# THE ROLE OF ACETYLCHOLINE NEUROTRANSMISSION IN THE VENTRAL TEGMENTAL AREA ON FOOD REWARD AND FOOD-RELATED LEARNING

by

RUTH SHARF

A dissertation submitted to the Graduate Faculty in Psychology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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|---|---|
| _____ | Robert Ranaldi, Ph.D._____ |
| Date | Chair of Examining Committee |
| _____ | Joseph Glick, Ph.D._____ |
| Date | Executive Officer |

Dr. Robert Ranaldi_____

Dr. Richard Bodnar_____

Dr. Susan Croll_____

Supervisory Committee

THE CITY UNIVERSITY OF NEW YORK

Abstract

THE ROLE OF ACETYLCHOLINE NEUROTRANSMISSION IN THE VENTRAL TEGMENTAL AREA ON FOOD REWARD AND FOOD-RELATED LEARNING

by

Ruth Sharf

Advisor: Professor Robert Ranaldi

The following dissertation is an investigation of the role of acetylcholine neurotransmission in the ventral tegmental area (VTA) in food reward and food-related learning. The role of VTA acetylcholine in food-rewarded operant conditioning, or lever-pressing to obtain a food-reward, was investigated. Here, during training, animals learn about a novel food stimulus, environment, and motoric response. Rats were trained to lever-press under a fixed ratio schedule of food reinforcement. Bilateral intra-VTA scopolamine (a muscarinic acetylcholine receptor, or mAChR, antagonist) or mecamylamine (a nicotinic acetylcholine receptor, or nAChR, antagonist) microinjections were administered prior to and following training. All rats that received the mecamylamine doses, and those that received the scopolamine vehicle dose, demonstrated daily increases in lever pressing until asymptote levels were reached, at which point these maximal levels were maintained. Those rats that had initially received an active dose of scopolamine during training failed to show daily increases until scopolamine treatment was terminated. Scopolamine and mecamylamine administration following training failed to affect responding. These results suggest that mAChR, but not nAChR, are necessary for acquiring a food-rewarded lever-pressing task, but neither are necessary for the performance of the task. The role of VTA acetylcholine in feeding-

related learning was examined. Here, animals were presented with a novel food-stimulus in a novel environment in which they learn to feed. Here, scopolamine, but not mecamylamine, treatment prevented daily increases in pellet consumption and both compounds failed to reduce consumption after the feeding-task was acquired (i.e., after demonstration of stable responding). These results suggest that mAChR, but not nAChR, are necessary for the acquisition, but not the expression, of feeding-related learning. The role of acetylcholine neurotransmission in food-related motivation, or in the amount of work an animal will perform to obtain a food-reward, was investigated. Here, neither scopolamine nor mecamylamine significantly affected lever-pressing. These results suggest that neither mAChR nor nAChR stimulation is necessary for the motivation to obtain a food reward in fully trained animals. Altogether, these data suggest a functional role of VTA mAChR in the acquisition of food-related learning but not in the expression of previously acquired behaviors.

# FORWARD

Portions of this dissertation have been accepted for publication. Chapter 4 consists of text and data that has been accepted for publication by the journal *Psychopharmacology* in February of 2006. This manuscript was co-authored with Jennifer McKelvey and Dr. Robert Ranaldi. Chapter 5 consists of text and data that has been accepted for publication by the journal *Psychopharmacology* in October of 2005. This manuscript was co-authored with Dr. Robert Ranaldi. Chapter 6 contains data that was published along with those presented in chapter 4 in the journal *Psychopharmacology.*

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| | |
|---|---|
| ANOVA | . Analysis of Variance |
| CS | ...Conditioned Stimulus |
| DA | ..Dopamine |
| i.p | Intraperitoneal |
| LDT | Laterodorsal Tegmental Nucleus |
| LTP | .Long-Term Potentiation |
| mPFC | ..Medial Prefrontal Cortex |
| mAChR | .. Muscarinic Acetylcholine Receptors |
| nAChR | Nicotinic acetylcholine receptors |
| NAcc | .. Nucleus Accumbens |
| PPN | Pedunculopontine Tegmental Nucleus |
| SEM | Standard Error of the Mean |
| SN | .. Substantia Nigra |
| US | .. Unconditioned Stimulus |
| VTA | Ventral Tegmental Area |

## Chapter 1: General Introduction

Survival strongly depends on the ability of an organism to engage in behaviors that allow the organism to seek and consume biologically relevant stimuli, such as food and water. Consumption of such stimuli is accompanied by an activation of brain reward systems, resulting in the reinforcement of the behaviors that preceded the presentation of rewarding stimuli. Hence, through this reinforcement process the probability of the re-occurrence of these behaviors in the future increases. In addition to the formation of a stimulus-response association, the organism is concurrently engaged in motor learning as it acquires new motoric responses and in perceptual learning as it forms associations among environmental stimuli. Altogether, reinforcement and reward-related learning are adaptive mechanisms that increase an organism s ability to acquire and ingest life-sustaining stimuli. Similarly to naturally rewarding stimuli, many drugs of abuse, such as cocaine and amphetamine, tap into the natural reward circuitry and therefore drug-related behaviors are acquired and reinforced.

To date, much of the literature in this area has focused on establishing the neural mechanisms underlying drug- and/or natural-reinforcement and reward-related learning. Thus far, it appears that an explanation based simply on the formation of stimulus-response associations is insufficient to understand reward-related learning. In order for a reward-associated behavior to be repeated, an organism must learn about the probability that a rewarding stimulus will follow a behavioral response (contingency) and the time frame between a behavioral response and the presentation of a stimulus (contiguity). Equal in importance is the ability of the organism to engage in perceptual learning, in which the organism learns about the environment and/or environmental stimuli that must

be present while the behavior occurs. Additionally, for reward-directed behaviors to occur, and therefore be reinforced, the organism s motivational state must be such to allow an organism to engage in such behaviors. Once an organism experiences stimulus-response associations while in the appropriate motivational state, reward-related learning, in which associations are formed among relevant environmental stimuli and relevant behaviors, is likely to occur. Such learning enhances the likelihood that in the future, when an organism is in a similar motivational state and is in the presence of similar environmental stimuli, it will engage in appropriate goal-directed behaviors.

Although the neural mechanisms underlying reward-related learning are not fully understood, they appear to involve the activation of the mesocorticolimbic and/or the nigrostriatal DA pathways. In particular, dopamine (DA) neurotransmission along the mesocorticolimbic pathway has been implicated in reward-related learning (Wise and Rompré, 1989; Wise and Bozarth, 1987; Berridge, 1995; Wise, 2004) and is thought to mediate the incentive motivational effects of rewards and conditioned environmental stimuli (Berridge and Robinson, 1998; Ikemoto and Panksepp, 1999; Wise, 2004). Although the precise role of DA in reward-related learning remains unclear, it is becoming evident that other neurotransmitter systems along these pathways also play a role.

