors were presented in 0.2% saccharin solutions (CS+, CS-). All training and testing in both paradigms took place in the rat’s home cage during the mid-light phase (~11 AM- 4 PM) of the light/dark cycle.

**2.3 BAC and Fructose-CFP Expression:** Seventeen rats were given ten daily 1-bottle training sessions (0.5 h/day) with 16 ml of the CS+/Fs solution presented on odd-numbered days, and 16 ml of the CS-/s solution presented on even-numbered days. On days 9 and 10, the rats had access to a second sipper tube containing water. This familiarized the rats to the presence of two sipper tubes used during the choice tests; water intake was negligible in these training trials. The left-right position of the CS and water sipper tubes was counterbalanced over the two days. The order of presentation of the CS+/Fs first followed by subsequent presentation of the CS-/s solution during training was identical to that used in our and other previous studies examining the pharmacological substrates of fructose-CFP (Baker et al., 2004; Golden and Houpt, 2007). Following training, the rats were given eight 2-bottle choice test sessions (0.5 h/day) with unlimited (~45 ml) access to the CS+ and CS- flavors mixed in 0.2% saccharin solutions. Solution intakes during training and testing were measured by weighing (0.1 g) the bottles before and after the sessions. The animals were limited to eight 2-bottle sessions because previous research (Baker et al., 2003, 2004; Yu et al., 2000) demonstrated that the magnitude of the preference did not change during this testing interval. Therefore, each animal received VEH, and three BAC doses. All 17 rats initially received a pair of VEH injections that were used to match the animals across subsequent pairs of BAC doses of 0.5 (n=14), 1.5 (n=15), 3 (n=14) and 5 (n=8) mg/kg 30 min prior to the two-bottle choice test. The 30-min interval for systemic BAC administration prior to the experimental condition in this and the other two paradigms was based
on this commonly-used interval in many other systemic studies. Thus, all groups of rats were tested in two consecutive daily sessions at VEH and three drug doses with the left–right position of the CS+ and CS- solutions counterbalanced across sessions to control for position effects. To control for drug dose order effects, half of the rats in each group were tested with an ascending dose order, and the remaining rats were tested with a descending dose order.

2.4 BAC and Fructose-CFP Acquisition: Three groups of rats, matched for their intakes of the unflavored 0.2% saccharin solution prior to training, were given ten 1-bottle training sessions (1 h/day) with 16 ml of the CS+/Fs solution presented on odd-numbered days, and 16 ml of the CS-/s solution presented on even-numbered days. The first group (VEH) of eight rats received daily VEH injections 30 min prior to each training session. The second (BAC 3) and third (BAC 5) groups received daily injections of BAC at doses of 3 (n=7) and 5 (n=7) mg/kg respectively, 30 min prior to each training session. Following training, all groups were given six daily 2-bottle choice sessions (1 h/day) with unlimited (~45 ml) access to the CS+ and CS- flavors mixed in 0.2% saccharin solutions; no drugs were administered prior to these sessions. The positions of the CS+ and CS- solutions were counterbalanced across sessions.

2.5 Quinine-CFA Initial Training and Test Solutions: During initial training in the quinine-CFA paradigm, rats were trained to drink an unflavored 8% fructose (Sigma Chemical Co.) and 0.2% sodium saccharin solution as previously described (Rotella et al., 2014, 2015); this initial unflavored training solution was the same concentrations as the flavored CS- solution used in the subsequent conditioning paradigms. Initial training was otherwise identical to the fructose-CFP paradigm. The two training solutions in the quinine-CFA acquisition study were 8% fructose + 0.2% saccharin with or without quinine (0.03%: Sigma Chemical Co.). Each solution was flavored with 0.05% unsweetened grape or cherry Kool-Aid (Rotella et al., 2014).
Half of the rats in each group had the cherry flavor added to the fructose + saccharin solution and the grape flavor added to the fructose + saccharin + quinine solution; the flavors were reversed for the remaining rats. In the two-bottle choice tests, the cherry and grape flavors were presented in 8% fructose + 0.2% saccharin solutions. The flavored fructose + saccharin + quinine solution is referred to as the CS+/FSQ, and the flavored fructose + saccharin solution as the CS-/FS; and the same flavors used in the two-bottle tests are referred to as CS+ and CS-, respectively (Rotella et al., 2014). All training and testing in this paradigm took place in the rat’s home cage during the mid-light phase (~11 AM- 4 PM) of the light/dark cycle.

2.6 BAC and Quinine-CFA Acquisition: Rats were trained over eight one-bottle training sessions (1 h) to drink the CS-/FS solution (16 ml) on odd-numbered days, and the CS+/FSQ solution (16 ml) on even-numbered days. The CS-/FS solution was presented first to minimize potential potent neophobic effects of the fructose-saccharin solution adulterated by quinine (CS+/FSQ), and encourage intake as performed previously (Rotella et al., 2014, 2015). The eight training trials were divided into four pairs of sessions with a one-day break between each pair. In the first three training pairs, only one bottle was presented. In the fourth pair of training sessions (days 7 and 8), a second sipper tube containing water was also presented to acclimate the rats to the presence of two sipper tubes used during the choice tests; water intake was negligible in these training trials. The left-right position of the CS and water sipper tubes was counterbalanced over the two days. The first group (VEH) of eighteen rats received daily VEH injections 30 min prior to each training session. The second (BAC 3) and third (BAC 5) groups received daily injections of BAC at doses of 3 (n=8) and 5 (n=9) mg/kg respectively, 30 min prior to each training session. Following training, all groups received six two-bottle sessions with the CS+ and CS-flavors mixed in 8% fructose + 0.2% saccharin (FS) solutions (unlimited (~45 ml) access). The
left–right position of the CS+ and CS- solutions were counterbalanced across sessions to control for position effects. Solution intakes during training and testing were measured by weighing (0.1 g) the bottles before and after the 1 h sessions.

2.7 Statistics: In fructose-CFP expression studies, training intakes were averaged over the five CS+/Fs and five CS-/s sessions and evaluated by a t-test. Intakes during the two-bottle preference tests were averaged over the two sessions (test pairs) at each dose and evaluated with two-way repeated-measures analyses of variance (ANOVA, CS condition vs. Dose). Separate ANOVAs evaluated percent CS+/g intakes and total intake as a function of dose. In fructose-CFP acquisition studies, training intakes were averaged over the five CS+/Fs and CS-/s sessions and were analyzed with a two-way randomized-blocks ANOVA (CS conditions x Groups). Intakes during the two-bottle preference tests were averaged over sessions 1-2, 3-4, and 5-6 (referred to as Tests 1, 2, and 3) to control for bottle position effects. A three-way randomized-blocks ANOVA compared the CS intakes of the three groups (Group x CS x Test). A separate two-way ANOVA evaluated percent CS+ intakes and total intakes of the three groups across the three tests. In quinine-CFA acquisition studies, training intakes were averaged over the four CS+/FSQ and CS-/FS sessions and were analyzed with a two-way randomized-blocks ANOVA (CS conditions x Groups). Intakes during the preference tests were averaged over sessions 1-2, 3-4, and 5-6 (referred to as Tests 1, 2, and 3) to control for bottle position effects. A three-way randomized-blocks ANOVA compared the CS intakes of the three groups (Group x CS x Test). A separate two-way ANOVA evaluated percent CS+ intakes and total intakes of the three groups across the three tests. When main or interaction effects were observed in any ANOVA, Bonferroni corrected comparisons (P<0.05) detected significant effects. Drug-induced changes in acquisition or expression preferences were operationally defined as a significant change in
percent CS+ intakes relative to VEH, and/or a failure to observe significant differences between CS+ and CS- intakes in the two-bottle preference tests.

3. Results:

3.1 BAC and Fructose-CFP Expression: Training intakes of CS+/Fs (11.3 g) and CS-(10.5 g) failed to differ (t(16)= 1.19). In the 2-bottle preference tests, overall, CS+ (11.8 g) intakes significantly exceeded CS- (3.5 g) intakes (F(1,16)= 349.59, P<0.0001), and intakes significantly differed for the interaction between conditions and doses (F(4,64)= 2.88, P<0.03), but not among BAC doses (F(4,64)= 0.85). CS+ intake was significantly higher than corresponding CS- intake following VEH and all BAC doses (Fig. 9). Percent CS+ preferences significantly differed (F(4,64)= 3.22, P<0.02) across BAC doses with the 3.0 mg/kg (66%) dose significantly reducing percent CS+ preferences relative to VEH (87%) (Fig. 9). Total saccharin intake failed to differ (F(4,64)= 0.31) among the 0.0 (15.7 g), 0.5 (14.6 g), 1.5 (16.1 g), 3.0 (15.7 g) and 5.0 (14.2 g) BAC doses.

3.2 BAC and Fructose-CFP Acquisition: Significant differences in training intakes failed to occur among groups (F(2,14)= 0.66), between CS+/Fs and CS-/s conditions (F(1,7)= 5.08) or for the interaction between groups and conditions (F(2,14)= 3.10): VEH (CS+/Fs: 12.0 g; CS-/s: 10.0 g), BAC 3 (CS+/Fs: 13.7 g; CS-/s: 10.5 g) and BAC 5 (CS+/Fs: 12.4 g; CS-/s: 12.5 g). The two-bottle preference tests produced significant differences among groups (F(2,14)= 3.96, P<0.04), among tests (F(2,14)= 3.74, P<0.05), between CS+ and CS- conditions (F(1,7)= 81.99, P=0.0001), but not for any of the two-way and three-way interactions. Fig. 10 illustrates the similar pattern of effects in CS+ and CS- intakes across the three tests in the VEH (Panel A), BAC 3 (Panel B) and BAC 5 (Panel C) groups. Significant differences in percent CS+ intake
Figure 9. (Baclofen effects upon fructose-CFP expression): Intakes (mean ±SEM, g/30 min) of CS+ and CS- solutions in two-bottle preference tests in animals pretreated (30 min) with systemic BAC. The percentages of CS+ intake over total intake are indicated above each pair of values. Significant differences are denoted between CS+ and CS- intake (*) as are significant BAC effects relative to vehicle (+).
Figure 10. (Baclofen effects upon fructose-CFP acquisition): Intakes (mean ±SEM, g/30 min) of CS+ and CS- solutions in three pairs of two-bottle preference tests (unlimited (~45 ml) access, Tests 1, 2, 3) in animals that received vehicle (VEH: Panel A) or baclofen at doses of 3 (BAC 3: Panel B) or 5 (BAC 5: Panel C) mg/kg during training. The percentages of CS+ intake over total intake are denoted above each pair of values.
failed to occur among groups (F(2,14)= 2.31), among tests (F(2,14)= 0.19) or for the interaction between groups and tests (F(4,28)= 1.18) (Fig. 10). Total saccharin intake significantly differed among groups (F(2,14)= 3.95, P<0.04) and among tests (F(2,14)= 3.77, P<0.05), but not for the interaction between groups and tests (F(4,28)= 1.82). Total saccharin intake in the third test was significantly higher in the BAC 5 (23.8 g) relative to the VEH (16.2 g) group.

3.3 BAC and Quinine-CFA Acquisition: In one-bottle training, CS-/FS intake significantly exceeded CS+/FSQ intake (14.1 vs. 2.8 g/1 h, F(1,17)= 1650.40, P<0.0001); there were also significant differences among groups (F(2,34)= 18.62, P<0.0001) and for the interaction between groups and CS (F(2,34)= 5.53, P=0.008). CS-/FS intake for all groups was significantly higher than CS+/FSQ intake (Fig. 11A). CS+/FSQ intake was significantly lower in the BAC 5 relative to the VEH group (Fig. 11A). In two-bottle choice tests, there were significant differences between CS+ (8.2 g) and CS- (15.9 g) intakes (F(1,17)= 65.47, P<0.0001) as well as among groups (F(2,34)= 7.17, P<0.003), across tests (F(2,34)= 97.95, p<0.0001), and for the interactions between groups and tests (F(4,68)= 7.54, P<0.01), between groups and conditions (F(2,34)= 7.06, P<0.003) and among groups, tests and conditions (F(4,68)= 6.29, P<0.0002), but not between tests and conditions (F(2,34)= 0.17). Within-group comparisons revealed that the VEH-trained group consumed significantly more CS- than CS+ only during Test 1 (Fig. 11B), consistent with the limited duration of quinine-CFA observed previously (Rotella et al., 2014, 2015). The BAC 3-trained group consumed significantly more CS- than CS+ during Test 3, and this pattern approached significance in Tests 1 and 2 (Fig. 11C). In contrast, CS- intake was significantly higher than CS+ intake across all three tests in the BAC 5-trained group (Fig. 11D). CS+ intake in the BAC 5-trained group was significantly lower than
Figure 11. (Baclofen effects upon quinine-CFA acquisition): One-bottle training intakes (mean ±SEM, g/60 min) of CS+/FSQ and CS-/FS solutions during training sessions (16 ml limit) in animals receiving systemic vehicle or baclofen at doses of 3 or 5 mg/kg 30 min prior to each training session (Panel A). Significant differences are denoted between CS+/FSQ and CS-/FS intake (*) as well as BAC effects relative to vehicle (+). Two-bottle choice test intakes (mean ±SEM, g/1 h, unlimited (~45 ml) access) of the CS+ and CS- flavors presented in fructose + saccharin solutions in Tests 1-3 in groups trained with VEH (Panel B), BAC 3 (Panel C) BAC 5 (Panel D). The percentages of CS+/FSQ intake over total intake are denoted above each pair of values. Significant differences are denoted between CS- and CS+ intakes (*) as well as BAC-induced effects relative to corresponding VEH (+).
CS+ intake of the VEH-trained group across all three tests (Fig. 11B, 11D). Significant differences in percent CS+ intake were observed among groups (F(2,34)= 10.41, P<0.0003), among tests (F(2,34)= 6.51, P<0.004) and for the interaction between groups and tests (F(4,68)= 2.71, P<0.04). Percent CS+ intakes were significantly lower across all three tests in the BAC 5 (Fig. 11D) relative to the VEH (Fig. 11B) group. The lower percent CS+ intakes in Tests 2 and 3 in the BAC 3 group did not differ from VEH (Fig. 11B, 11C). Total fructose and saccharin intake significantly differed among groups (F(2,34)= 7.20, P<0.003), among tests (F(2,34)= 98.09, P<0.0001) and for the interaction between groups and tests (F(4,68)= 7.58, P<0.0001). Total fructose and saccharin intake in the first test was significantly lower in the BAC 3 (20.0 g) and BAC 5 (15.6 g) groups relative to the VEH (24.5 g) group.