A plausible explanation opts for the possibility that reward-related learning depends on an interaction among various neurotransmitter systems, including DA, in mesencephalic and telencephalic regions. More specifically, it is suspected that there is an interaction among the DA, acetylcholine, and glutamate neurotransmitter systems in various brain regions including the medial prefrontal cortex (mPFC), the nucleus

accumbens (NAcc), the amygdala, and the ventral tegmental area (VTA). One hypothesis posits that Hebbian synapses (Hebb, 1949), in which synaptic strengthening occurs when input is received by an already active postsynaptic neuron (see below), play a primary role. It has been suggested that Hebbian synapses via *N*-methyl-*D*-aspartate (NMDA) glutamate receptors (Kelley et al., 1997; Smith-Roe et al., 1999; Baldwin et al., 2000) and/or acetylcholine muscarinic receptors (Steriade et al., 1990; Winn et al., 1997; Miller et al., 2002) are involved in strengthening the efficacy of synapses on DA cells.

The purpose of this dissertation research is to examine the role of acetylcholine neurotransmission in the VTA in food reward and food-related learning. The following is a review of the literature regarding DA s role in reward and reward-related learning and the involvement of acetylcholine in activating DA neurons and, thus, the cholinergic contribution to reward-related learning. But first, the Hebbian synapse is reviewed as a general model that may account for synaptic plasticity as a result of the acetylcholine-dopamine interaction.

The Hebbian Synapse

Hebb (1949) provided a hypothetical model depicting the possible mechanisms of synaptic modifications that underlie synaptic plasticity in the brain. According to the Hebb rule, the effectiveness of an excitatory synapse is increased if the postsynaptic neuron is active at the same time that input to the synapse is received. That is, a weak synaptic connection may be strengthened if a strong synaptic connection is activated simultaneously. The Hebb rule has been demonstrated empirically to contribute to modifications in synaptic efficacy in slices of the hippocampus. In these experimental manipulations, a short series of high frequency electric pulses is applied to either the

Schaffer collateral pathway (whose axons project from the CA3 region of the hippocampus to the CA1 region) or the perforant pathway (whose axons arise primarily from the entorhinal cortex and project to various areas of the hippocampus). As a result of the brief electric stimulation, the synapses in CA1 and CA3, respectively, became strengthened, or potentiated, so that these synapses were now active for a longer period of time (Lomo, 1966; Bramham, 1992; Johnston et al., 1992; Bliss and Collingridge, 1993; Nicoll and Malenka, 1995). This long-term enhancement in synaptic efficacy following repeated stimulation of a synapse has been termed long term potentiation (LTP) (Bliss and Lomo, 1973). Subsequently, LTP has been found to occur in various regions throughout the brain.

The physiological mechanisms responsible for potentiation of synapses involve activation of the glutamate *N*-methyl-*D*-aspartate (NMDA) receptor. A unique feature of the NMDA receptor is that its ion channel is gated by a $Mg^{2+}$ block in a voltage-dependent manner (Nowak et al., 1984). Thus, activation of the NMDA receptor depends on a depolarization of the postsynaptic membrane via stimulation of non-NMDA glutamate receptors, such as AMPA receptors, in order to remove of the $Mg^{2+}$ block (Herron et al., 1986). Furthermore, NMDA receptors are highly permeable to $Ca^{2+}$ (MacDermott et al., 1986), and once activated, $Ca^{2+}$ initiates a cascade of intracellular events that result in the induction of LTP. Due to these unique properties, the NMDA receptor is considered to be a coincidence detector for concurrent activation of pre- and postsynaptic receptors (Herron et al., 1986). That is, activation of the NMDA receptors depends on the coincident activity of two incoming signals within a limited time frame.

Two forms of LTP have been classified: The Hebbian, or associative, LTP requires coincident activation of pre- and postsynaptic neurons, whereas non-Hebbian, or non-associative, LTP requires only activation of a pre- or a postsynaptic neuron (Hebb, 1949; McNaughton and Barnes, 1990; Jaffe and Johnston, 1990; Derrick and Martinez, 1994a). Interestingly, Hebbian LTP appears to be NMDA-dependent (Grover and Teyler, 1992; Johnston et al., 1992), whereas non-Hebbian LTP does not require activation of NMDA receptors (Harris and Cotman, 1986).

The concept of the Hebbian synapse, or Hebbian LTP, has been beneficial in accounting for neural modifications that accompany learning (Berger, 1984; Lynch and Baudry, 1984; Bliss and Collingridge, 1993). For example, classical conditioning, in which a conditioned stimulus (CS) acquires the ability to elicit a response similar to one elicited by an unconditioned stimulus (US), has been explained in terms of the Hebb rule. According to this model, a US forms strong synaptic connections with motor neurons, and hence its presentation results in an unconditioned response (UR). Unlike a US, prior to conditioning, neutral stimuli form weak synaptic connections with motor neurons, and hence their presentation fails to elicit a response. However, after repeated pairing of a US with a neutral stimulus, the neutral stimulus becomes a conditioned stimulus (CS) capable of eliciting a similar, conditioned response (CR). During the pairing, it is presumed that the weak synapse between the CS and the motor neurons is active at the same time as the strong synapse from the US is active. This co-activation leads to a Hebbian strengthening of the weak synapse - enhancing the signal from the CS so that its presentation in the future will be able to produce a response. A classic demonstration of the importance of Hebbian synapses in classical conditioning comes from demonstrations

that the gill withdrawal reflex of *Aplysia* can be classically conditioned by pairing a light tactile stimulus to the siphon (CS) with a strong electric shock to the tail (US) (Carew et al., 1981). Prior to training, presentation of a shock to the tail results in serotonin release onto sensory neurons. During training, when sensory neurons are activated simultaneously by both the tactile stimulation and the shock, the presence of serotonin results in intracellular changes that enhance activation of the gill withdrawal reflex in the presence of subsequent CSs (Hawkins et al., 1983; Walters and Byrne, 1983).

Similarly, reward-related learning is presumed to involve Hebbian synapses along various components of the reward-circuitry in a similar manner. The signal provided by natural rewards, or the US signal, is thought to be co-activated with signals provided by neutral environmental stimuli. This concurrent activation leads to the strengthening of the synaptic inputs provided by environmental signals and over time, these environmental signals are capable of guiding behavior independently of primary rewards. Due to the role of DA in reward-related behaviors, it is hypothesized that learning-associated synaptic modifications occur along the DA pathways.

Behavioral Measures of Reward

There are many applications of the term reward, including its delineation of a reinforcer, which is defined as a stimulus that increases the frequency of any behavior that precedes its presentation. However, reward has also been defined in terms of the hedonic, or pleasurable, effects of a stimulus (Cannon and Bseikri, 2004) and in terms of the environmental incentives that are directly related to the rewarding stimulus (Wise, 2002). The term reward can be used as a noun (e.g., a stimulus, such as a food pellet), as a verb (e.g., a reinforcer), an adverb (e.g., a reinforcing stimulus), or an adjective (e.g.,

a rewarding stimulus). Thus, the term reward connotes more than reinforcement and studies measuring reward, and brain mechanisms underlying reward, typically seek to investigate its role in its entirety rather than simply its ability to increase behavior. The term reward will be used throughout this dissertation, rather than the term reinforcement, as it refers to both stimuli with motivational effects as well as to the behavioral modifications that accompany the presentation of such stimuli.

Most behavioral, and other, measures of reward assess the hedonic, or rewarding, effects of stimuli indirectly. For instance, most measures rely on the assumption that reward relevant behaviors (such as lever-pressing to obtain a reward stimulus) are directly related to the rewarding value of the stimulus and that changes in their performance levels reflect changes in the reward value of the stimulus. In a typical operant conditioning experiment, a schedule of reinforcement is applied to assess reward. For instance, in a fixed ratio schedule of reinforcement, the rewarding stimulus is presented following a pre-specified number of responses, whereas in a progressive ratio schedule of reinforcement, the response requirements to obtain successive stimuli are progressively increased so that the work requirement eventually becomes so great that the animal ceases to respond. Using various schedules of reinforcement, classic behavioral studies have demonstrated that the rate of responding is directly related to the size (Baron and Herpolsheimer, 1999; Crossman, 1968) and the magnitude (Perone and Courtney, 1992) of the rewarding stimulus.

In order to assess brain mechanisms involved in reward, animals are first trained to lever-press under a particular schedule of reinforcement and then pharmacological manipulations, via injections of agonist and antagonist compounds, are made. Changes

in behavior as a result of psychopharmacological agents are presumed to represent changes in the reward process. However, interpretation of results is problematic as pharmacological agents may alter non-reward related factors, such as general level of arousal or motor capacity, which may affect lever-pressing rates (Roll, 1970; Miliaressis et al., 1986). Therefore, other techniques are often used either in conjunction or independently to assess reward. The following is an overview of several of the designs that are cited throughout this dissertation.

The conditioned place preference (CPP) test has become a popular paradigm for the measure of reward-related behavior. An animal, usually a rodent, is placed in an enclosed environment, which is divided into two or more distinct compartments. Each compartment contains distinctive environmental cues that differentiate it from the other(s). After the animal is allowed to move freely among the compartments, it is confined to one of the compartments and is presented with a rewarding stimulus. The animal is assumed to develop an association between the rewarding stimulus (or the US) and the environmental cues (or the CS) during this time. After the pairing, when allowed to roam freely among the compartments, the animal will spend more time in the reward-paired chamber if the US was rewarding. However, drug-induced motoric or other deficits remain problematic for proper interpretation. Garcia et al. (1957), the first to use the conditioned place preference paradigm, demonstrated that following exposure to ionizing ration, animals display spatial aversion to contextual cues present in that environment.

The conditioned reward paradigm combines operant and Pavlovian conditioning to assess reward. In such paradigms, a US is paired with some environmental cue (CS),

such as a tone or a light stimulus. It is assumed that once pairing has occurred, the CS adopts rewarding effects and acquires the ability to elicit behaviors independently of the primary reward. As the CS adopts rewarding effects, it becomes a conditioned reward. Following pairing, the animal is presented with an opportunity to perform an operant response (such as a lever pressing task) that will provide the CS, and generally this operant is maintained by the conditioned reward. The conditioned reward paradigm attempts to resolve the motor problem by presenting animals with two levers, one of which provides the CS while the other produces nothing or a neutral stimulus. When pharmacological manipulations selectively affect responding on the CS-producing lever, and not on the other, it is concluded that changes in performance reflect changes in the rewarding value of the CS. In one of the first conditioned reward experiments, Skinner (1938), by pairing the sound of a feeder clicker with food, trained animals to press a lever to obtain the feeder clicker.

Measures of reward-related learning utilize the same behavioral paradigms, such as conditioned place preference and conditioned reward, but rather than making pharmacological manipulation during the performance of a task (e.g., after an organism exhibits stable responding), these agents are applied as an animal acquires the behavioral response (e.g., during training sessions). If performance differs as a result of psychopharmacological manipulations, it is assumed that the drugs interfere with the acquisition of the response. This methodology of testing before and after training can resolve the motor problem in the event that pharmacological manipulations alter behavioral responding only during training but not after, and vice versa.

Dopamine s mediation of reward

To date, the majority of the literature examining neurotransmitter mechanisms involved in reinforcement and reward-related learning has focused on DA neurotransmission in several brain regions. Three main pathways have been explored: The mesolimbic DA pathway, which originates in the ventral tegmental area (VTA) and terminates in limbic structures such as the nucleus accumbens (NAcc) and amygdala; the mesocortical DA pathway, which originates in the VTA and terminates in cortical structures, such as the medial prefrontal cortex (mPFC); and the nigrostriatal pathway, which originates in the substantia nigra (SN) and terminates in the neostriatum (Ungerstedt, 1971; Lindvall and Bjorklund, 1974; Fallon and Moore, 1978). Generally, DA concentrations in terminal regions of these pathways are maintained at a tonic basal level (Finlay et al., 1995; Westerink, 1995; Wilson et al., 1995); however, upon presentation of behaviorally-relevant stimuli, phasic increases occur (Finlay et al., 1995; Westerink, 1995; Wilson et al., 1995; Bassareo and Di Chiara, 1997; Rebec et al., 1997) due to burst firing of DA neurons (Strecklet and Jacobs, 1987; Schultz et al., 1997). The rewarding effects of natural rewards, such as food and water, and of drugs of abuse, such as cocaine, are frequently attributed to dopaminergic function (Wise, 1978; Beninger, 1983) in one or more of these pathways.

The role of DA in reward has been established by studies demonstrating that rewards elevate extracellular DA concentrations in mesocorticolimbic and mesostriatal systems (Hernandez and Hoebel, 1988; Bradberry and Roth, 1989; Pettit and Justice, Jr., 1989; Chen and Reith, 1994; Ranaldi et al., 1999) and DA neurotransmission in these pathways appears critical for their rewarding effects. Studies have shown the importance of DA for cocaine and food reward by demonstrating that blockade of DA receptors

results in attenuation of cocaine- or food-maintained operant responding (de Wit and Wise, 1977; Wise et al., 1978). The pattern of responding following blockade of DA receptors resembles the pattern typically seen in extinction (Fouriezos and Wise, 1976, Rolls et al., 1974, Wise et al., 1978), in which there is a gradual and progressive decline in responding, suggesting that DA antagonism acts to attenuate the reward associated with the stimuli, rather than by producing motoric deficits that prevent the animal from carrying-out the behavioral response. Subsequently, DA neurotransmission has been repeatedly implicated in the reinforcing effects of various rewarding stimuli, such as drugs of abuse (Koob, 1992; Wise, 1996; Woolverton and Johnson, 1992), food (de Wit and Wise, 1977; Wise et al., 1978), electrical brain stimulation (Bardo, 1998; Wise and Rompré, 1989), sexual contact (Blackburn et al., 1992), and conditioned rewards (Beninger and Ranaldi, 1994).