4. Discussion

The present study examined whether GABA<sub>B</sub> receptor activation with systemic BAC would alter the expression and acquisition of fructose-CFP or the acquisition of quinine-CFA. GABA or its agonists administered into limbic and hypothalamic sites increase food intake (e.g., Arnt et al., 1979; Echo et al., 2002; Grandison and Guidotti, 1977; Miner et al., 2010; Soderpalm and Berridge, 2000; Stratford and Kelley, 1997; Ward et al., 2000; Wirtshafter et al., 1993). Further, whereas peripheral BAC administration increased chow and fat intake in non-deprived rats and mice (e.g., Bains and Ebenezer, 2013; Ebenezer and Patel, 2011), it decreased food intake in diabetic and diet-induced obese mice (Sato et al., 2007). Moreover, peripheral BAC reduced fat relative to chow intake under normal, limited-access and “binge-type” conditions in rats (e.g., Buda-Levin et al., 2005; Rao et al., 2008; Wojnicki et al., 2006), particularly intakes of pure fat or a sugar-fat mixture (e.g., Avena et al., 2014; Berner et al., 2009; Corwin et al., 2009; Wong et al., 2009).
Although the present study focused on the role of BAC in sugar preference and quinine avoidance, BAC effects upon total intake could be analyzed for saccharin intake in the fructose-CFP expression 2-bottle testing paradigm, for fructose + saccharin and saccharin intake in the fructose-CFP 1-bottle training paradigm, and for fructose + saccharin and quinine + fructose + saccharin intake in the quinine-CFA acquisition 1-bottle training paradigm. In the fructose-CFP expression paradigm, systemic BAC failed to alter total saccharin intake across the 0.5-5 mg/kg BAC dose range. In the fructose-CFP acquisition paradigm, neither the 3 nor 5 mg/kg doses of BAC administered during training significantly altered fructose + saccharin or saccharin intake relative animals receiving VEH. The inability of systemic BAC to alter saccharin intake per se is in contrast to the ability of the same dose range to impair gustatory discrimination of 0.3% and 0.6% saccharin solutions (Wilson et al., 2011). In the quinine-CFA paradigm, fructose + saccharin intake was similar among the three training groups, but quinine-adulterated fructose and saccharin intake was significantly lower in the rats receiving the 5 mg/kg BAC dose during training. It should be noted that animals in all three conditioning paradigms were food-restricted and received multiple injections of systemic BAC, factors that alter BAC-induced orexigenic actions on chow intake (e.g., Patel and Ebenezer, 2008). Thus, these data are in agreement with the general failure to observe differences in sweet intake per se following systemic BAC (Avena et al., 2014; Berner et al., 2009; Corwin and Wojnicki, 2009).

4.1 GABA<sub>B</sub> Receptor Agonism and Fructose-CFP Expression: In the fructose-CFP expression paradigm, CS+ intake was significantly higher than corresponding CS- intake in the two-bottle preference tests following VEH and all (0.5-5 mg/kg) BAC doses. The magnitude of the fructose-CFP measured by percent CS+ preference was significantly, but marginally lowered by the 3 mg/kg BAC dose (66%) relative to VEH; lower (0.5, 1.5) and higher (5.0) BAC doses
failed to exert effects. This marginal reduction in fructose-CFP expression following systemic GABA_B receptor activation was similar and comparable to that observed following systemic administration of AM-251, a cannabinoid CB_1 receptor inverse agonist (Miner et al., 2008) and muscarinic (scopolamine) and nicotinic (mecamylamine) cholinergic antagonists (Rotella et al., 2015). In contrast, systemic administration of dopamine D1 (SCH23390) or D2 (raclopride) receptor antagonists eliminated expression of fructose-CFP in real-feeding rats and sucrose-CFP in sham-feeding rats (Baker et al., 2003; Yu et al., 2000). Further, neither systemic NMDA (MK-801) nor opioid (naltrexone) receptor antagonism affected expression of fructose-CFP in real-feeding rats or sucrose-CFP in sham-feeding rats (Baker et al., 2004; Golden and Houpt, 2007; Yu et al., 1999).

4.2 GABA_B Receptor Agonism and Fructose-CFP Acquisition: In the fructose-CFP acquisition paradigm, the patterns of CS+ and CS- intake in all three two-bottle preferences tests were similar in rats receiving VEH or the two (3 or 5 mg/kg) BAC doses during training. Correspondingly, the magnitude of fructose-CFP measured by percent CS+ intake failed to differ among these groups. The inability of systemic GABA_B receptor activation to alter the acquisition (learning) of fructose-CFP stands in marked contrast to the abilities of systemic administration of DA D1, DA D2, NMDA or muscarinic receptor antagonists to eliminate acquisition of fructose-CFP in real-feeding rats and/or sucrose-CFP in sham-feeding rats (Baker et al., 2003; Golden and Houpt, 2007; Rotella et al., 2015; Yu et al., 2000). Rather, the effects of systemic BAC in failing to affect fructose-CFP acquisition were similar to that observed following NTX (Baker et al., 2004; Yu et al., 1999), AM-251 (Miner et al., 2008) or MEC (Rotella et al., 2015).

4.3 GABA_B Receptor Agonism and Quinine-CFA Acquisition: In the quinine-CFA paradigm, systemic BAC dose-dependently enhanced and prolonged the magnitude of quinine-
CFA with rats receiving the 5 mg/kg BAC dose during training eliciting significantly higher CS-than CS+ intake across all three tests. As observed previously (Rotella et al., 2014, 2015), the VEH-trained group consumed significantly more CS- than CS+ only during Test 1. The BAC 3-trained group consumed significantly more CS- than CS+ during Test 3, and this pattern approached significance in Tests 1 and 2. Consequently, the magnitudes of quinine-CFA as measured by percent CS+ intake takes were significantly lower across all three tests in rats trained with the 5 mg/kg BAC dose (15-20-25%) relative to VEH-trained rats (34-48-47%). The dose-dependent ability of systemic GABA$_B$ receptor activation to enhance and prolong quinine-CFA is similar to that observed following systemic dopamine D1, NMDA, opioid or nicotinic, but not dopamine D2 or muscarinic antagonists (Rotella et al., 2014, 2015). A role for GABA$_B$ receptor systems in conditioned aversions is supported by the ability of the GABA$_B$ agonist, BAC, but not the GABA$_B$ antagonist, saclofen, to suppress saccharin-induced drinking following pairing (Echo et al., 2002; Wilson et al., 2011). However, another study using a similar dose range found that systemic BAC failed to induce an aversion or affect ethanol-induced aversions (Chester and Cunningham, 1999). Further, although systemic BAC failed to alter operant responding to quinine-adulterated solutions (Petry and Heyman, 1997), it did enhance the discriminative abilities of D-amphetamine in a conditioned taste aversion procedure (Miranda et al., 2009). The present findings that systemic BAC enhanced and prolonged quinine-CFA extends the role of GABA$_B$ receptor signaling in both orosensory and post-ingestive processes related to avoidance and aversion, respectively.

In conclusion, these data implicate GABA$_B$ receptor signaling in the acquisition of quinine avoidance with minimal or no effects upon fructose preferences. Further studies investigating systemic BAC effects upon fat-CFP are warranted given the reductions in fat intake
following systemic administration of GABA$_B$ receptor agonists (e.g., Avena et al., 2014; Berner et al., 2009; Buda-Levin et al., 2005; Corwin et al., 2009; Rao et al., 2008; Wojnicki et al., 2006; Wong et al., 2009).
Chapter 6: Muscarinic, nicotinic and GABAergic receptor signaling differentially mediate fat-conditioned flavor preferences in rats.

1. Introduction:

The pharmacological substrates of the acquisition and expression of sugar- and fat-CFP have been examined for DA, opioid and NMDA receptor systems. Systemic administration of DA D1 (SCH23390) or D2 (raclopride), but not opioid (naltrexone), receptor antagonists eliminated the acquisition and expression of flavor-flavor conditioning studies elicited by sucrose in sham-feeding rats (Yu et al., 1999, 2000a, 2000b) or fructose in real-feeding rats (Baker et al., 2003, 2004), implicating both DA receptor families, but not opioids in both the learning and maintenance of these responses. Yet DA D1, but not D2 or opioid receptor antagonists eliminated acquisition, and to a lesser degree, reduced expression of flavor-nutrient conditioning elicited by IG sucrose infusions (Azzara et al., 2000, 2001), implicating only the DA D1 receptor in the learning of this response. In contrast, fat (CO)-CFP acquisition and expression was only attenuated by DA D2, but not D1 or opioid receptor antagonists (Dela Cruz et al., 2012a, 2012b), indicating that combined flavor-flavor and flavor-nutrient mechanisms reduce the effectiveness of DA antagonism to affect the response. The non-competitive NMDA receptor antagonist, MK-801 eliminated the acquisition, but not expression of CFP induced by fructose (Golden and Houpt, 2007) or CO (Dela Cruz et al., 2012a), implicating glutamatergic signaling in the learning of this response. The more limited pharmacological effects on CO-CFP is similar to that observed following glucose that also activates both flavor-flavor and flavor-nutrient processes. Thus, oral glucose-CFP was significantly though marginally attenuated in expression studies by DA D1, DA D2 or NMDA receptor antagonism (Dela Cruz et al., 2014).
Cholinergic muscarinic and nicotinic receptor signaling and GABA<sub>B</sub> receptor signaling have been recently implicated in the mediation of fructose-CFP (Rotella et al., 2015, 2016). Fructose-CFP expression was significantly reduced by systemic administration of muscarinic (SCOP: 2.5-10 mg/kg: 65-68%) and nicotinic (MEC: 4-8 mg/kg: 67-73%) cholinergic receptor antagonists, but only at doses that reduced total saccharin intake. However, this occurred despite the fact that CS+ preference intakes were significantly higher than CS- preference intakes, indicating minimal actions of cholinergic signaling on expression of this response. Further, fructose-CFP acquisition was eliminated by SCOP at doses of 1 (40-54%) and 2.5 (45-58%) mg/kg, and was accompanied by a failure to observe CS+ and CS- intake differences. On the other hand, MEC failed to alter fructose-CFP acquisition, indicating a critical role for muscarinic cholinergic receptors in the learning of this response. In contrast, MEC, but not SCOP enhanced the magnitude and persistence of quinine-induced conditioned flavor avoidance for a fructose solution (Rotella et al., 2015), indicating different cholinergic receptor mechanisms in preference and avoidance responses. Whereas systemic administration of the GABA<sub>B</sub> receptor agonist, BAC minimally reduced the expression, but not the acquisition of fructose-CFP, the magnitude and persistence of quinine-induced conditioned flavor avoidance for a fructose solution was enhanced (Rotella et al., 2016), indicating a limited role for GABA<sub>B</sub> signaling in expression of this response.

Cholinergic receptor signaling has also been implicated in the mediation of food and fat intake. Food increases Ach release in the AMY and the NAc (Avena et al., 2006, 2008a, 2008b, 2008c; Hajnal et al., 1998; Mark et al., 1992, 1995). Consumption of a high-fat diet for one week reduced acetylcholinesterase activity in the frontal cortex, hypothalamus and midbrain, as well as increased both β2-nAChR binding in the medial prefrontal cortex and substantia nigra, in
addition to α7-nAChR binding in the lateral and ventromedial hypothalamus (Morganstern et al., 2012). MEC blocked the enhancements in exploratory and novelty-seeking behaviors induced by high-fat consumption (Morganstern et al., 2012). Chronic nicotine reduced body weight in mice, particularly those maintained on a high-fat diet, an effect blocked by MEC co-treatment (Mangubat et al., 2012). Accumbal microinjections of SCOP markedly reduced fat intake elicited by accumbal administration of the mu-opioid receptor agonist, DAMGO, and also reduced food intake in food-deprived rats (Perry et al., 2009; Will et al., 2006). However, accumbal SCOP failed to affect fat intake itself (Will et al., 2006).

GABA_B receptor signaling has also been implicated in the mediation of fat intake. BAC administered into limbic and hypothalamic sites increased food intake (e.g., Arnt et al., 1979; Echo et al., 2002; Stratford and Kelley, 1997; Ward et al., 2000; Wirtshafter et al., 1993), and was reported to be mediated through GABA receptor interactions between the ventral tegmental area and nucleus accumbens (Miner et al., 2010). Systemic BAC increased fat intake under normal conditions (e.g., Bains and Ebenezer, 2013; Ebenezer and Patel, 2011), but suppressed fat intake under “binge-type” conditions (Avena et al., 2014; Berner et al., 2009; Buda-Levin et al., 2005; Corwin and Wojnicki, 2009; Rao et al., 2008; Wojnicki et al., 2014; Wong et al., 2009). Therefore, the present study examined whether systemic administration of muscarinic (SCOP) and nicotinic (MEC) cholinergic receptor antagonists and a GABA_B receptor agonist (BAC) would alter expression and acquisition of fat-CFP elicited ingestion of a flavor (e.g., cherry) paired with a higher (3.5%) CO concentration relative to a flavor (e.g., grape) paired with a lower (0.9%) CO concentration.
2. Methods:

2.1 Subjects: Male Sprague-Dawley rats (260-300 g, Charles River Laboratories, Wilmington, MA) were housed individually in wire mesh cages and maintained on a 12:12 h light/dark cycle with chow (5001, PMI Nutrition International, Brentwood, MO) and water available ad libitum, except as noted below. The experimental protocols were approved by the Queens College Institutional Animal Care and Use Committee certifying that all subjects and procedures are in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

2.2 Test Solutions: The training fluids consisted of 3.5% and 0.9% corn oil (CO: Sigma Chemical Co., St. Louis, MO) flavored with 0.05% unsweetened grape or cherry Kool-Aid (General Foods, White Plains, NY) and prepared as suspensions using 0.3% xanthan gum (Sigma) as described previously (Dela Cruz et al., 2012a, 2012b). Half of the rats in each group had the cherry flavor added to the 3.5% CO and the grape flavor added to the 0.9% CO; the flavors were reversed for the remaining rats. In the two-bottle preference tests, the 0.05% cherry and grape flavors were each presented in similar 0.9% CO + 0.3% xanthan gum suspensions. The CO + Kool-Aid + gum mixtures with the flavored training solutions are hereafter referred to as CS+/3.5% CO and CS-/0.9% CO, and the flavored 0.9% CO two-bottle test solutions are referred to as CS+ and CS-. All testing took place in the rat’s home cage during the mid-light phase of the light:dark cycle. In the two weeks prior to testing, the rats were placed on a food restriction schedule that maintained their body weights at 85-90% of their ad libitum level to increase approach behavior to the solutions. This procedure has been consistently applied in all of our and other prior CFP studies using oral intake or intragastric infusions of sugars and fats (e.g., Azzara et al., 2000, 2001; Baker et al., 2003, 2004; Dela Cruz et al., 2012a, 2012b; 2014;
Golden and Houpt, 2007; Rotella et al., 2015, 2016; Yu et al., 199, 2000a, 2000b). It is important to note that examination of these effects in food-deprived rats not only increases motivation to drink, but also produces an energy deficit. Many prior studies have explored both taste and consumption of palatable diets under both sated and deprived conditions, and have found dissociable behavioral and neurochemical processes. In other words, distinct hedonic and homeostatic processes can govern approach and consumption, and can presumably differentially alter preference for flavored solutions (see review: Baldo et al., 2013). For instance, administration of naloxone into the basolateral nucleus of the amygdala blocked increased fat intake induced by accumbal administration of DAMGO, but not increased fat intake induced by food deprivation (Parker et al., 2010). In contrast, administration of naloxone into the central nucleus of the amygdala blocked food deprivation-induced increases in fat intake, but not fat intake induced by accumbal DAMGO (Parker et al., 2014). Further, whereas intra-accumbal DAMGO increased c-Fos activation within the hypothalamic perifornical area that was blocked by baso-lateral amygdala inactivation, inta-accumbal DAMGO increased c-Fos activation in the ventral tegmental area that was unaffected by baso-lateral amygdala inactivation (Parker et al., 2015). Our laboratory (Yu et al., 1999) performed one CFP study that tested the ability of naltrexone to affect acquisition and expression of sucrose intake in sham-feeding rats under sated and deprived conditions. We found that the feeding condition failed to alter naltrexone’s inability to reduce acquisition or expression of sucrose-CFP in sham-feeding rats. Thus, the fact that these studies were done under food restriction should serve as a caveat.