More specifically, DA neurotransmission in the terminal regions of the mesocorticolimbic DA pathway, such as the Nacc and mPFC, is implicated in reward-related behavior (Wise and Bozarth, 1987; Wise and Rompré, 1989; Berridge, 1995; Bardo, 1998). For instance, administration of cocaine is accompanied by increases in DA transmission in the NAcc due to a blockade of the dopamine re-uptake transporter (Ritz et al., 1987), and this increase appears to be directly related to its reinforcing effect. Similarly, feeding or lever pressing for food is associated with increased DA release in the NAcc (Joseph and Hodges, 1990; Joseph et al., 1989). The important role of the NAcc in cocaine reward was established by studies in which NAcc DA terminals (Roberts et al., 1977), NAcc intrinsic neurons (Zito et al., 1985), or the cells of origin of the mesolimbic DA system (Roberts and Koob, 1982) were lesioned with neurotoxins

resulting in decreased cocaine self-administration. Evidence for the role of DA projections from the VTA to the NAcc in food reward comes from studies showing that lesions of DA terminals disrupt food-rewarded operant responding (Salamone et al., 1991). Furthermore, electrophysiological studies reveal activation of NAcc neurons in monkeys lever pressing for juice (Bowman et al., 1996; Schultz et al., 1997; Hollerman et al., 1998) and in rats lever pressing for water (Carelli and Deadwyler, 1994). Subsequent supporting evidence comes from microdialysis studies showing that DA levels in the NAcc are elevated by self-administered cocaine (Pettit and Justice, Jr., 1989) and lever-pressing for food (Hernandez and Hoebel, 1988).

Not only is mesocorticolimbic DA implicated in primary reward, it is also implicated in the motivational effects of reward-associated conditioned stimuli. Environmental stimuli that are associated with primary rewards, such as cocaine or food, can themselves become rewarding stimuli, or conditioned rewards. Presentation of conditioned rewards is associated with increased VTA DA cell firing (Schultz, 1997) and release of DA in the NAcc (Blackburn and Phillips, 1989; Gratton and Wise, 1994). Pharmacological manipulations of DA neurotransmission alter responding maintained by conditioned rewards, suggesting that the increases in cell firing and DA release are functionally related to the reinforcing effects of conditioned rewards. For instance, amphetamine, an indirect DA agonist, enhances responding for conditioned rewards (Robbins et al., 1983; Mazurski and Beninger, 1986; Beninger and Ranaldi, 1992). This amphetamine-induced enhancement of responding maintained by a conditioned reward is reduced following injections of SCH 23390, a DA D1 antagonist (Chu and Kelley, 1992). Additionally, DA infused directly into the NAcc of rats enhances responding for

conditioned rewards (Cador et al., 1991). A functional relation between conditioned stimuli and mesocorticolimbic DA is suggested by studies showing that activation of mesolimbic DA reinstates extinguished reward-related responding (Stewart, 1984; Ranaldi et al., 1999) and that increases in mesocorticolimbic DA occur immediately prior to reward-related lever presses (Gratton and Wise, 1994; Kiyatkin and Gratton, 1994; Richardson and Gratton, 1996). Thus, mesocorticolimbic DA neurons appear to come under the control of reward-associated stimuli.

Although the literature has focused primarily on DA neurotransmission in the NAcc, it is becoming more evident that other brain regions along the mesocorticolimbic and nigrostrial pathways are also involved in reward. For instance, reductions in DA neurotransmission in the mPFC, VTA, striatum or SN reduce responding maintained by food (Beninger et al., 1993; Beninger and Ranaldi, 1993; Aberman et al., 1998; Smith-Roe and Kelley, 2000; Baldwin et al., 2002; Sharf et al., 2005), cocaine (McGregor and Roberts, 1993; Ranaldi and Wise, 2001; Quinlan et al., 2004) or VTA brain stimulation (Kurumiya and Nakajima, 1988). Voltammetry studies implicate DA neurotransmission in both the NAcc and neostriatum in food reward as DA or DA metabolites increase in both regions following feeding or lever pressing for food (Blackburn et al., 1986; Church et al., 1986; Heffner et al., 1980). Lesions of amygdala DA terminals reduce responding maintained by conditioned reward (Cador et al., 1989) and injections of DA antagonists into the amygdala or mPFC reduce the rewarding effectiveness of cocaine (McGregor and Roberts, 1995).

Furthermore, DA release is not limited to dopamine terminals, and the actions of DA are not limited to inhibition of terminal regions. DA can also be dendritically

released (Cheramy et al., 1981; Nissbrandt et al., 1989), and thus may modulate dopaminergic cell activity and DA-related behaviors. Previous studies have demonstrated a functional role of dendritically-released DA in reward. For instance, Ranaldi and Wise (2001) demonstrated that dendritically released DA at the level of the VTA plays a significant role in intravenous cocaine reward; intra-VTA microinjections of a D1 antagonist decreased cocaine self-administration in a dose-related manner. Subsequently, it was found that dendritic DA release in the SN also contributes to intravenous cocaine reward (Quinlan et al., 2004), and dendritic DA release in the VTA contributes to food reward (Sharf et al., 2005).

Theories of the role of dopamine in reward-related learning

Although the precise role of DA in reward-related learning is controversial, it is clear that organisms are capable of learning to make operant responses to obtain rewarding stimuli, such as food, water, drugs of abuse, and electrical brain stimulation, which activate one or more of the DA pathways. Many theories have been proposed to address the question of how DA plays a role in such learning. The anhedonia hypothesis suggests that DA mediates the hedonic or pleasurable effects of rewards (Wise et al., 1978; Wise, 1978). Such viewpoints attest that reductions in reward-related behaviors following blockade of DA receptors are reflective of blunted rewarding effects of reward stimuli. However, such an approach may be insufficient to explain the rewarding effects of electrical brain stimulation or psychostimulants (Robinson and Berridge, 1993), which may be better understood in terms of an approach process, in which DA attributes incentive salience to reward-related stimuli (Berridge, 1995; Berridge and Robinson, 1998), rather than a consummatory process. Studies in which blockade of DA receptors

disrupts operant responses for food and water, but does not affect the consumption of these stimuli (Blackburn et al., 1987, Fibiger et al., 1976; Ljunberg, 1987; Rolls et al., 1974), support the theory that DA mediates incentive-motivation.

One of the primary components of reward-related learning is the capability of rewarding stimuli to alter the ability of reward-associated stimuli to elicit approach or consummatory behaviors in future events (Bolles, 1972; Bindra, 1974; Beninger, 1983). Incentive motivation learning refers to the ability of stimuli that have been previously associated with a reward to acquire motivational effects; therefore, previously neutral stimuli become reward-signaling stimuli that are capable of independently eliciting behavior (Beninger, 1983). A prime example of the incentive motivational effects of reward-associated stimuli has been demonstrated by a study by Pickens and Harris (1968), in which the strength of association between environmental cues in a runway alley and a primary reward determined the speed at which an animal will run. Thus, environmental cues serve as incentive motivational stimuli that are learned predictors of reward (Wise, 2004). Similarly, incentive motivational stimuli have acquired the ability to act as rewarding stimuli, or as conditioned rewards. Conditioned rewards, like primary rewards, are capable of altering the ability of stimuli that are associated with their presentation to elicit behaviors in future events. The incentive motivational hypothesis of dopamine suggests that dopamine is essential for the reinforcement of, and in the establishment of, incentive stimuli during initial conditioning. Furthermore, DA acts to maintain these incentive motivational stimuli through repeated reinforcement.

Other theories propose that DA functions to reinforce stimulus-response associations (Beninger, 1983; White and Milner, 1992; Kelley, 1999). Support for this

theory includes findings that show that animals treated with DA antagonists do not learn to press a lever for rewards, such as food (Wise et al., 1978), water (Gorber et al., 1981), and cocaine (de Wit and Wise, 1977), and if the DA antagonists are administered after the animals have already learned the task, operant responding is not maintained following antagonism (Wise et al., 1978; McFarland and Ettenberg, 1995; Dickinson et al., 2000; Wise et al., 1978). Similarly, DA blockade prevents the formation of preferences in a conditioned place preference paradigm for environments associated with reward (Spyraki et al., 1982; Bozarth and Wise, 1981; Spyraki et al., 1987). Altogether these findings demonstrate a role for DA in positive reinforcement; DA release is associated with more than a mere stamping-in of these stimulus-response associations.

The motor theory posits that the DA system activates sensory-motor connections (Koob, 1982; Salamone and Correa, 2002) and DA acts to stamp-in motoric responses. The GABAergic medium spiny neurons of the NAcc project to the ventral pallidum, the SN, and the VTA (Heimer et al., 1991; Nauta and Domesick, 1984), regions that modulate motoric output. Therefore, a fully functioning DA system is necessary for an organism to engage in an appropriate series of motoric outputs necessary to obtain a rewarding stimulus. This theory is supported by findings in which responding for reward is attenuated following DA receptor blockade (Rolls et al., 1974; Fibiger et al., 1976). That is, these data can support the motoric hypothesis by interpreting behavioral reductions as a result of motoric impairments. However, blockade of DA does not prevent an animal from initiating or reinstating a normal response, rather it reduces the probability that a particular response will be maintained (Fouriezos et al., 1976; Fouriezos and Wise, 1976; Wise et al., 1978), arguing against the explanation that all

deficits are purely motoric in nature. Nonetheless, it would be inaccurate to conclude that DA does not play any role in reward-related motor behavior. It has been argued that DA elicits goal-directed approach behaviors (Wise and Bozarth, 1987; White and Milner, 1992).

Regardless of the discrepancy among the various theories, all suggest that DA plays a role in learning; however, the precise role(s) remains unknown. One commonality shared by these theories is that DA plays some role in the *incentive motivation* for rewarding stimuli. It does so by establishing conditioned incentive motivational stimuli, eliciting goal-directed behaviors, and allowing performance of behaviors that enable an organism to come in contact with rewarding stimuli.

Dopamine s contribution to reward-related learning

DA is involved in reward-related learning. Most likely, it does so through an intricate neural network that involves activation of DA cells along one or more of the dopaminergic pathways (Lindvall et al., 1978; Beckstead et al., 1979). It has been shown that prior to operant or classical conditioning training, DA cells fire in response to unexpected and novel stimuli. During the initial phases of the training process, in which an animal learns that some environmental stimuli serve as reward-predicting cues, DA cells appear to respond to both environmental cues and to reward (Ljungberg et al., 1992; Schultz et al., 1993; Mirenowicz and Schultz, 1994; Fiorillo et al., 2003; Takikawa et al., 2004). Following training, the same DA cells now respond primarily to environmental reward-predicting sensory cues (Miller et al., 1981; Kosobud et al., 1994; Kiyatkin and Rebec, 2001; Hyland et al., 2002) rather than to the predicted reward, and in highly trained animals, DA cell responses to reward were largely diminished (Ljungberg et al.,

1992). Recently, it was discovered that cue- induced DA cell firing is contingent upon the association among the environmental cues and the reward, and the ability of cues to increase cell firing develops during the acquisition of classically conditioned behavior (Pan et al., 2005). Altogether, these results suggest that DA, by concurrently signaling the rewarding stimulus and reward-predicting stimuli during training, plays a role in stamping-in associations among these stimuli, and once these associations have been formed, DA acts to signal the salience of environmental cues more so than to signal the primary reward (Robinson and Berridge, 1993; Schultz et al., 1993; Berridge and Robinson, 1998; Horvitz, 2000).

Further supporting evidence of the role of DA in reward-related learning is provided by studies demonstrating that DA neurotransmission in the NAcc and mPFC is essential for the acquisition of operant behaviors (Sawaguchi and Goldman-Rakic, 1991; Salamone, 1994; Beninger and Miller, 1998; Smith-Roe and Kelley, 2000; Baldwin et al., 2002). Presentations of primary rewards during initial operant- or classical- conditioning training sessions increase DA in the NAcc and mPFC (Richardson and Gratton, 1996; Izaki et al., 1998). Furthermore, presentation of a novel food is associated with increased DA concentrations in the mPFC, while presentation of the same food following its habituation did not produce increases in mPFC DA levels, and resulted in smaller increases in the NAcc (Bassareo and Di Chiara, 1997). Behavioral evidence comes from studies demonstrating that blockade of NAcc (Smith-Roe and Kelley, 2000) or mPFC (Baldwin et al., 2002) DA neurotransmission at D1 receptors, in conjunction with blockade of glutamate neurotransmission at NMDA receptors in these same regions, attenuates the rate of acquisition of a food-rewarded operant response.

It has been suggested that DA action at D1 receptors is responsible for the synaptic plasticity that accompanies instrumental learning. For instance, blockade of dopamine D1 receptors in the mPFC attenuates learning of an operant response (Baldwin et al., 2002). Stimulation of D1 receptors is associated with stimulation of cyclic-AMP (Kebabian and Calne, 1979), which acts on cyclic-AMP dependent protein kinases to phosphorylate other proteins responsible for synaptic modifications.