The rats were initially adapted to drink an unflavored 0.2% saccharin solution from sipper tubes during daily 2-h sessions. The sipper tube was mounted on the front of the cage held by a taut steel spring, and was positioned 3-6 cm above the cage floor. This training procedure
was repeated daily until all rats approached the sipper tubes with short (< 1 min) latency, typically within three days. The limited food rations were given 30 min after each training session.

### 2.3 Experiment 1: SCOP, MEC and BAC CO-CFP: Expression Study:

Twenty-four male rats were given ten 1-bottle training sessions (2 h/day) with 24 ml of the CS+/3.5% CO solution presented on odd-numbered days, and 24 ml of the CS-/0.9% CO solution presented on even-numbered days. On days 9 and 10, the rats had access to a second sipper tube containing water. This familiarized the rats to the presence of two sipper tubes used during the choice tests; water intake was negligible in these training trials. The left-right position of the CS and water sipper tubes was counterbalanced over the two days. Following training, all rats were given eight daily two-bottle choice test sessions (2 h/day) with the CS+ (45 ml) and CS- (45 ml) solutions. Thirty min prior to the first two sessions, all rats were given vehicle injections (1 ml 0.9% saline/kg body weight, intraperitoneally (ip)). The animals were then divided into three equal (n=8) groups matched for the magnitude of their CO preferences observed following vehicle treatment. The SCOP group received three doses (1, 5 and 10 mg/kg, ip, Sigma Chemical Co., St. Louis, MO) prior to the remaining six sessions; half of the rats were tested with an ascending dose order, and the remaining rats were tested with a descending dose order. The rats were tested in two consecutive daily sessions at each drug dose with the left-right position of the CS+ and CS- solutions counterbalanced across sessions to control for bottle position effects. The MEC group received three doses (1, 6 and 8 mg/kg, ip, Sigma Chemical Co.), and the BAC group received three doses (1.5, 3 and 5 mg/kg, ip, Sigma Chemical Co.) prior to the remaining six sessions. The dose ranges were similar to those used in our prior conditioning studies with sugars (Rotella et al., 2015, 2016). Care was taken to minimize spillage due to the fact that some of the effects
could be potentially small. After initially weighing each bottle, it was gently shaken to insure appropriate flow of the viscous CO solutions. Any effluent from the bottle (~ 0.5-1.0 g) was collected and appropriate spillage adjustments were made to obtain an accurate pre-weight measurement. The taut steel spring prevented movement of the bottles during the sessions. Visual inspection of the bottles during the study revealed minimal if any spillage because of the viscosity of the solutions. The session length of 2 h was identical to that previously used in assessing fructose-CFP (Baker et al., 2003, 2004), and CO-CFP (Dela Cruz et al., 2012a, 2012b).

2.4 Experiment 2: SCOP, MEC and BAC and CO-CFP: Acquisition Study. Eight groups of naïve male rats were matched for their intakes of an unflavored 0.2% saccharin solution prior to training. The rats were given ten 1-bottle training sessions (2 h/day, 24 ml) with the CS+/3.5% CO solution presented on odd-numbered sessions, and the CS-/0.9% CO solution presented on even-numbered sessions, and all intraperitoneal (ip) injections were administered 30 min prior to each training session. Seven of the eight groups received vehicle (VEH, n=11, 1 ml 0.9% saline/kg body weight), SCOP at doses of 1 (SCOP1, n=10) and 2.5 (SCOP2.5, n=8) mg/kg, MEC at doses of 4 (MEC4, n=8) and 6 (MEC6, n=11) mg/kg, BAC at doses of 3 (BAC3, n=8) and 5 (BAC5, n=8) mg/kg. Because some of the drugs reduced overall CS intakes, an eighth group (Limited VEH, n=10) received vehicle injections, and their intakes were limited to approximate the reduced intakes observed in the different drug dose groups. These doses were similar to those employed in acquisition studies with sugars (Rotella et al., 2015, 2016). Following training, all eight groups were given six daily two-bottle choice sessions (2 h/day) with unlimited access to the CS+ (45 ml) and CS- (45 ml) solutions; no drugs were administered prior to these sessions. The positions of the CS+ and CS- solutions were counterbalanced across sessions.
2.5 Data analysis: In the expression studies, training intakes were averaged over the five CS+/3.5% CO and five CS-/0.9% CO sessions and evaluated by t-tests. Intakes during the preference tests were averaged over the two sessions at each dose and evaluated with two-way repeated-measures analyses of variance (ANOVA, CS condition vs. Dose) for the SCOP, MEC and BAC groups, respectively. Separate ANOVAs evaluated percent CS+ intakes and total intakes as a function of drug doses. In the acquisition study, training intakes were averaged over the five CS+/3.5% CO and CS-/0.9% CO sessions, and were analyzed separately in a two-way randomized-blocks ANOVA (CS x Groups). Intakes during the preference tests were averaged over sessions 1-2, 3-4, and 5-6 (referred to as Tests 1, 2, and 3). A three-way randomized-blocks ANOVA compared the CS intakes of the drug and control groups (Group x CS x Test). Separate two-way ANOVAs evaluated percent CS+ intakes and total intakes of the groups. When main or interaction effects were found, Bonferroni corrected comparisons (p<0.05) detected significant effects.

3. Results:

3.1 SCOP, MEC and BAC and expression of CO-CFP: The mean 1-bottle training intake of the CS+/3.5% CO (21.8 ±0.3 g/2 h) was significantly greater (t(23)= 4.03, p<0.0005) than the CS-/0.9% CO (18.2 ±1.0 g/2 h). In the two-bottle choice tests in SCOP-tested rats, CS+ was consumed significantly more (F(1,28)= 72.06, p<0.0001) than CS-, and significant differences were observed among doses (F(3,28)= 17.94, p<0.0001) and for the CS x Dose interaction (F(3,28)= 22.24, p<0.0001). CS+ intakes significantly exceeded CS- intakes following VEH and the 1 and 10, but not the 5 mg/kg SCOP doses (Figure 12A). Rats consumed significantly less CS+ at all SCOP doses compared to VEH, whereas CS- intakes failed to be significantly affected (Figure 12A). Total intake (g/2 h) significantly (F(3,24)= 24.75, p<0.0001) declined following
Figure 12. (Expression Study): Intakes (mean (g/2 h), ±SEM) of CS+ and CS− flavored 0.9% corn oil (CO) solutions in two-bottle preference tests in animals receiving systemic injections of the muscarinic cholinergic antagonist, scopolamine (SCOP: Panel A), the nicotinic cholinergic antagonist, mecamylamine (MEC, Panel B) or the GABAB receptor agonist, baclofen (BAC: Panel C) 30 min prior to testing. Significant differences are denoted between CS+ and CS− intake within an injection condition (*) and between CS+ intake following a drug dose relative to vehicle treatment (+). The percentages of CS+ intake over total intake are denoted above each pair of values with significant differences relative to vehicle treatment (+) noted.
the 1 (11.3 ±3.5 g), 5 (6.0 ±1.4 g) and 10 (4.6 ±1.3 g) mg/kg SCOP doses relative to VEH (27.6 ±3.3 g). Significant differences in the percent CS+ intakes were observed (F(3,21)= 5.00, p<0.009), and the 70% preference at the 5 mg/kg SCOP dose was significantly lower than the 98% preference following VEH (Figure 12A). Preferences at the 1 (84%) and 10 (82%) mg/kg SCOP doses were intermediate, but did not differ from the VEH test.

In the two-bottle choice tests in MEC-tested rats, CS+ was consumed significantly more (F(1,28)= 229.44, p<0.0001) than CS-, and significant differences were observed among doses (F(3,28)= 21.52, p<0.0001) and for the CS x Dose interaction (F(3,28)= 30.05, p<0.0001). CS+ intakes significantly exceeded CS- intakes following VEH and all three MEC doses (Figure 12B). Rats consumed significantly less CS+ at the two higher MEC doses compared to VEH, whereas CS- intakes were not affected (Figure 12B). Total intake (g/2 h) significantly (F(3,21)= 43.17, p<0.0001) declined following the 6 (8.4 ±1.3 g) and 8 (8.8 ±1.2 g) mg/kg MEC doses relative to VEH (31.8 ±4.0 g). Significant differences in the percent CS+ intakes were observed (F(3,21)= 4.62, p<0.01), and the 85% preference at the 8 mg/kg MEC dose was significantly lower than the 97% preference following VEH (Figure 12B). Preferences at the 1 (95%) and 6 (91%) mg/kg MEC doses were intermediate, and did not differ from the VEH test.

In the two-bottle choice tests in BAC-tested rats, CS+ was consumed significantly more (F(1,28)= 37.71, p<0.0001) than CS-, but no differences were observed among doses (F(3,28)= 0.24) or for the CS x Dose interaction (F(3,28)= 1.08). CS+ intakes significantly exceeded CS- intakes following VEH and the 1.5 and 5, but not the 3 mg/kg BAC doses (Figure 12C). BAC-induced CS+ and CS- intakes did not differ from VEH values (Figure 12C). Total intake (g/2 h) also did not differ (F(3,21)= 1.03) across the VEH and BAC conditions. The percent CS+ intakes approached significance (F(3,21)= 2.68, p=0.073), and a post-hoc comparison revealed
that the 74% preference at the 3 mg/kg BAC dose was significantly lower than the 97% preference following VEH (Figure 12C). Preferences at the 1.5 (85%) and 5 (86%) mg/kg BAC doses were intermediate, and did not differ from the VEH test.

3.2 SCOP, MEC and BAC and acquisition of CO-CFP: During 1-bottle training, overall CS+/3.5% CO intake (13.7 g/2 h) significantly (F(1,132)= 4.23, p<0.0001) exceeded CS-/0.9% CO intake (11.6 g/2 h), and significant differences were observed among groups (F(7,132)= 12.07, p<0.0001) but not for the Group x CS interaction (F(7,132)= 0.31). Total training intakes (2 h/g) were significantly greater in the VEH group (20.3 g) than the LMTD VEH (8.6 g), SCOP1 (12.8 g), SCOP2.5 (9.4 g), MEC4 (8.7 g) and MEC6 (6.9 g), but not the BAC3 (18.3 g) and BAC5 (16.2 g) groups (Figure 13A). Intakes of the SCOP and MEC groups did not differ from the LMTD VEH group. CS+/3.5% CO and CS-/0.9%CO intakes of the SCOP2.5, MEC4, MEC6 LMTD VEH groups were significantly lower than the corresponding VEH animals, whereas CS+/3.5%CO intake of the SCOP1 group was significantly lower than corresponding VEH animals. However, CS+/3.5% CO and CS-/0.9% CO intakes of these groups did not differ from those of the LMTD VEH group. In the two-bottle preference tests, overall, rats consumed significantly more (F(1,396)= 538.31, p<0.0001) CS+ than CS- solution, and significant differences were observed among the eight groups (F(7,396)= 2.51, p<0.016), and for the Groups x CS interaction (F(7,396)= 11.59, p<0.0001), but not among tests (F(2,396)= 0.58) or for the Groups x Tests (F(14,128)= 0.42), Tests x CS (F(2,396)= 1.89) and Groups x Tests x CS (F(14,396)= 0.52) interactions. CS+ intakes significantly exceeded CS- intakes across all three tests in the VEH (Figure 13B), Limited VEH (Figure 13C), SCOP1 (Figure 13D), MEC4 (Figure 13F), MEC6 (Figure 13G), BAC3 (Figure 13H) and BAC5 (Figure 13I) groups. In contrast, CS+ and CS- intakes did not differ across any test in the SCOP2.5 group (Figure 13E). Significant
Figure 13. (Acquisition Study): Training intakes (mean (g/2 h), ±SEM) of rats exposed to ten 1-bottle sessions of flavored 3.5% corn oil solutions (CS+/3.5% CO, Days 1, 3, 5, 7, 9) or 0.9% corn oil solutions (CS-/0.9% CO, Days 2, 4, 6, 8, 10) 30 min following systemic injections of vehicle (VEH), a limited vehicle (LTD VEH) control, scopolamine at doses of 1 (SCOP1) or 2.5 (SCOP2.5) mg/kg, mecamylamine at doses of 4 (MEC4) or 6 (MEC6) mg/kg or baclofen at doses of 3 (BAC3) or 5 (BAC5) mg/kg. Significant differences are denoted between CS+/3.5% CO or CS-/0.9% CO intake following a drug dose relative to VEH (+) treatment (Panel A).

Intakes (mean (g/2 h), ±SEM) of CS+ and CS− flavored 0.9% CO solutions in three two-bottle preference tests in the VEH (Panel B), LTD VEH (Panel C), SCOP1 (Panel D), SCOP2.5 (Panel E), MEC4 (Panel F), MEC6 (Panel G), BAC3 (Panel H) or BAC5 (Panel I) groups. Significant differences (*) are denoted between CS+ and CS−intake within each test and each group, and for percent CS+ intake in a given drug group relative to VEH (+) conditions.
differences in the percent CS+ intakes were observed among groups (F(7,198)= 19.16, p<0.0001), but not among tests (F(2,198)= 2.0), or for the interaction between Groups x Tests (F(14,198)= 0.41). Percent CS+ intake were significantly lower in the SCOP2.5 (Figure 13E) relative to the VEH group (Figure 13B) in tests 1 (59% vs. 88%), 2 (55% vs. 87%) and 3 (41% vs. 84%). All other groups did not differ from VEH values across tests in percent CS+ intake. Significant differences in total intake were observed among groups (F(7,198)= 2.36, p<0.024), but not among tests (F(2,198)= 0.56), or for the interaction between Groups x Tests (F(14,198)= 0.39). Whereas total intake was typically similar for most groups relative to the VEH group, significant reductions were noted in the first two tests in the MEC6 group and in the third test in the LMTD VEH group.

4. Discussion:

The present study examined whether muscarinic (SCOP) or nicotinic (MEC) cholinergic receptor antagonism or GABA_B (BAC) receptor activation would alter expression and acquisition of fat (CO)-CFP. Using percent CS+ intake as a measure of the magnitude of a preference, expression of CO-CFP was significantly, but marginally reduced by the 5 mg/kg SCOP dose (70%), by the 8 mg/kg MEC dose (85%) and by the 3 mg/kg BAC dose (74%) relative to comparable vehicle values (97-98%). These mild reductions in CO-CFP expression occurred despite sizable dose-dependent reductions in total CS intake following SCOP and MEC, but not BAC. Acquisition of CO-CFP was selectively and dose-dependently eliminated by administration of SCOP, but not MEC or BAC during one-bottle training. Training intakes of the CS+ and CS- CO solutions were significantly lower in groups receiving SCOP and MEC, but not BAC. To control for the non-specific actions of reduced solution consumption during training, an additional Limited VEH group had their intakes limited to approximate the reduced intakes
observed in drug groups. In contrast to the robust CO-CFP observed across the three tests in the VEH (86%) and Limited VEH (95%) groups, the SCOP2.5 group failed to display differences between CS+ and CS- intakes across tests, and their overall percent CS+ intake approached indifference (52%). Whereas total intake during the preference tests failed to differ among the VEH, Limited VEH and SCOP2.5 groups, the CS+ and CS- intakes respectively decreased and increased across preference tests in the SCOP2.5 group, demonstrating a selective loss of the salience of the CS+ solution. In contrast, a lower dose of SCOP (SCOP1 group) failed to affect the significant CS+ preferences demonstrated by analyses of CS+ and CS- intakes and percent CS+ intake (78%). Moreover, neither MEC at doses of 4 or 6 mg/kg nor BAC at doses of 3 or 5 mg/kg administered during training affected CO-CFP acquisition in analyses of either CS+ and CS- intakes or percent CS+ intake. The following sections examine cholinergic and GABA
 receptor involvement in preference conditioning and palatable intake.

4.1 Muscarinic and Nicotinic Cholinergic and GABA Receptor Involvement in Fat-CFP Expression: Expression of CO-CFP, like that of fructose-CFP (Rotella et al., 2015) was minimally affected only at certain doses over a wide dose range of either SCOP (fructose-CFP: 65-68% (2.5-10 mg/kg; CO-CFP: 70% (5 mg/kg)) or MEC (fructose-CFP: 67-73% (4-8 mg/kg); CO-CFP: 80% (8 mg/kg)). In both paradigms, these reductions were accompanied by reductions in total, and particularly CS+, intake following SCOP and MEC, but not BAC. The intake reductions of saccharin CS solutions in the fructose-CFP paradigm by SCOP and MEC are similar to reductions of sweet intake following systemic nicotinic receptor antagonism (Taraschenko et al., 2011). The intake reductions of 0.9% corn oil CS solutions in the CO-CFP paradigm by SCOP and MEC differ from the inability of accumbal SCOP to affect fat intake
(Will et al., 2006), but correspond to the finding that accumbal SCOP microinjections reduced fat intake elicited by accumbal DAMGO administration (Perry et al., 2009; Will et al., 2006).

Coincidentally, expression of CO-CFP, like that of fructose-CFP (Rotella et al., 2016), was minimally affected only at one dose (3 mg/kg) over a wide dose range of BAC (fructose-CFP: 66%; CO-CFP: 74%). The inability of the BAC dose range to alter total fat intake in the present study stands in contrast to its previously-reported increases in fat intake under normal ad-libitum conditions (e.g., Bains and Ebenezer, 2013; Ebenezer and Patel, 2011), and suppression of fat intake under “binge-type” conditions (Avena et al., 2014; Berner et al., 2009; Buda-Levin et al., 2005; Corwin and Wojnicki, 2009; Rao et al., 2008; Wojnicki et al., 2014; Wong et al., 2009). This may be due to the fact that animals in the present study were chronically food-restricted to encourage short latency sampling of the solutions during the preference paradigm.

The patterns of very limited inhibition of expression of CO-CFP by muscarinic or nicotinic cholinergic receptor antagonism or GABA_B receptor agonism are similar to DA D1, DA D2, opioid and NMDA receptor antagonism of CO-CFP expression (Dela Cruz et al., 2012a, 2012b). Despite evaluating a wide dose range (50-800 nmol/kg) of SCH23390 and raclopride, CO-CFP expression was reduced only by intermediate (200 nmol/kg) DA D1 (56%) and DA D2 (61%) antagonist doses. Similarly, despite evaluating a wide naltrexone dose range (0.1-5 mg/kg), CO-CFP expression was modestly reduced by only the 0.1 (69%) and 1 mg/kg (71%) doses. Further, despite evaluating a wide MK-801 dose range (50-200 µg/kg), the loss of preference (49%) following the highest NMDA antagonist dose was accompanied by dramatic decreases in total fat intake. Antagonist effects upon fructose-CFP are more striking in that SCH23390 or raclopride dose-dependently eliminated the expression of both fructose-CFP in real-feeding rats and sucrose-CFP in sham-feeding rats (Baker et al., 2003; Hsiao and Smith,
In contrast, neither NMDA nor opioid receptor antagonism affected expression of fructose-CFP (Baker et al., 2004; Golden and Houpt, 2007). It is important to note that whereas glucose and sucrose are capable of conditioning flavor preferences through both flavor-flavor and flavor-nutrient processes, fructose elicits flavor preferences through flavor-flavor, but not flavor-nutrient processes in short-term tests (Sclafani and Ackroff, 1994; Sclafani et al., 1993, 1999). Therefore, comparisons of the present limited cholinergic and GABA_B receptor signaling effects on CO-CFP with sugar-CFP may be more pertinent for oral glucose-CFP because, like CO, glucose activates both flavor-flavor and flavor-nutrient processes. Rats displayed a robust oral glucose-CFP (94-95%) which was significantly though marginally attenuated in expression studies by SCH23390 (67-70%), raclopride (77%) or MK-801 (70%) at doses that also markedly reduced overall CS intake (Dela Cruz et al., 2014). Given the imperfection of direct fructose and oil comparisons and given that parallel glucose data using the present agents is not yet available, other future studies need to address this issue as well as others examining oral only and oral + post-ingestive mechanisms. Finally, other future studies should address whether the oral effects of fat are more important in these procedures than fat-related post-ingestive effects by examining whether pharmacological manipulation of preferences elicited by short-term exposure to intragastric oil are similar to or different from that of the present data.

4.2 Muscarinic and Nicotinic Cholinergic and GABA_B Receptor Involvement in Fat-CFP Acquisition: Acquisition of CO-CFP was selectively and dose-dependently eliminated by administration of SCOP, but not MEC during one-bottle training despite the fact that both antagonists lowered training intakes. The SCOP2.5 group failed to display differences between CS+ and CS- intakes across tests, and their overall percent CS+ intake approached indifference.
(52%). CS+ and CS- intakes respectively decreased and increased across preference tests in the SCOP2.5 group, demonstrating a selective loss of the salience of the CS+ solution. Further, the SCOP dose (2.5 mg/kg) capable of eliminating CO-CFP acquisition is lower than the dose (5 mg/kg) that only mildly reduced CO-CFP expression. In contrast, the SCOP1, MEC4 and MEC6 groups failed to display changes in CO-CFP acquisition. The pattern of selective muscarinic, but not nicotinic cholinergic receptor involvement parallels the ability of SCOP, but not MEC to eliminate fructose-CFP acquisition (Rotella et al., 2015). The selective actions by which muscarinic, but not nicotinic cholinergic receptor blockade blocks both CO- and fructose-CFP acquisition (Rotella et al., 2015) are similar to other previously-cited cue-related feeding responses (Carballo-Marquez et al., 2009; Pratt et al., 2007; Sharf and Ranaldi, 2006). It does not appear that SCOP is eliminating fat- and fructose-CFP acquisition through some aversive quality. Reasons include that the doses necessary to eliminate acquisition of these responses are far lower than the SCOP doses that marginally reduce the maintenance of these responses in expression studies. Second, and more importantly, whereas SCOP, but not MEC blocks the acquisition of fructose-CFP, MEC, but not SCOP enhances the conditioned flavor avoidance induced by quinine adulteration (Rotella et al., 2015). Finally, acquisition of CO-CFP was unaffected by administration of BAC at doses of 3 or 5 mg/kg during one-bottle training, and these doses also failed to affect training intakes. The inability of GABA<sub>B</sub> receptor agonism to affect CO-CFP acquisition parallels its inability to alter fructose-CFP acquisition (Rotella et al., 2016). This failure by BAC to affect acquisition of sugar- or fat-CFP stands in contrast to its ability to enhance and prolong the magnitude of quinine-CFA (Rotella et al., 2016).

The elimination of acquisition of CO-CFP by muscarinic cholinergic antagonism is similar to NMDA receptor antagonist effects (Dela Cruz et al., 2012a), and is more potent than
DA D1 and D2 receptor antagonist effects (Dela Cruz et al., 2012b). In contrast, the inability of nicotinic cholinergic receptor antagonism or GABA<sub>B</sub> receptor agonism to alter CO-CFP acquisition is similar to the relative lack of opioid antagonist effects (Dela Cruz et al., 2012a). That GABA<sub>B</sub> agonists and opioid antagonists similarly fail to affect CO-CFP acquisition stand in contrast to the ability of BAC and naltrexone to strongly suppress fat intake under “binge-eating” and other similar conditions (Avena et al., 2014; Berner et al., 2009; Buda-Levin et al., 2005; Corwin and Wojnicki, 2009; Rao et al., 2008; Wang et al., 2011; Wong et al., 2009). Thus, it would appear that the regulatory processes underlying opioid and GABA<sub>B</sub> regulation of “binge-eating” is unrelated to the lack of involvement of these systems in the learning of these strong fat preferences.

The present and previous (Rotella et al., 2015, 2016) data indicate that whereas SCOP, MEC and BAC minimally affect fat-CFP expression, SCOP, but not MEC or BAC effectively eliminates the acquisition of fructose-CFP and fat-CFP. The use of systemic cholinergic receptor antagonist treatment or GABA<sub>B</sub> agonist treatment in examining fructose- and fat-CFP followed our previous strategy of initial systemic evaluation of DA receptor involvement in fructose- and fat-CFP (Baker et al., 2003; Dela Cruz et al., 2012a, 2012b Yu et al., 2000a, 2000b) followed by DA antagonist administration into central candidate limbic sites for fructose-CFP (Amador et al., 2014; Bernal et al., 2008, 2009; Malkusz et al., 2012). Much of the evidence implicating cholinergic involvement in complex aspects of food intake appears to be due to activity in a limbic circuit (specifically the VTA and NAc) in which cholinergic signaling can act directly upon sweet preferences and avoidances either in and of itself, or through interactions with brain DA. Sweet solutions appear to affect both DA and Ach release, particularly in the NAc and related sites. In addition to the ability of general food intake to increase DA and Ach in the NAc
shell (Mark et al., 1992), daily bingeing on sugar repeatedly and initially released NAc shell DA followed by Ach NAc shell release (Rada et al., 2005). Whereas real-feeding sucrose increased NAc DA and Ach, sucrose intake in sham-drinking rats displayed the DA, but not the Ach elevations in the NAc (Avena et al., 2006). Correspondingly, increases in NAc DA were observed in normal-weight and underweight rats, whereas NAc Ach was increased in the former, but not the latter group (Avena et al., 2008c). In contrast, animals trained to binge on a sucrose solution display increased Ach and decreased DA release in the NAc shell following food deprivation (Avena et al., 2008a). Consistent with the ability of sugars to produce greater CFP relative to saccharin, sucrose-predictive cues evoked greater NAc DA release than saccharin-predictive cues (McCutcheon et al., 2012). Administration of morphine, the mu-opioid agonist, DAMGO or galanin into the paraventricular nucleus of the hypothalamus increased DA and decreased Ach in the NAc shell (Rada et al., 1998, 2010). A parallel system might be proposed for mediation of fat-CFP given the present data, and that accumbal microinjections of SCOP markedly reduced fat intake elicited by accumbal administration of the mu-opioid receptor agonist, DAMGO, and also reduced food intake in food-deprived rats (Perry et al., 2009; Will et al., 2006). However, accumbal SCOP failed to affect fat intake itself (Will et al., 2006).

Given these relationships, a central site of action at which muscarinic receptor antagonism might reduce sugar- and fat-CFP and interact with brain DA is the NAc shell. Such Ach-DA interactions would presumably occur through DA terminal innervation of Ach-containing interneurons in the NAc (de Rover et al., 2002; Witten et al., 2010; Zhou et al., 2002), although cholinergic PPT/LDT innervation is found there as well (Dautan et al., 2014). NAc cholinergic-DA interactions act through local DA D2 receptors (Alcantara et al., 2003), mediate accumbal DA release that also involves glutamate signaling (Cachope et al., 2012; Chuhma et
al., 2014; Threlfell and Cragg, 2011), and provide feedback control of VTA DA release (Rahman and McBride, 2002). Further studies with accumbal cholinergic receptor antagonists are necessary to confirm this hypothesis for fat-CFP.
Chapter 7: General Discussion

The final chapter will briefly summarize the effects of muscarinic and nicotinic cholinergic receptor antagonism on the acquisition and expression of fructose-CFP or CO-CFP (e.g., Specific Aims 1 and 2) as well as the effects of GABAB receptor agonism (e.g., Specific Aims 3 and 4). Our novel flavor avoidance paradigm (quinine-CFA) will then be reviewed in terms of the effects of DA D1, DA D2, opioid and NMDA receptor antagonism (Specific Aim 5) and muscarinic and nicotinic cholinergic receptor antagonism and GABAB receptor agonism (Specific Aim 6) on the acquisition of quinine-CFA. Finally, there will be a general discussion of the following topics: A) what is the relationship between preference and avoidance, and how theoretically would receptor antagonists affect the two forms of learning, B) future directions on the proposed research as well as C) implications of animal based CFP and CFA investigations for human consumption behaviors and pathology.