Similarly, DA has also been implicated in classical conditioning, in which associations between reward-associated stimuli and reward lead to the formation of conditioned rewards. The acquisition of conditioned rewards, and of responding maintained by conditioned rewards, has also been attributed to DA signals at D1 and D2 DA receptors (Beninger, 1983; Miller et al., 1990; Beninger, 1991; Beninger and Ranaldi, 1992). The blockade of D2 receptors, via treatment with pimozide, during training in which a food reward is paired with a lights-off stimulus, prevented the conditioned stimulus from acting as such when the animals were later presented with an opportunity to lever-press for the conditioned stimulus (Beninger and Phillips, 1980; Hoffman and Beninger, 1985). Furthermore, blockade of both D1 and D2 receptors, via treatment with *cis*-flupenthixol, following US-CS paring, prevented the acquisition of responding for conditioned rewards (Beninger and Phillips, 1980; Robbins et al., 1983). Altogether, these findings suggested that an intact DA system is necessary for a reward-paired stimulus to adopt rewarding effects and for the acquisition of new behaviors maintained by the conditioned reward (Beninger and Ranaldi, 1994).

Altogether, previous studies suggest that intact DA neurotransmission is critical for the acquisition of both instrumental and classical conditioning and the establishment

of conditioned rewards. Furthermore, intact DA neurotransmission is necessary for conditioned rewards to act as such.

Glutamate s contribution to reward-related learning

Glutamate transmission via NMDA receptors has long been acknowledged in drug-related learning. For instance, the role of glutamate in reward-related learning has been demonstrated by studies in which blockade of NMDA receptors via infusions of MK-801 disrupted the *acquisition* of cocaine self-administration (Schenk et al., 1993a), an operant response assumed to require associative, stimulus-response, learning. Moreover, systemic glutamate antagonists block both the acquisition (Tzschentke and Schmidt, 1995; Kim et al., 1996) and expression (Tzschentke and Schmidt, 1997) of morphine conditioned place preference and blockade of NMDA receptors in the VTA and NAcc prevents the expression of morphine conditioned place preference (Popik and Kolasiewicz, 1999). Altogether, these findings suggest that glutamate neurotransmission is involved in the acquisition and expression of stimulus-controlled reward-associations.

It has long been suggested that glutamate-induced plasticity may be responsible for reward-related learning. Activation of NMDA receptors has been associated with the induction of LTP, which is the primary component underlying synaptic plasticity. LTP is defined as a long-lasting strengthening of synaptic efficacy following repeated stimulation of a synapse. That is, activation of NMDA receptors is the primary component of associative learning - NMDA receptors act as coincidence detectors that signal the convergent activity of two independent synaptic inputs and thereby they strengthen a weaker synapse. The synaptic connections that are strengthened are most likely those that lie along the trajectories of the mesocorticolimbic or nigrostriatal

pathways. The glutamatergic inputs to these pathways originate in various cortical and subcortical regions and are presumed to convey sensory information about conditioned environmental stimuli (Schultz, 1998; Bespalov et al., 2000). There is evidence that activation of NMDA receptors in the mPFC and NAcc is critical for the acquisition of various types of associative learning. For instance, blockade of NMDA receptors in these regions impairs the acquisition, but not the expression, of operant learning (Kelley et al., 1997; Smith-Roe et al., 1999; Baldwin et al., 2000).

Previous studies suggest that the glutamatergic activation within the VTA is associated with the induction of LTP in the DA neurons. LTP appears to occur at excitatory synapses in the VTA (Bonci and Malenka, 1999; Overton et al., 1999; Mansvelder and McGehee, 2000) and may be influenced by DA neurotransmission (Wolf et al., 2004). The VTA appears to be a critical site for synaptic modifications involved in the conditioning of environmental stimuli to drug rewards. For instance, Harris et al. (2004) have shown that intra-VTA injections of AMPA and NMDA receptor antagonists block the acquisition of conditioned place preference to morphine and cocaine, suggesting that the VTA may play a critical role in the integration of information, allowing environmental stimuli to become associated with rewarding stimuli.

The VTA receives inputs from glutamate afferents that synapse directly onto the DA cells (Beninger, 1983, 1991, 1993; Wickens, 1990) from the mPFC (Sesack and Pickel, 1992; Smith et al., 1996), the amygdala and the bed nucleus of the stria terminalis (Hopkins and Holstege, 1978; Pillipson, 1979) and the pedunculopontine nucleus (Charara et al., 1996). These afferents terminate on the DA cells of the VTA and activate NMDA, non-NMDA, and metabotropic glutamate receptors (mGluR), which excite these

cells (Robinson and Camp, 1990; Iversen, 1993; Waters et al., 1993). DA cell firing appears to be dependent in part on some glutamatergic activation. For instance, blockade of glutamate receptors suppress activity of DA cells (Grenhoff et al., 1988; Charlety et al., 1991; Overton and Clark, 1992; Chergui et al., 1993). However, anatomical and electrophysiological studies have revealed that glutamatergic afferents, especially those originating in the mPFC, form monosynaptic connections with both DA- and GABA-containing neurons (Sesack and Pickel, 1992; Tong et al., 1998), suggesting a complicated circuitry in which glutamate projections can modulate the activity of DA neurons directly and indirectly.

Glutamate s contribution to reward and reward-related learning may in fact be mediated via dopaminergic actions in the VTA. The VTA has a dense concentration of DA D1 receptors on glutamate and GABA feedback afferents (Lu et al., 1997) originating in the forebrain. The effect of DA at D1 receptor sites is to facilitate the release of GABA and glutamate (Cameron and Williams, 1993; Kalivas and Duffy, 1995; Starr, 1987), which in turn modulate dopaminergic and GABAergic neurons in the VTA (Carr and Sesack, 2000). Therefore, DA released dendritically in the VTA acts on the D1 receptors located on these afferent terminals to modulate GABA and glutamate release which in turn modulates dopaminergic and GABA output cell activity. The behavioral functions of forebrain DA and GABA are, therefore, determined by the effects of a complex interaction between a short-loop feedback system - the dendritic DA GABA glutamate interactions and a long-loop feedback system the cortico-mesencephalic glutamate and striato-mesencephalic GABA afferents on the activity of DA and GABA output cells themselves.

Hypothesized model of food-related learning

Thus far, the model proposed offers that during food-related learning, an intact DA signal along the trajectory of mesocorticolimbic pathway is necessary for animals to experience food reward and to attribute incentive-motivational properties to environmental stimuli. Following learning, these environmental stimuli can act as reward-predicting cues that have adopted both rewarding effects and the ability to elicit goal-directed behaviors. The neural changes that allow for incentive motivational cues to act as such are presumed to undergo synaptic plasticity as repeated synaptic co-activity enhances the ability of environmental stimuli to activate DA cells. The precise mechanisms that induce these synaptic changes remain unclear.

As mentioned above, the environmental reward-predicting signal is presumed to originate in the PFC, which has been shown to excite DA cells in the VTA. However, for environmental signals to successfully activate DA cells, synaptic strengthening must occur by co-activation of environmental signals (CSs) and reward signals (US). The signal that is concurrently activated (or the US signal) remains to be discovered. One possibility is that cholinergic inputs onto the VTA are necessary for synaptic strengthening of glutamatergic inputs.