1A. Summary of Muscarinic and Nicotinic Cholinergic Receptor Antagonism: Fructose- and Fat-CFP (Specific Aims 1 & 2)

The expression of fructose-CFP failed to be affected by a wide dose range of the muscarinic antagonist, SCOP or the nicotinic antagonist, MEC. However, the magnitude of fructose-CFP expression, defined by changes in percent CS+ intake, was significantly reduced by high doses of SCOP (2.5-10 mg/kg: 65-68%) and MEC (4-8 mg/kg: 67-73%) relative to VEH (89-90%). These reductions were accompanied by reductions in total, and particularly CS+, intake. These intake reductions of saccharin by systemic SCOP and MEC correspond to previous reductions of sweet intake following systemic nicotinic receptor antagonism (Taraschenko et al., 2011) and chow intake following systemic of muscarinic and nicotinic receptor antagonism (Dandekar et al., 2011; Dickson et al., 2010; Perry et al., 2009; Pratt and Blackstone, 2009; Will
et al., 2006). The very limited inhibition of systemic muscarinic or nicotinic cholinergic receptor antagonism of expression of fructose-CFP stands in contrast to previously-observed systemic pharmacological effects. Thus, systemic DA D1 (SCH23390) or D2 (raclopride) receptor antagonists dose-dependently eliminated the expression of both fructose-CFP in real-feeding rats and sucrose-CFP in sham-feeding rats (Baker et al., 2003; Hsiao and Smith, 1995; Yu et al., 2000a, 2000b). Whereas a CB1 receptor inverse agonist (AM-251) reduced expression of fructose-CFP (Miner et al., 2008), neither NMDA (MK-801) nor opioid (naltrexone) receptor antagonism affected expression of fructose-CFP (Baker et al., 2004; Bernal et al., 2010; Golden and Houpt, 2007). Thus, muscarinic or nicotinic cholinergic receptor antagonism plays a minimal role in the maintenance of an already-acquired CFP for fructose.

In contrast, acquisition of fructose-CFP was eliminated by systemic SCOP, but not MEC administered during training and the magnitude of fructose-CFP relative to VEH-trained (85-92%) rats was eliminated in SCOP-trained rats receiving 1 (40-54%) and 2.5 (45-58%) mg/kg doses, effects indicative of indifference. However, these SCOP doses significantly reduced CS+/Fs and CS-/s intakes during training, lending to the possibility that this loss of preference could be due to specific primary actions of muscarinic cholinergic receptor antagonism or to secondary actions of reducing the opportunities to learn the preference due to reduced intake per se. To control for this second possibility, the addition of the LMTD VEH group receiving vehicle injections, but limited in comparable CS+ and CS- intakes to those of the drug groups, displayed comparable, significant fructose-CFP preferences relative to vehicle-trained rats suggesting that the inability of SCOP-trained rats to display preferences was thus due to its cholinergic receptor antagonism. Further, while lower SCOP doses eliminated fructose-CFP acquisition, they failed to affect fructose-CFP expression. In contrast, MEC-trained rats
continued to display significant preferences with CS+ intake significantly higher than CS- intake despite MEC resulting in significant reductions in CS+/Fs and CS-/s intakes during training to levels observed for SCOP. Hence, systemic muscarinic, but not nicotinic receptor antagonism administered during training eliminated fructose-CFP acquisition. The systemic SCOP-induced elimination of fructose-CFP acquisition is similar in magnitude to the abilities of systemic DA D1, DA D2 and NMDA receptor antagonists to eliminate fructose-CFP acquisition in real-feeding rats (Baker et al., 2003; Golden and Houpt, 2007; Hsiao and Smith, 1995) and sucrose-CFP acquisition in sham-feeding rats (Yu et al., 2000a, 2000b).

Given the similar, albeit weaker effects of DA D1, DA D2, and NMDA receptor antagonists on the acquisition and expression of CO-CFP relative to fructose-CFP (Dela Cruz et al., 2012a, 2012b) in addition to the research suggesting interactions amongst these systems and the cholinergic system in the mediation of reward and learning (Avena & Rada, 2012), it was hypothesized and addressed in Specific Aim 2 that blockade of muscarinic and nicotinic cholinergic receptors would also influence the acquisition and expression of CO-CFP, and that these effects would be smaller in magnitude relative to the effects observed for fructose-CFP.

The expression of CO-CFP, like that of fructose-CFP (Rotella et al., 2015) was minimally affected only at certain doses over a wide dose range of either SCOP (fructose-CFP: 65-68% (2.5-10 mg/kg; CO-CFP: 70% (5 mg/kg)) or MEC (fructose-CFP: 67-73% (4-8 mg/kg); CO-CFP: 80% (8 mg/kg)). In both paradigms, these reductions were accompanied by reductions in total, and particularly CS+, intake following SCOP and MEC. The intake reductions of saccharin CS solutions in the fructose-CFP paradigm by SCOP and MEC are similar to reductions of sweet intake following systemic nicotinic receptor antagonism (Taraschenko et al., 2011). The patterns of very limited inhibition of expression of CO-CFP by muscarinic or nicotinic cholinergic
receptor antagonism is similar to DA D1, DA D2, opioid and NMDA receptor antagonism of CO-CFP expression (Dela Cruz et al., 2012a, 2012b). Despite evaluating a wide dose range (50-800 nmol/kg) of SCH23390 and raclopride, CO-CFP expression was reduced only by intermediate (200 nmol/kg) DA D1 (56%) and DA D2 (61%) antagonist doses. Similarly, despite evaluating a wide NTX dose range (0.1-5 mg/kg), CO-CFP expression was modestly reduced by only the 0.1 (69%) and 1 mg/kg (71%) doses. Further, despite evaluating a wide MK-801 dose range (50-200 µg/kg), the loss of preference (49%) following the highest NMDA antagonist dose was accompanied by dramatic decreases in total fat intake. Antagonist effects upon fructose-CFP are more striking in that SCH23390 or raclopride dose-dependently eliminated the expression of both fructose-CFP in real-feeding rats and sucrose-CFP in sham-feeding rats (Baker et al., 2003; Hsiao and Smith, 1995; Yu et al., 2000a, 2000b). In contrast, neither NMDA nor opioid receptor antagonism affected expression of fructose-CFP (Baker et al., 2004; Golden and Houpt, 2007). It is important to note that whereas glucose and sucrose are capable of conditioning flavor preferences through both flavor-flavor (orosensory) and flavor-nutrient (post-ingestive) processes, fructose elicits flavor preferences through flavor-flavor, but not flavor-nutrient processes in short-term tests (Sclafani and Ackroff, 1994; Sclafani et al., 1993, 1999). Therefore, comparisons of the present limited cholinergic receptor signaling effects on CO-CFP with sugar-CFP may be more pertinent for oral glucose-CFP because, like CO, glucose activates both flavor-flavor and flavor-nutrient processes. Rats displayed a robust oral glucose-CFP (94-95%) which was significantly though marginally attenuated in expression studies by SCH23390 (67-70%), raclopride (77%) or MK-801 (70%) at doses that also markedly reduced overall CS intake (Dela Cruz et al., 2014). Given the imperfection of direct fructose and oil comparisons and given that parallel glucose data using the present agents is not yet available, other future studies need to
address this issue as well as others examining oral only and oral + post-ingestive mechanisms. Finally, other future studies should address whether the oral effects of fat are more important in these procedures than fat-related post-ingestive effects by examining whether pharmacological manipulation of preferences elicited by short-term exposure to intragastric oil are similar to or different from that of the present data.

Acquisition of CO-CFP was selectively and dose-dependently eliminated by administration of SCOP, but not MEC during one-bottle training despite the fact that both antagonists lowered training intakes. The SCOP2.5 group failed to display differences between CS+ and CS- intakes across tests, and their overall percent CS+ intake approached indifference (52%). CS+ and CS- intakes respectively decreased and increased across preference tests in the SCOP2.5 group, demonstrating a selective loss of the salience of the CS+ solution. Further, the SCOP dose (2.5 mg/kg) capable of eliminating CO-CFP acquisition is lower than the dose (5 mg/kg) that only mildly reduced CO-CFP expression. In contrast, the SCOP1, MEC4 and MEC6 groups failed to display changes in CO-CFP acquisition. The pattern of selective muscarinic, but not nicotinic cholinergic receptor involvement parallels the ability of SCOP, but not MEC to eliminate fructose-CFP acquisition (Rotella et al., 2015). The selective actions by which muscarinic, but not nicotinic cholinergic receptor blockade blocks both CO- and fructose-CFP acquisition (Rotella et al., 2015) are similar to other previously-cited cue-related feeding responses (Carballo-Marquez et al., 2009; Pratt et al., 2007; Sharf and Ranaldi, 2006). It does not appear that SCOP is eliminating fat- and fructose-CFP acquisition through some aversive quality. Reasons include that the doses necessary to eliminate acquisition of these responses are far lower than the SCOP doses that marginally reduce the maintenance of these responses in expression studies. Second, and more importantly, whereas SCOP, but not MEC blocks the
acquisition of fructose-CFP, MEC, but not SCOP enhances the conditioned flavor avoidance induced by quinine adulteration (Rotella et al., 2015).

The elimination of acquisition of CO-CFP by muscarinic cholinergic antagonism is similar to NMDA receptor antagonist effects (Dela Cruz et al., 2012a), and is more potent than DA D1 and D2 receptor antagonist effects (Dela Cruz et al., 2012b). Thus in relation to **Specific Aim 2**, whereas both SCOP and MEC partially mediate fat-CFP expression, only SCOP is involved in the acquisition of fat-CFP.

1B. **Summary of GABAβ Receptor Agonism: Fructose- and Fat-CFP (Specific Aims 3 & 4)**

Although the present study focused on the role of BAC in sugar preference, BAC effects upon total intake could be analyzed for saccharin intake in the fructose-CFP expression 2-bottle testing paradigm, for fructose + saccharin and saccharin intake in the fructose-CFP 1-bottle training paradigm. In the fructose-CFP expression paradigm, systemic BAC failed to alter total saccharin intake across the 0.5-5 mg/kg BAC dose range. In the fructose-CFP acquisition paradigm, neither the 3 nor 5 mg/kg doses of BAC administered during training significantly altered fructose + saccharin or saccharin intake relative animals receiving VEH. The inability of systemic BAC to alter saccharin intake per se is in contrast to the ability of the same dose range to impair gustatory discrimination of 0.3% and 0.6% saccharin solutions (Wilson et al., 2011). It should be noted that animals in these conditioning paradigms were food-restricted and received multiple injections of systemic BAC, factors that alter BAC-induced orexigenic actions on chow intake (e.g., Patel and Ebenezer, 2008). Thus, these data are in agreement with the general failure to observe differences in sweet intake per se following systemic BAC (Avena et al., 2014; Berner et al., 2009; Corwin and Wojnicki, 2009).
In the fructose-CFP expression paradigm, CS+ intake was significantly higher than corresponding CS- intake in the two-bottle preference tests following VEH and all (0.5-5 mg/kg) BAC doses. The magnitude of the fructose-CFP was significantly, but marginally lowered by the 3 mg/kg BAC dose (66%) relative to VEH; lower (0.5, 1.5) and higher (5.0) BAC doses failed to exert effects. This marginal reduction in fructose-CFP expression following systemic GABA_B receptor activation was similar and comparable to that observed following systemic administration of AM-251, a cannabinoid CB1 receptor inverse agonist (Miner et al., 2008) and muscarinic (scopolamine) and nicotinic (mecamylamine) cholinergic antagonists (Rotella et al., 2015). In contrast, systemic administration of dopamine D1 (SCH23390) or D2 (raclopride) receptor antagonists eliminated expression of fructose-CFP in real-feeding rats and sucrose-CFP in sham-feeding rats (Baker et al., 2003; Yu et al., 2000). Further, neither systemic NMDA (MK-801) nor opioid (naloxone) receptor antagonism affected expression of fructose-CFP in real-feeding rats or sucrose-CFP in sham-feeding rats (Baker et al., 2004; Golden and Houpt, 2007; Yu et al., 1999).

In the fructose-CFP acquisition paradigm, the patterns of CS+ and CS- intake in all three two-bottle preferences tests were similar in rats receiving VEH or the two (3 or 5 mg/kg) BAC doses during training. Correspondingly, the magnitude of fructose-CFP measured by percent CS+ intake failed to differ among these groups. The inability of systemic GABA_B receptor activation to alter the acquisition of fructose-CFP stands in marked contrast to the abilities of systemic administration of dopamine D1, dopamine D2, NMDA or muscarinic receptor antagonists to eliminate acquisition of fructose-CFP in real-feeding rats and/or sucrose-CFP in sham-feeding rats (Baker et al., 2003; Golden and Houpt, 2007; Rotella et al., 2015; Yu et al., 2000). Rather, the effects of systemic BAC in failing to affect fructose-CFP acquisition were
similar to that observed following NTX (Baker et al., 2004; Yu et al., 1999), AM-251 (Miner et al., 2008) or MEC (Rotella et al., 2015). Thus in relation to Specific Aim 3, while BAC marginally reduced fructose-CFP expression, it failed to block fructose-CFP acquisition.

The expression of CO-CFP, like that of fructose-CFP (Rotella et al., 2015), was minimally affected only at one dose (3 mg/kg) over a wide dose range of BAC (fructose-CFP: 66%; CO-CFP: 74%). The inability of the BAC dose range to alter total fat intake in these series of studies stands in contrast to its previously-reported increases in fat intake under normal ad-libitum conditions (e.g., Bains and Ebenezer, 2013; Ebenezer and Patel, 2011), and suppression of fat intake under “binge-type” conditions (Avena et al., 2014; Berner et al., 2009; Buda-Levin et al., 2005; Corwin and Wojnicki, 2009; Rao et al., 2008; Wojnicki et al., 2014; Wong et al., 2009). This may be due to the fact that animals in the present study were chronically food-restricted to encourage short latency sampling of the solutions during the preference paradigm. The patterns of very limited inhibition of expression of CO-CFP by GABA_B receptor agonism are similar to DA D1, DA D2, opioid, NMDA, muscarinic or nicotinic cholinergic receptor antagonism on CO-CFP expression (Dela Cruz et al., 2012a, 2012b). It is important to note that whereas glucose and sucrose are capable of conditioning flavor preferences through both flavor-flavor (orosensory) and flavor-nutrient (post-ingestive) processes, fructose elicits flavor preferences through flavor-flavor, but not flavor-nutrient processes in short-term tests (Sclafani and Ackroff, 1994; Sclafani et al., 1993, 1999). Therefore, comparisons of the present limited GABA_B receptor signaling effects on CO-CFP with sugar-CFP may be more pertinent for oral glucose-CFP because, like CO, glucose activates both flavor-flavor and flavor-nutrient processes. Rats displayed a robust oral glucose-CFP (94-95%) which was significantly though marginally attenuated in expression studies by SCH23390 (67-70%), raclopride (77%) or MK-
801 (70%) at doses that also markedly reduced overall CS intake (Dela Cruz et al., 2014). Given the imperfection of direct fructose and oil comparisons and given that parallel glucose data using the present agents is not yet available, other future studies need to address this issue as well as others examining oral only and oral + post-ingestive mechanisms. Finally, other future studies should address whether the oral effects of fat are more important in these procedures than fat-related post-ingestive effects by examining whether pharmacological manipulation of preferences elicited by short-term exposure to intragastric oil are similar to or different from that of the present data.

The acquisition of CO-CFP was unaffected by administration of BAC at doses of 3 or 5 mg/kg during one-bottle training, and these doses also failed to affect training intakes. The inability of GABA_B receptor agonism to affect CO-CFP acquisition parallels its inability to alter fructose-CFP acquisition (Rotella et al., 2016). This failure by BAC to affect acquisition of sugar- or fat-CFP stands in contrast to its ability to enhance and prolong the magnitude of quinine-CFA (Rotella et al., 2016).

That GABA_B agonists and opioid antagonists similarly fail to affect CO-CFP acquisition stand in contrast to the ability of BAC and NTX to strongly suppress fat intake under “binge-eating” and other similar conditions (Avena et al., 2014; Berner et al., 2009; Buda-Levin et al., 2005; Corwin and Wojnicki, 2009; Rao et al., 2008; Wang et al., 2011; Wong et al., 2009). Thus, it would appear that the regulatory processes underlying opioid and GABA_B regulation of “binge-eating” is unrelated to the lack of involvement of these systems in the learning of these strong fat preferences.

The inability of nicotinic cholinergic receptor antagonism or GABA_B receptor agonism to alter CO-CFP acquisition is similar to the relative lack of opioid antagonist effects (Dela Cruz et
al., 2012a), but differs from the ability of muscarinic cholinergic (Rotella et al., 2016b) and NMDA receptor antagonist effects (Dela Cruz et al., 2012a). Thus, in relation to **Specific Aim 4**, these data indicate that BAC minimally affects fat-CFP expression and fails to effectively eliminate the acquisition of fructose-CFP and fat-CFP.

**1C. Summary of the Pharmacology of Quinine-CFA (Specific Aims 5 & 6)**

Under an evolutionary perspective, it could be said that the activation of certain taste receptors for sweets and fats and the following “pleasant” perceptions aided in the survival of man and many species and thus were selected for and subsequently passed to following generations due to the energy and nutrients these substances provided. By contrast, it could be said that the activation of certain taste receptors for bitter toxins and the following “unpleasant” perceptions aided in the survival of man and many species and thus were selected for and subsequently passed to following generations due to the harm or death that was prevented. Those failing to learn to avoid these toxic substances presumably did not survive and the genes coding for the taste receptors and underlying neurochemical substrates mediating this learning were also not passed down. Therefore, not only is important to understand the neurochemical and/or neuroanatomical substrates mediating these adaptive approach behaviors, but it is equally important to understand the same processes underlying adaptive avoidant behaviors. As such, another primary goal of this dissertation was to determine whether the same neurotransmitter systems mediating the learning and maintenance of preferences conditioned by primary rewards (e.g. sugars and fats) also mediated the underlying learning processes of avoidances conditioned by primary aversive stimuli (e.g. bitter taste of quinine).

In order to assess the proposed effects on **Specific Aim 5**, we first demonstrated that a CFA could be produced by adulterating a fructose+saccharin solution with quinine depending
upon the concentration of the bitterant. At very low concentrations (0.001-0.008%), the added quinine failed to reduce CS+/FSQ training intake relative to the CS-FS, and did not produce significant differences in CS+ and CS- intakes in the two-bottle choice tests. Although adulteration with 0.012% or 0.016% quinine significantly depressed CS+/FSQ training intake, it was insufficient to produce a CFA in the two-bottle tests. However, when quinine concentration was raised to 0.03%, CS+/FSQ intake was significantly reduced relative to CS-/FS intake in one-bottle training trials, and a quinine-CFA was observed in two-bottle Test 1 but not in Tests 2 and 3. The transitory nature of the 0.3% quinine-induced CFA was also observed in the VEH groups of Experiments 2 and 3 that were tested with CS flavors presented in fructose+saccharin and saccharin-only solutions, respectively. The data from Experiment 3 indicates that the transitory quinine CFA observed in the first two experiments was not due to the use of palatable fructose+saccharin solutions in the choice tests. Instead, the quinine-CFA was found to be related to the concentration of the bitter adulterant. The VEH.06% rats trained with a CS+FSQ containing 0.06% quinine consumed less solution during training and displayed a more persistent CS+ avoidance (Tests 1 and 2) than did the VEH rats with the CS+FSQ containing 0.03% quinine (Test 1 only), indicating that the persistence of the quinine-CFA could be increased by increasing quinine concentration.

In CFP studies, systemic DA D1 and D2 antagonists reduce the acquisition of flavor-taste sugar preferences (Baker et al., 2003; Hsiao & Smith, 1995; Yu et al., 2000a; 2000b), whereas only DA D1 antagonists reduce the acquisition of flavor-nutrient sugar preferences (Azzara et al., 2001). One interpretation of how DA antagonists block sugar-based CFPs is that the drugs reduce the reward value of the sweet taste or post-oral actions of sugars. An extension of this hypothesis would be that DA antagonists also reduce the negative reward value of aversive tastes.
or post-oral aversive states. In fact, DA D1 receptor antagonists administered into either the lateral hypothalamus (Caulliez et al., 1996) or shell of the NAc (Fenu et al., 2001) disrupted the acquisition of a flavor-toxin CFA induced by LiCl in a manner similar to reductions in the acquisition of flavor-nutrient CFP elicited by IG glucose infusions (Touzani et al., 2008; 2009b). The selective effects of SCH23390, but not raclopride injections on quinine-induced CFA is similar to reports that only SCH23390 altered flavor-toxin avoidance produced by LiCl injections (Fenu et al., 2005; 2009). However, whereas SCH23390 blocked the development of the LiCl-CFA, the drug enhanced the quinine-CFA in the present study. The quinine and LiCl conditioning procedures differed in several important respects which complicate comparisons between the different drug effects. Note, in particular, that in one experiment, SCH23390 significantly attenuated a LiCl-induced CFA when injected 5 min after the CS training sessions but not when injected 30 min prior to the CS training sessions as in the present study (Fenu et al., 2001). Thus, to determine if SCH23390, or other drugs, differentially influence the learning of a bitter taste (quinine) or toxic drug (LiCl) CFA, it is essential to use similar training paradigms (drug dose, injection timing, CS flavor, etc.).

Conceivably, DA D1 antagonism may enhance quinine-conditioned flavor avoidance because it selectively increases the aversiveness of bitter adulterants. However, there is little evidence concerning the impact of systemic SCH23390 on quinine avoidance; indeed, one study reported that SCH microinfusions into the ventral pallidum suppressed saccharin, but not quinine intake (Shimura et al., 2006). In Experiments 2 and 3, SCH23390 reduced the intake of both the FS and FSQ solutions so the drug effect on quinine avoidance per se cannot be differentiated. The failure of raclopride injections to alter CS+FSQ training intake or CS+ preference, relative
to the vehicle treatment, is consistent with one report that this DA D2 antagonist did not alter quinine solution intake in rats (Phillips et al., 1991).

In a CFP study, systemic NMDA receptor antagonism eliminated the acquisition, but not expression of a flavor-taste fructose preference (Golden & Houpt, 2007). Yet the present study demonstrated that systemic treatment with MK-801 significantly prolonged the flavor-taste CFA induced by quinine. During training, MK-801-treated rats displayed significant reductions in CS+FSQ, but not CS-FS intake as compared to VEH-treated rats, suggesting that it enhanced the avoidance of the quinine adulterated solution. Yet, a previous study (Vardigan et al., 2010) reported that systemic MK-801 did not reduce quinine solution intake in thirsty rats. Golden and Houpt (2007) hypothesized that NMDA receptor signaling mediates the learning process by which a flavor CS is associated with the reward value of a gustatory US. Thus, it is not clear why systemic NMDA receptor antagonism with MK-801 blocks a fructose-CFP (Golden & Houpt, 2007), but enhances a quinine-CFA. CFA is induced by MK-801 as well as other NMDA antagonists (Fowler et al., 2011; Jackson & Sanger, 1989; Traverso et al., 2003; 2012; Turgeon et al., 2000), and it enhances ethanol-induced CFA (Blenkowski et al., 1998).

In CFP studies, neither systemic nor central (NAc) opioid receptor antagonism altered the acquisition or expression of flavor-taste or flavor-nutrient sugar preferences (Azzara et al., 2000; Baker et al., 2004; Bernal et al., 2010; Yu et al., 1999). Yet the present study demonstrated that NTX significantly prolonged the flavor-taste CFA induced by quinine. This effect may be related to the finding that opioid antagonism increased quinine aversion in rats (Cagniard & Murphy, 2013; Le Magnen et al., 1980; Siviy & Reid, 1983), although this is not a consistent result (Ferraro et al., 2002; Goodwin et al., 2001; Levine et al., 1982; Parker et al., 1992). Opioid receptor antagonism with naloxone is also reported to enhance taste aversions elicited by LiCl.
(Davis et al., 2009; Micelli et al., 1979; Smurthwaite et al., 1992). NTX as well as delta-opioid agonists and antagonists produce conditioned flavor avoidance by themselves (Hutchinson et al., 2000; Kautz et al., 1989; Parker & Rennie, 1992). Yet, opioid antagonism blocks morphine-induced taste aversions (Fox et al., 2006; Stevenson et al., 1992).

In relation to Specific Aim 6, the last set of studies addressed the roles of muscarinic, nicotinic or GABA<sub>B</sub> receptor signaling in the acquisition of quinine-CFA.

Systemic administration of nicotinic (MEC), but not muscarinic (SCOP) cholinergic receptor antagonism significantly enhanced and prolonged the acquisition of quinine-CFA. VEH-trained rats displayed a significant, transitory quinine-CFA as demonstrated by a significant aversion observed after the first (34%), but not second (48%) or third (47%) pairs of tests. Systemic nicotinic cholinergic antagonism significantly increased the magnitude and duration of quinine-CFA as indicated by rats trained with the 4 (18-24%) and 6 (11-13%) mg/kg MEC doses. In contrast, the SCOP 1 group displayed a significant aversion after the first test pair (17%), but failed to differ from VEH-trained rats thereafter, whereas the SCOP 2.5 group failed to display quinine-CFA after any preference test. It should be noted that both systemic SCOP and MEC significantly reduced CS-/FS intake and CS+/FSQ intake during training relative to VEH. This raises the possibility that the greater subsequent avoidance responses could be due to non-specific malaise brought about by pairing the antagonist with the flavored solutions during training. Both lithium chloride-induced conditioned taste aversions and attenuation of neophobic responses were blocked by central SCOP pretreatment into the insular cortex (Ferreira et al., 2002; Gutierrez et al., 2003a, 2003b; Naor and Dudai, 1996) and NAc shell (Ramirez-Lugo et al., 2006), but not when SCOP was administered after the presentation of the new taste.

Although nicotinic receptor antagonists have not been evaluated in this paradigm, galantamine,
an acetylcholinesterase inhibitor and positive allosteric modulator of nicotinic Ach receptors, reduced nicotine seeking without producing malaise (Hopkins et al., 2012).

The inability of SCOP to affect quinine-CFA acquisition is consistent with its previously-described inability to alter quinine aversion while attenuating LiCl-induced CFA (Coil et al., 1978). Although systemic SCOP elicited a flavor-taste CFA (Ossenkopp et al., 1986), the present data indicate that SCOP- and quinine-CFA fail to synergize. The ability of MEC to enhance and prolong quinine-CFA is consistent with previous observations that nicotine depressed quinine-evoked responses in the nucleus tractus solitarius that were blocked by MEC (Simons et al., 2006), and CFAs induced by nicotine and quinine generalized to each other in mice (Gyekis et al., 2012) that were blocked by MEC (Oliveira-Maia et al., 2009). However, the ability of MEC to enhance and prolong quinine-CFA appears specific to this type of “inhibitory” learning. Recent studies demonstrated that chronic co-treatment with systemic MEC prevented the occurrence of depressive-like behavior elicited by chronic restraint stress as measured by the forced swim test, sucrose preference and body weight control (Aboul-Fotouh, 2015). Moreover, whereas nicotine facilitated a version of “inhibitory” learning called negative occasion setting, systemic MEC co-treatment extended the number of training sessions to elicit this behavior (Meyer et al., 2015).

These data suggest that systemic nicotinic, but not muscarinic receptor antagonism, enhances and prolongs quinine-CTA by acting on its specific cholinergic signaling mechanism, and not through non-specific malaise-induced effects. These effects extend our previous (Rotella et al., 2014) findings demonstrating that the persistence of quinine-CFA was significantly enhanced by systemic administration of DA D1, NMDA and opioid, but not DA D2 receptor antagonists administered prior to training. Thus, systemic nicotinic, DA D1, NMDA and
opioid, but not muscarinic, or DA D2 receptor antagonists administered during training enhanced and prolonged the acquisition of quinine-CFA.

In the quinine-CFA paradigm, systemic BAC dose-dependently enhanced and prolonged the magnitude of quinine-CFA with rats receiving the 5 mg/kg BAC dose during training eliciting significantly higher CS- than CS+ intake across all three tests. As observed previously (Rotella et al., 2014, 2015), the VEH-trained group consumed significantly more CS- than CS+ only during Test 1. The BAC 3-trained group consumed significantly more CS- than CS+ during Test 3, and this pattern approached significance in Tests 1 and 2. Consequently, the magnitudes of quinine-CFA as measured by percent CS+ intake takes were significantly lower across all three tests in rats trained with the 5 mg/kg BAC dose (15-20-25%) relative to VEH-trained rats (34-48-47%). The dose-dependent ability of systemic GABA_B receptor activation to enhance and prolong quinine-CFA is similar to that observed following systemic dopamine D1, NMDA, opioid or nicotinic, but not dopamine D2 or muscarinic antagonists (Rotella et al., 2014, 2015).