Acetylcholine receptors

Thus far, 5 different subtypes of muscarinic acetylcholine receptors (mAChR) (M1-M5) (Nadler et al., 1999) and 17 different subunits of the nicotinic acetylcholine receptors (nAChR) (Picciotto et al., 2000) have been discovered. The mAChR are metabotropic receptors that are coupled to GTP-binding proteins (G-proteins) and their activation leads to activation of second messenger pathways (Bonner, 1992). The M1-

type receptors (M1, M3, and M5) are positively coupled to phospholipase C; thereby activation of these receptors catalyzes the production of inositol triphosphate ($IP_3$) and diacylglycerol, which result in the release of calcium from intracellular stores and activation of protein kinase C, respectively. Activation of the M2-type receptors (M2 and M4) results in the inhibition of adenylyl cyclase and calcium channels (Caulfield, 1993). Unlike the metabotropic mAChR, the nAChR are ionotropic and surround an ion channel (Wagner et al., 1991).

Both nAChR and mAChR are localized near DA cells in the VTA and SN, and mRNA for these receptors is lost following lesions of DA cells via 6-hydroxydopamine (Clarke and Pert, 1985; Vilaro et al., 1990; Weiner et al., 1990), suggesting that these receptors are localized on the DA cells. However, the only muscarinic receptor subtype localized in the vicinity of DA cells of the VTA and SN is the M5 subtype as the M5 mRNA is the only mAChR subtype marker to be localized on the DA cell bodies (Vilaro et al., 1990; Weiner et al., 1990; Reever et al., 1997). The M5 receptor is the last receptor to have been discovered and no high-affinity ligands or toxins for this receptor have been identified as of yet (Flynn et al., 1997). Therefore, unlike the other muscarinic receptors, the M5 receptor has yet to undergo pharmacological characterization.

Acetylcholine excitation of dopamine cells

The VTA, the origin of both the mesocortical and the mesolimbic systems, and the SN, the origin of the nigrostriatal system, are heavily innervated by ACh-secreting neurons originating in the mesopontine tegmentum, which has been divided into two relatively homogeneous regions: the pedunculopontine tegmental (PPN) and the laterodorsal tegmental (LDT) nuclei. Both regions appear to be morphologically and

physiologically similar (Satoh et al., 1983; el Mansari et al., 1989; Steriade et al., 1990b; Clements et al., 1991; Kayama et al., 1992; Semba and Fibiger, 1992; Ford et al., 1995; Datta and Siwek, 2002). Innervation from the PPN and LDT is the only known cholinergic input to SN (A9) and VTA (A10) dopamine neurons (Woolf, 1991). Innervation of these midbrain regions by the mesopontine tegmentum appears to be topographically organized the LDT and caudal PPN innervate mainly the VTA, whereas the rostral regions of the PPN innervate the substantia nigra (SN) (Oakman et al., 1995). These cholinergic neurons form monosynaptic, excitatory connections with DA neurons (Oakman et al., 1995; Clarke and Pert, 1985; Smith and Bolam, 1991; Lacey et al., 1990). It appears that cholinergic PPN neurons that project to the VTA can stimulate DA neurons (Blaha et al., 1996), and enhance mesostriatal dopamine transmission (Lokwan et al., 1999; Blaha and Winn, 1993). Similarly, there is an excitatory cholinergic LDT innervation of the VTA DA cells (Omelchenko and Sesack, 2005). In accordance with the topographical arrangement, the elevation in extracellular DA in the NAcc is selectively attenuated by excitotoxic lesions of the LDT, but not the PPN (Blaha et al., 1996) whereas the increase in DA in the neostriatum is selectively attenuated by excitotoxic lesions of the PPN (Blaha and Winn, 1993).

In addition, stimulation of the muscarinic M2 autoreceptors on LDT and PPN cell bodies also increases DA neurotransmission (Vilaro et al., 1994). In situ hybridization studies demonstrate that M2 mRNA outweighs M3 and M4 receptors in PPT and LDT neurons, while M1 and M5 receptors are nonexistent in these regions (Levey, 1993; Curro Dossi et al., 1991). Blockade of the M2 autoreceptors enhances the activity of the cholinergic-secreting neurons, resulting in increased DA release in terminal regions

(Chapman et al., 1997). On the other hand, activation of M2 receptors hyperpolarizes these PPN and LDT cholinergic neurons (Luebke et al., 1993; Leonard and Llinas, 1994). Furthermore, electrical stimulation of the mesopontine cholinergic neurons leads to burst-firing and depolarization of DA cells (Kelland et al., 1993; Futami et al., 1995; Lokwan et al., 1999).

The most prominent excitatory inputs from the LDT synapse specifically onto the mesoaccumbal DA neurons in the VTA that project to the NAcc and not to mesoaccumbal GABA cells that are co-localized in this region, which receive a predominantly inhibitory signal from the LDT (Omelchenko and Sesack, 2005). These GABAergic/non-dopaminergic cells make up a small proportion of neurons in the mesoaccumbens pathway (Swanson, 1982; Van Bockstaele and Pickel, 1995; Steffensen et al., 1998); however, they do influence the physiology of the circuitry by inhibiting DA cells through a network of local connections (Johnson and North, 1992).

It has been demonstrated that glutamate-secreting neurons are co-localized with acetylcholine-secreting neurons in the PPN and LDT in squirrel monkeys (Lavoie and Parent, 1994), and it is likely that the same occurs in the rat mesopontine region (Clarke et al., 1997). This glutamatergic innervation excites dopamine cells via ionotropic and metabotropic glutamate receptors in the VTA and SN (Scarnati et al., 1986; Di Loreto et al., 1992; Kelland et al., 1993; Futami et al., 1995; Lokwan et al., 1999).

Mesopontine tegmentum acetylcholine-secreting neurons form monosynaptic connections with VTA DA neurons (Oakman et al., 1995; Clarke and Pert, 1985; Smith and Bolam, 1991; Lacey et al., 1990) and excite these cells via stimulation of either mAChR or nAChR. Stimulation of both receptor subtypes produces burst firing in DA

neurons (Gronier and Rasmussen, 1998) as well as DA release in the NAcc and frontal cortex (Nisell et al., 1994). These findings suggest that acetylcholine neurotransmission in the VTA controls the activity of DA cells.