A role for GABA_B receptor systems in conditioned aversions is supported by the ability of the GABA_B agonist, BAC, but not the GABA_B antagonist, saclofen, to suppress saccharin-induced drinking following pairing (Echo et al., 2002; Wilson et al., 2011). However, another study using a similar dose range found that systemic BAC failed to induce an aversion or affect ethanol-induced aversions (Chester and Cunningham, 1999). Further, although systemic BAC failed to alter operant responding to quinine-adulterated solutions (Petry and Heyman, 1997), it did enhance the discriminative abilities of D-amphetamine in a conditioned taste aversion procedure (Miranda et al., 2009). The present findings that systemic BAC enhanced and prolonged quinine-CFA extends the role of GABA_B receptor signaling in both orosensory and post-ingestive processes related to avoidance and aversion, respectively. These data implicate GABA_B receptor
signaling in the acquisition of quinine avoidance and extend our findings to that of systemic nicotinic, DA D1, NMDA and opioid, but not muscarinic, or DA D2 receptor antagonism, as demonstrated by the enhancement and prolonged duration of the acquisition of quinine-CFA.

2A. CFP and CFA: Theoretical Perspective of Pharmacological Effects

The series of studies conducted for this dissertation involved the pharmacological substrates of both the learning and maintenance of two forms of food-related preference learning in rats, fructose-CFP and corn oil-CFP, as well as the learning of a form of food-related avoidance, quinine-CFA. Much evidence supports the roles of the D1, D2 and NMDA receptor antagonism in the acquisition and expression of both fructose- and corn oil-CFP and the present series of studies provide even further elucidation of the pharmacology of these learned food-related preferences. By contrast, the pharmacological substrates of learned food-related avoidances are far less studied, and as a result the series of studies conducted for this dissertation extended the pharmacology of learned preferences to learned avoidances, demonstrating stark behavioral and pharmacological differences between the two. These findings are summarized in Table 1.

With respect to learned food-related preferences (fructose-CFP and fat-CFP), we show that muscarinic receptor signaling is involved in the associative learning phase of both food-related preferences that were under investigation, but not the expression phase of this learning. We reported that systemic administration of SCOP eliminated the acquisition of both fructose- and corn oil-CFP acquisition, with slightly more robust effects for fructose-CFP (40-54% indifference) than CO-CFP (41%-59% indifference). These results lend behavioral support for a cholinergic hypothesis of learning and memory (Hasselmo, 1999, 2006; Hasselmo et al., 2002;
Table 1
Summary of systemic pharmacological findings in the fructose-conditioned flavor preference (CFP) expression and acquisition, quinine-conditioned flavor avoidance (CFA) acquisition and corn oil-CFP acquisition and expression paradigms.

<table>
<thead>
<tr>
<th>Paradigm</th>
<th>Neurotransmitter system</th>
<th>Receptor sub-type: drug</th>
<th>Effect</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Fructose-CFP</td>
<td>Dopamine</td>
<td>DA D1 antagonist (SCH)</td>
<td>Blocked</td>
<td>Blocked*</td>
<td>Baker et al., 2003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DA D2 antagonist (RAC)</td>
<td>Blocked</td>
<td>Blocked*</td>
<td>Baker et al., 2003</td>
</tr>
<tr>
<td></td>
<td>Glutamate</td>
<td>NMDA antagonist (MK801)</td>
<td>Blocked</td>
<td>No Effect</td>
<td>Golden &amp; Houpt, 2007</td>
</tr>
<tr>
<td></td>
<td>Opioid</td>
<td>General antagonist (NTX)</td>
<td>No Effect</td>
<td>No Effect</td>
<td>Baker et al., 2004</td>
</tr>
<tr>
<td></td>
<td>Acetylcholine</td>
<td>mACh antagonist (SCOP)</td>
<td>Blocked</td>
<td>Attenuated</td>
<td>Rotella et al., 2015</td>
</tr>
<tr>
<td></td>
<td></td>
<td>nACh antagonist (MEC)</td>
<td>No Effect</td>
<td>Attenuated</td>
<td>Rotella et al., 2015</td>
</tr>
<tr>
<td></td>
<td>GABA</td>
<td>GABA &lt;sub&gt;B&lt;/sub&gt; agonist (BAC)</td>
<td>No Effect</td>
<td>Attenuated</td>
<td>Rotella et al., 2016a</td>
</tr>
<tr>
<td>B. Quinine-CFA</td>
<td>Dopamine</td>
<td>DA D1 antagonist (SCH)</td>
<td>Enhanced/prolonged</td>
<td>n/a</td>
<td>Rotella et al., 2014</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DA D2 antagonist (RAC)</td>
<td>No Effect</td>
<td>n/a</td>
<td>Rotella et al., 2014</td>
</tr>
<tr>
<td></td>
<td>Glutamate</td>
<td>NMDA antagonist (MK801)</td>
<td>Enhanced/prolonged</td>
<td>n/a</td>
<td>Rotella et al., 2014</td>
</tr>
<tr>
<td></td>
<td>Opioid</td>
<td>General antagonist (NTX)</td>
<td>Enhanced/prolonged</td>
<td>n/a</td>
<td>Rotella et al., 2014</td>
</tr>
<tr>
<td></td>
<td>Acetylcholine</td>
<td>mACh antagonist (SCOP)</td>
<td>No Effect</td>
<td>n/a</td>
<td>Rotella et al., 2015</td>
</tr>
<tr>
<td></td>
<td></td>
<td>nACh antagonist (MEC)</td>
<td>Enhanced/prolonged</td>
<td>n/a</td>
<td>Rotella et al., 2015</td>
</tr>
<tr>
<td></td>
<td>GABA</td>
<td>GABA &lt;sub&gt;B&lt;/sub&gt; agonist (BAC)</td>
<td>Enhanced/Prolonged</td>
<td>n/a</td>
<td>Rotella et al., 2016a</td>
</tr>
<tr>
<td>B. Corn-oil-CFP</td>
<td>Dopamine</td>
<td>DA D1 antagonist (SCH)</td>
<td>Attenuated</td>
<td>Blocked</td>
<td>Dela Cruz et al., 2012a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DA D2 antagonist (RAC)</td>
<td>Attenuated</td>
<td>Blocked</td>
<td>Dela Cruz et al., 2012a</td>
</tr>
<tr>
<td></td>
<td>Glutamate</td>
<td>NMDA antagonist (MK801)</td>
<td>Blocked</td>
<td>Attenuated</td>
<td>Dela Cruz et al., 2012b</td>
</tr>
<tr>
<td></td>
<td>Opioid</td>
<td>General antagonist (NTX)</td>
<td>Attenuated</td>
<td>Attenuated</td>
<td>Dela Cruz et al., 2012b</td>
</tr>
<tr>
<td></td>
<td>Acetylcholine</td>
<td>mACh antagonist (SCOP)</td>
<td>Blocked</td>
<td>Attenuated</td>
<td>Rotella et al., 2016b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>nACh antagonist (MEC)</td>
<td>No Effect</td>
<td>Attenuated</td>
<td>Rotella et al., 2016b</td>
</tr>
<tr>
<td></td>
<td>GABA</td>
<td>GABA &lt;sub&gt;B&lt;/sub&gt; agonist (BAC)</td>
<td>No Effect</td>
<td>Attenuated</td>
<td>Rotella et al., 2016b</td>
</tr>
</tbody>
</table>

DA: dopamine; NMDA: N-methyl-D-aspartate; SCH; SCH23390; RAC: raclopride; MK801: MK-801; NTX: naltrexone; SCOP: scopolamine; MEC: mecamylamine, BAC: baclofen.

* Blocked: Significant %CS effect present (≈ 50%) absent of significant differences in CS+ and CS- intakes.

* Very low CS intakes at higher doses.
This hypothesis proposes that oscillations in cholinergic activity within a network including the entorhinal cortex, dentate gyrus, CA3, CA1 and medial septum (Meeter et al., 2004) shifts to allow optimal levels of information encoding, consolidation and retrieval, where decreases in cholinergic activity would impair encoding and increases would impair consolidation and retrieval. Our findings lend support to this hypothesis in that SCOP administration differentially blocked the acquisition (encoding) phase of fructose- and CO-CFP. These findings are similar to that of other studies demonstrating these effects on learning and memory using SCOP (Winters et al., 2007, 2006, 2008; Young et al., 1995); however, theses studies demonstrated that consolidation could even be enhanced by SCOP administration, which would suggest an enhanced expression of CFP. Although we failed to demonstrate significant blockade or enhancement of expression of CFP in these studies, it could be due to methodological differences between the studies.

Interestingly, these effects on acquisition were also seen for systemic blockade of DA and NMDA receptors in rats. In contrast, nicotinic receptor antagonism or GABA<sub>B</sub> receptor agonism failed to affect either type of preference acquisition. Given this, it could be hypothesized that muscarinic receptor signaling is required for acquiring a food-related association. However, preferences are not the only form of associative learning animals and humans may acquire, avoidances are also possible. Thus, it could be said that if muscarinic blockade simply interferes with all forms of food-related learning, it would also block learned food-related avoidances. The current series of studies found a contrasting effect, in which systemically administered SCOP failed to affect the acquisition of quinine-CFA. Therefore, the ability of SCOP to block the learning of a food-related association transcends the nutrient used to condition the behavior, but
may be related to the hedonic valence of the stimulus used to condition the behavior, where fructose and CO act as appetitive stimuli with positive hedonic valence and quinine acts an avoidant stimulus with negative hedonic valence.

Given these effects, it may be hypothesized that the pharmacological agents administered eliminate or reduce the salience of the stimulus used to condition the behavior, leading to indifference during extinction testing. For example, with respect to CFPs, the positive hedonic qualities (e.g. sweet taste of fructose or fatty taste/texture of CO) play a role in their ability to condition flavor preferences, resulting in significant % CS+ preferences relative to CS-. The current findings support the role of SCOP to eliminate the positive hedonic salience of fructose and CO given that administration of the drug blocked their acquisition as measured by indifference (about 50% CS+ relative to CS- intake) during two-bottle testing. If this hypothesis were true of food-learning despite hedonic salience, the same would hold for avoidances, leading a taste avoidance to shift to indifference. With respect to quinine-CFA, the negative hedonic quality (e.g. bitter taste of quinine) plays a role in its ability to condition a flavor avoidance, resulting in significant %CS+ avoidances relative to CS-. By contrast, the current findings fail to support the role of SCOP in its ability to eliminate the negative hedonic salience of quinine by shifting the learned avoidance to indifference, given its failure to eliminate quinine-CFA acquisition. Therefore it would seem that the effects of SCOP on food-related learning are restricted to conditioning occurring through the pairing of stimuli of positive hedonic salience.

Another interesting result was that nicotinic and GABA_b receptor signaling, despite failing to mediate the acquisition of fructose- and CO-CFP, were found to significantly affect the acquisition of quinine-CFA, demonstrating a pharmacological and behavioral distinction between these and muscarinic signaling. Here, we report that systemically administered MEC
and BAC significantly prolonged the duration of quinine-CFA. If the above hypothesis were to hold true, it would be assumed that the acquisition of quinine-CFA would be eliminated rather than prolonged. As a result, the totality of our findings does NOT support this hypothesis relating to the elimination of the salience of the stimulus used to condition the behavior.

A more encompassing hypothesis to the findings reported in this dissertation would be that the pharmacological agents administered serve to shift the acquired association in the negative direction, such that taste preferences would shift to indifferences and taste avoidances would become more avoided. The results of SCOP administration provide support for this hypothesis in that systemic SCOP lead to %CS+ indifference for both fructose- and CO-CFP acquisition, also supported by similar effects reported for DA and glutamatergic NMDA receptor blockade, but not nicotinic, GABAB or opioid receptor blockade. On the other hand, while systemic MEC or BAC administration caused an already avoided solution to become more avoided or avoided for longer periods of time, similar to opioid, DA and glutamatergic NMDA receptor blockade, muscarinic receptor blockade failed to affect quinine-CFA acquisition. Thus although not all pharmacological agents administered affected the learning of these behaviors, most served to shift the acquired association in the negative direction, leading preferences to indifference and avoidances to become more avoided or for increased duration.

2B. Future Directions of Research

Cholinergic Effects: The present data indicate that whereas systemic muscarinic (SCOP) and nicotinic (MEC) cholinergic receptor antagonism minimally affects fructose- and fat-CFP expression, systemic muscarinic, but not nicotinic cholinergic receptor antagonism effectively eliminates the acquisition of fructose- and fat-CFP. In contrast, systemic nicotinic, but not muscarinic cholinergic receptor antagonism enhanced and prolonged the acquisition of quinine-
CFA. The use of systemic cholinergic receptor antagonist treatment followed our previous strategy of initial systemic evaluation of DA receptor involvement in fructose-CFP (Baker et al., 2003; Yu et al., 2000a, 2000b) followed by antagonist administration into central candidate limbic sites (Amador et al., 2014; Bernal et al., 2008, 2009; Malkusz et al., 2012). Thus, we propose the central candidate sites for muscarinic receptor antagonist effects on fructose- and fat-CFP acquisition and nicotinic receptor antagonists for quinine-CFA acquisition.

**Muscarinic Acetylcholine Effects:** Much of the evidence implicating cholinergic involvement in complex aspects of food intake appears to be due to activity in a limbic circuit (specifically the VTA and NAc) in which cholinergic signaling can act directly upon preferences and avoidances either in and of itself, or through interactions with brain DA. Sweet solutions appear to affect both DA and Ach release. One Ach-DA neuroanatomical interaction presumably occurs through Ach inputs from the PPT/LDT nuclei to identified DA cells in the VTA (Holmstrand and Sesack, 2011; Maskos, 2008; Omelchenko and Sesack, 2005; Woolf et al., 1990). The second Ach-DA interaction presumably occurs through DA terminal innervation of Ach-containing interneurons in the NAc (de Rover et al., 2002; Witten et al., 2010; Zhou et al., 2002), although cholinergic PPT/LDT innervation is found there as well (Dautan et al., 2014). NAc cholinergic-DA interactions act through local DA D2 receptors (Alcantara et al., 2003), mediate accumbal DA release that also involves glutamate signaling (Cachope et al., 2012; Chuhma et al., 2014; Threlfell and Cragg, 2011), and provide feedback control of VTA DA release (Rahman and McBride, 2002).

In addition to the ability of general food intake to increase DA and Ach in the NAc shell (Mark et al., 1992), daily bingeing on sugar repeatedly and initially released NAc shell DA followed by Ach NAc shell release (Rada et al., 2005). Whereas real-feeding sucrose increased
NAc DA and Ach, sucrose intake in sham-drinking rats displayed the DA, but Ach elevations in the NAc (Avena et al., 2006). Correspondingly, increases in NAc DA were observed in normal-weight and underweight rats, whereas NAc Ach was increased in the former, but not the latter group (Avena et al., 2008c). In contrast, animals trained to binge on a sucrose solution display increased Ach and decreased DA release in the NAc shell following food deprivation (Avena et al., 2008a). Consistent with the ability of sugars to produce greater CFP relative to saccharin, sucrose-predictive cues evoked greater NAc DA release than saccharin-predictive cues (McCutcheon et al., 2012). Administration of morphine, a mu-opioid agonist, DAMGO or galanin into the paraventricular nucleus of the hypothalamus increased DA and decreased Ach in the NAc shell (Rada et al., 1998, 2010). VTA Ach and NAc DA are concomitantly released by the orexigenic peptide, ghrelin (Jerlhag et al., 2012). Given these relationships, a central site of action at which muscarinic receptor antagonism might reduce fructose- and fat-CFP and interact with brain DA is the NAc shell.

Furthermore, multiple studies implicate the VTA as a potential site in which muscarinic antagonist effects may block the acquisition of CFPs, given not only the interaction of DA and Ach here, but also NMDA receptors. NMDA receptor antagonism by MK-801 was found to block both fructose- (Golden & Houpt, 2007) and fat-CFP (Dela Cruz et al., 2012b) and (2R)-amino-5-phosphonomopentanoate (AP-5) in the VTA impaired acquisition of lever pressing for food, but not when injected dorsal to the VTA suggesting region specificity (Zellner et al., 2009). Further, VTA NMDA receptor stimulation is necessary for both the acquisition of reward-related learning and acquisition by the CS to activate dopamine terminal regions, as measured by c-fos expression in forebrain structures such as prefrontal cortex area 2, nucleus accumbens core and shell as well as medial and lateral caudate (Ranaldi et al., 2011). It has also been reported that
primary rewarding UCSs activate VTA DA neurons and NSs associated with these UCSs acquire the ability to act as CSs since they also come to acquire the ability to activate VTA DA neurons through NS-UCS associations (Zellner & Ranaldi, 2010), as measured by c-fos activity in the VTA (Kest et al., 2012).

This dissertation reported that systemic antagonism of muscarinic cholinergic receptors blocks the acquisition of both fructose- and corn oil-CFP, but failed to affect the acquisition of quinine-CFA suggesting that SCOP primarily interferes with reward-related learning. These effects are support by studies demonstrating that SCOP in the VTA blocks the acquisition of reward-related learning in the absence of performance deficits (Galaj et al., 2017). Similar effects were reported for the ability of VTA SCOP to block the acquisition of food-related learning (Sharf & Ranaldi, 2006) or the acquisition of food-rewarded operant responding, but not the performance of the task in rats as measured by a failure to affect lever pressing break points under a progressive ratio schedule of reinforcement (Sharf et al., 2006). Taken together, these findings suggest that SCOPs ability to block acquisition of fructose- and corn oil-CFP may be due to interfering with the ability of the NSs to acts as CSs by preventing the CS to either activate VTA DA on its own or to prevent NMDA-dependent VTA DA activation. Thus, muscarinic cholinergic receptor activation is necessary for a NS to acquire the ability to act as a CS through associating with a UCS in the absence of performance deficits.

Given the pronounced effects of muscarinic cholinergic receptor antagonism on the acquisition of fructose-CFP, the process whereby these effects occurred may be discussed in relation to whether SCOP blocked the rewarding effects of fructose, or whether SCOP blocked the learned association. A recent study reported that SCOP significantly reduced intakes of both a more preferred 16% sucrose solution as well as a less preferred 0.2% saccharin solution in
C57BL/6 and BALB/c mice, but not SWR mice (Olsson et al., 2017), suggesting not only a genetic difference in the extent to which muscarinic cholinergic receptor blockade influences sweet intake, but more importantly that SCOPs effects on intake did not depend on the rewarding quality of the stimulus in C57BL/6 and BALB/c mice. Additionally, whereas SCOP dose-dependently reduced the expression of sucrose-CFP in C57BL/6 and BALB/c mice it failed to affect expression in SWR mice, and while SCOP dose-dependently reduced the acquisition of sucrose-CFP in BALB/c mice it marginally affected acquisition in C57BL/6 and SWR mice (Bourie et al., 2017). Therefore, the reductions induced by SCOP on acquisition and expression of sugar-CFP particularly in SWR mice appear due to interference with the associative process underlying the behavior (Bourie et al., 2017), and not the inherent reward value of the UCS (Olsson et al., 2017). Further studies are necessary to examine this “reward” vs. “learning” issue.

**Nicotinic Acetylcholine Effects:** With respect to avoidances, DA D1, but not D2 antagonism disrupted the acquisition of a LiCl-induced CFA following systemic administration, and following central administration into the LH or NAc shell (Caulliez et al., 1996; Fenu et al., 2001, 2005, 2009), sites also involved in preference–related learning. Blockade of NMDA, AMPA and metabotropic glutamate receptors in the AMY disrupted LiCl-induced CFA (Yasoshima et al., 2000). SCOP and nicotine are capable of eliciting flavor-taste CFAs with the former abolished and the latter enhanced by lesions placed in the area postrema (Ossenkopp et al., 1986, Ossenkopp and Gugno, 1990). Further, nicotine depressed quinine-evoked responses in the nucleus tractus solitarius that were blocked by MEC (Simons et al., 2006). Given these relationships, central sites of action at which nicotinic receptor antagonism might enhance quinine-CFA is through either by mediation of brain DA reward circuitry, acting within the NAc
shell, or by mediation of medullary toxin-detecting structures, acting within the area postrema or solitary nucleus.

It is possible that the greater subsequent avoidance responses could be due to non-specific malaise brought about by pairing the antagonist with the flavored solutions during training. Both LiCl-induced conditioned taste aversions and attenuation of neophobic responses were blocked by central SCOP pretreatment into the insular cortex (Ferreira et al., 2002; Gutierrez et al., 2003a, 2003b; Naor and Dudai, 1996) and NAc shell (Ramirez-Lugo et al., 2006), but not when SCOP was administered after the presentation of the new taste. Although nicotinic receptor antagonists have not been evaluated in this paradigm, galantamine, an acetylcholinesterase inhibitor and positive allosteric modulator of nicotinic Ach receptors, reduced nicotine seeking without producing malaise (Hopkins et al., 2012). These data suggest that systemic nicotinic, but not muscarinic receptor antagonism enhances and prolongs quinine-CTA by acting on its specific cholinergic signaling mechanism, and not through non-specific malaise-induced effects. Further studies altering the timing of cholinergic antagonist injections are necessary to completely rule out participation by this non-specific effect.

**GABA<sub>B</sub> Effects:** The present data indicate that whereas systemic BAC administration failed to significantly affect fructose- and fat-CFP, it enhanced and prolonged the acquisition of quinine-CFA. The use of systemic GABA<sub>B</sub> receptor agonist treatment followed our previous strategy of initial systemic evaluation of DA receptor involvement in fructose-CFP (Baker et al., 2003; Yu et al., 2000a, 2000b) followed by antagonist administration into central candidate limbic sites (Amador et al., 2014; Bernal et al., 2008, 2009; Malkusz et al., 2012). Thus, we propose the central candidate central sites for GABA<sub>B</sub> receptor agonist effects for quinine-CFA acquisition.
BAC administered into limbic and hypothalamic sites increased food intake (e.g., Arnt et al., 1979; Echo et al., 2002; Stratford and Kelley, 1997; Ward et al., 2000; Wirtshafter et al., 1993), and was reported to be mediated through GABA receptor interactions between the VTA and NAc (Miner et al., 2010). Feeding elicited by the GABA_B agonist, BAC is mediated through GABA receptor interactions between the VTA and NAc (Miner et al., 2010). Indeed, brain DA and cholinergic systems modulate medium spiny NAc GABA output and VTA DA output (see reviews: Avena & Rada, 2012; Hoebel et al., 2007). Given these relationships, a central site of action at which GABA_B receptor agonism might enhance quinine-CFA is the VTA. A summary of the proposed neuroanatomical system mediating sugar-CFP is presented in Figure 14.

2C. Clinical Implications

**Food-Related Pathology:** Although the etiology of obesity is complex, including both environmental and genetic factors, obesity in humans tends to occur where cheap, highly palatable, and energy-dense foods are readily available (Dragone, 2009). Furthermore, it has been proposed that the apparent bias in favor of weight gain rather than weight loss may be a result of not the body’s energy homeostasis systems, but instead due to a bias towards meal initiation as a result of learning, sensory and emotional cues as well as certain societal variables (Saper et al., 2002; Schwartz et al., 2003). As such, sensory, emotional and societal stimuli associated with palatable foods can activate potent reward systems that can override the homostatic energy system and lead to over-consumption. Thus, understanding of the behavioral pharmacology of learned food preferences and avoidances may be translatable to human ingestive studies with the goal of determining the correct drug targets for therapeutic intervention in individuals with obesity or other food-related behavioral pathology. Accordingly, muscarinic
Figure 14. A model neuroanatomical system of a “distributed brain network” mediating the pharmacological effects of sugar-CFP. The model system begins with the ventral tegmental area (VTA) and its two major dopamine (DA) projections through the meso-limbic and meso-cortical pathways. In this model, the meso-limbic DA projection terminates in the nucleus accumbens (NAC) and amygdala (AMY) both of which possess DA D1 and D2 receptors (D1/2 R). The meso-cortical DA projection terminates in the medial prefrontal cortex (mPFC) and medial orbital frontal cortex (MOFC) both of which possess D1/2 R. The NAC sends GABA projections to the mPFC and MOFC, and in turn receives glutamate (Glu) projections from those cortical sites. Major Glu projections reciprocally innervate the AMY with the mPFC and MOFC. Further, mixed GABA/Glu projections reciprocally innervate the NAC and AMY. Finally, the VTA receives input from the NAC through the ventral pallidum (VP), from the AMY through a GLU pathway, and from the lateral hypothalamus (LH) through an orexin (Or) projection that is in turn innervated by a DA pathway originating in the zona incerta.
receptor antagonists such as SCOP may be used to help reduce the associations made between unhealthy foods or their associated environmental cues and their rewarding flavors in patients diagnosed with pre-diabetic symptoms. Further, nicotinic receptor antagonists and GABA$_B$ receptor agonists may be used to help enhance the negative qualities or environmental cues associated with binge- or over-eating, effectively combatting the development of diabetes.

**Food and Addictions:** Animal models of consumption have shown that high-fat and/or high-sugar foods lead to neurobiological and behavioral changes that are similar to those due to consumption of addictive drugs including their associations with reward dysfunction (Gearhardt et al., 2011; Davis et al., 2013) and behavioral factors such as craving and impulsivity (Murphy et al., 2014; Meule & Kubler, 2012; Meule & Gearhardt, 2014). Accordingly, although the behaviors assessed in this dissertation involved primarily orosensory properties, similar associative processes play a role in the development of various addictions, for example, alcoholism. BAC (Colombo et al., 2004) or GABA$_B$ positive allosteric modulators (Maccioni et al., 2015) were reported to suppress the acquisition and maintenance of alcohol self-administration in rats by reducing their reinforcing properties. Further, BAC has been reported to be effective in reducing the craving aspect of alcohol addiction in a clinical study of men (Rozatkar et al., 2016). In our studies, we found that while BAC administration failed to affect preference learning, it significantly enhanced and prolonged quinine-CFA, suggesting its role was to increase quinine’s avoidant properties. Given this, perhaps this dissertation research can help explain BAC’s effectiveness in reducing alcohol self-administration by means of enhancing avoidance to the negative and toxic qualities of alcohol.

With respect to nicotine addiction, MEC has been reported to increase cessation rates in smokers most effectively when combined with a nicotine patch (Lancaster & Stead, 2000). We
report here that while MEC administration failed to affect preference learning, it significantly enhanced and prolonged quinine-CFA, demonstrating the pharmacological difference between cholinergic receptor subtype on behavior type. Our results suggest that MEC’s role was to increase quinine’s avoidant properties, thus enhancing and prolonging the avoidance. Together these findings suggest that MEC is a potential pharmacological agent to aid in smoking cessation during periods of reduced intake and that this behavioral result could be also due to MEC enhancing the negative and toxic qualities of nicotine when paired with a nicotine patch, such that it may act to enhance the actions of the nicotine patch without having to apply it as often or in higher doses.

In addition, a recent study reported that ADX71441, a novel GABA\textsubscript{B} receptor positive allosteric modulator, decreased alcohol self-administration preferentially in alcohol-dependent versus non-dependent rats and further blocked cue- and stress- induced relapse (Augier et al., 2017), which are a result of underlying associative processes with alcohol consumption. In comparison to BAC, ADX71441 achieved these effects in the absence of significant secondary non-specific effects. Given the similaries in the effects of BAC versus ADX71441 in alcohol-related behaviors, but the benefit of ADX71441’s lack of secondary non-specific effects, it would be interesting to see how ADX71441 influences the orosensory learning procedures we evaluated in this dissertation. If the secondary non-specific effects of BAC were interfering with its central effects on learning, use of ADX71441 may help to rule out this issue.

In conclusion, the results from our series of studies suggest that whereas muscarinic cholinergic receptor signaling mediates acquisition of preferences associated with sweet and fatty tastants, nicotinic cholinergic receptor signaling mediates acquisition of avoidances associated with bitter tastants, in addition to GABA\textsubscript{B} receptor signaling. These results expand on
previous literature supporting the roles of DA D1, D2 and NMDA receptor signaling in the acquisition of preferences associated with sweet and fatty tastants and the roles of DA D2, NMDA and opioid receptor signaling in the acquisition of avoidances associated with bitter tastants, ultimately lending support for theories proposing the interaction of these major neurotransmitter systems in the development of food-related learning behaviors. Interestingly, none of the antagonists administered significantly affected the expression of these preferences or avoidances, similar to more sparse effects for DA, glutamate and opioid systems, suggesting that the maintenance of these learned effects maybe be more isolated with respect to neurochemistry. Although these series of studies are limited in the extent that they examined pharmacological effects rather than anatomical effects, they do provide support for future studies in which anatomical substrates may be targeted. Much of the underlying processes of learning and memory remain unclear, but it can be thought that these processes are mediated by a distributed neural network (Figure 14), and the results of these series of studies add yet another piece to this puzzle.
References:


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