Muscarinic receptors are densely concentrated in the A10 region of the VTA (Rotter et al., 1979) and stimulation of mAChR in the ventral midbrain activates DA neurons (Scarnati et al., 1986; Gronier and Rasmussen, 1998) by initiating an inward current (Lacey et al., 1990). Additionally, nAChR are localized near the vicinity of DA-secreting neurons in the VTA (Clarke and Pert, 1985). Both nicotine, a nAChR agonist (Calabresi et al., 1989), and muscarine, a mAChR agonist (Lacey et al., 1990), enhance the firing rates of DA neurons in the VTA and SN via depolarizing currents. Similarly, stimulation of mAChR in the VTA results in DA release in both the terminal and the somatodendritic regions of the VTA (Gronier et al., 2000). However, neural activation via nAChR stimulation seems to rely in part on presynaptic receptors located on glutamatergic terminals (Schilstrom et al., 1998). The nicotinic current is relatively short lasting, whereas the muscarinic current is longer in duration (Gronier and Rasmussen, 1998). Microdialysis and voltammetry studies have demonstrated that stimulation of muscarinic receptors in the VTA and SN stimulates DA release in mesocorticolimbic areas (Gongora-Alfaro et al., 1991; Grenhoff and Svenson, 1992). Stimulation of nAChR via intra-VTA or SN infusions of nicotine also results in the activation of the DA cells and in the efflux of dopamine in the NAcc (Blaha and Winn, 1993; Nisell et al., 1994a; Blaha et al., 1996)

Additionally, high concentrations of choline acetyl transferase, an ACh synthesizing enzyme, and acetylcholinestrase, an ACh degradative enzyme, have been

found in the vicinity of the DA neurons in the VTA and SN (Greenfield, 1991; Butcher, 1977ab; Butcher and Woolf, 1982). Other cholinergic markers are present in these regions as well, such as a sodium-dependent choline uptake system (Massey and James, 1978) and the amine base choline (Kobayashi et al., 1975), suggesting the existence of a system well prepared to interact with acetylcholine.

Altogether, these findings suggest that stimulation of either mAChR or nAChR in the VTA produces burst firing in DA neurons (Gronier and Rasmussen, 1998) as well as DA release in the NAcc and frontal cortex (Nisell et al., 1994ab). Thus, acetylcholine neurotransmission via both mAChR and nAChR has been shown to control dopaminergic neurons in the VTA.

Although both nicotinic and muscarinic receptors are found on DA cells in the VTA (Clarke and Pert, 1985), it is the muscarinic receptors on the DA cells that play a critical role in some types of reward. For instance, blockade of nAChR via intra-VTA infusions of the nicotinic receptor antagonists hexamethonium and mecamylamine fail to affect lever-pressing maintained by hypothalamic brain stimulation (Yeomans et al., 1993). Recently, it has been reported that cholinergic stimulation of the M1-type receptors, primarily the M5 mAChR, is critical for dopamine release in the NAcc (Forster et al., 2001). Muscarinic excitation of DA cells appears to be mediated via the M1-type receptors, which excite DA cells by causing a membrane depolarization and, in voltage-clamp, an inward current (Lacey et al., 1990). Interestingly, the only mAChR subtype to exist on the DA cells in the VTA appears to be the M5 mAChR as the M5 mRNA is the only mAChR subtype marker to be localized on the DA cell bodies (Vilaro et al., 1990; Weiner et al., 1990; Reever et al., 1997) and neurotoxic lesions of the VTA DA cells via

6 hydroxydopamine are followed by a loss of M5 mRNA (Vilaro et al., 1990; Reever et al., 1997). These findings suggest that the M5 receptors are the only muscarinic receptors that mediate the excitatory actions of muscarine on DA neurons in the VTA. A functional role for M5 mAChR has been established by studies demonstrating that these receptors are important for hypothalamic stimulation reward in rats (Yeomans et al., 2000).

Acetylcholine mediation of reward

Acetylcholine activity in the mesopontine tegmentum has been shown to be involved in reward-related behaviors. For instance, lesions of the PPN attenuate self-administration of opiates (Olmstead et al., 1998) and nicotine (Lanca et al., 2000). Moreover, blockade of reward is demonstrated in studies in which lesions of the PPN are accompanied by reduced conditioned place preference for food, opiates (Bechara and van der Kooy, 1992), morphine (Olmstead and Franklin, 1993) and amphetamine (Olmstead and Franklin, 1994). Similarly, lesions of the PPN raise thresholds for hypothalamic brain stimulation reward (Buscher et al., 1989). Pharmacological evidence implicating acetylcholine transmission in reward comes from studies demonstrating that injections of carbachol, a cholinergic agonist, directly into the PPN decrease sensitivity to brain stimulation reward in rats, whereas intra-PPN infusions of scopolamine, a cholinergic antagonist, increase it (Yeomans, 1995).

Cholinergic stimulation of other brain regions is also implicated in reward. For instance, activation of the nigrostriatal pathway via cholinergic axons arising in the PPN plays a role in feeding and lever-pressing for food (Blackburn et al., 1986; Church et al., 1986). Acetylcholine neurotransmission in the VTA has also been implicated in reward-

related behavior. Intra-VTA nicotine injections can result in conditioned place preference (Museo and Wise, 1994), and extracellular concentrations of acetylcholine increase in the VTA following eating, drinking and self-stimulation of the lateral hypothalamus (Rada et al., 2000).

These rewarding effects are achieved via stimulation of both nAChR and mAChR receptors. Intra-VTA nicotine injections increase DA release in the NAcc (Blaha et al., 1996) and facilitate brain stimulation reward (Bauco and Wise, 1994). Both of these effects are dependent on cholinergic stimulation of nAChR in the VTA (Nisell et al., 1994a; Nisell et al., 1994b). Furthermore, blockade of the α7 nAChR in the VTA decreased both nicotinic- and food-induced DA release in the NAcc (Schilstrom et al., 1998), although no general behavioral or feeding changes were observed following reductions in DA levels. Intra-VTA microinjections of mAChR agonists enhance the rewarding effect of brain stimulation (Redgrave and Horrell, 1976) while similar microinjections of antagonists reduce it (Yeomans et al., 1985; Kofman and Yeomans, 1988; Yeomans et al., 1993; Yeomans and Baptista, 1997). In addition, mAChR antagonists in the VTA were shown to reduce eating in one study (Rada et al., 2000) and approach and consummatory responses for food in another (Ikemoto et al., 1996). These findings suggest that VTA mAChR stimulation is involved in mediating the incentive motivational effects of reinforcers.

Cholinergic agonists administered in the VTA are directly rewarding. For instance, cytosine (Museo and Wise, 1994), a nAChR agonist, and carbachol (Yeomans et al., 1985), a non-specific cholinergic agonist infused directly into the VTA induced conditioned place preferences. Lever-pressing for carbachol or neostigmine, an

acetylcholinesterase inhibitor, is reduced following blockade of mAChR, nAChR, or DA receptors in the VTA (Ikemoto and Wise, 2002).

Based on these findings, it is probable that rewarding stimuli, including food and brain stimulation reward, achieve their rewarding effects via activation of DA cells. How these DA cells become active remains unclear, but converging evidence points to a cholinergic mediation of DA cell firing, and thus suggest that at least some rewarding stimuli depend on cholinergic activation. Interestingly, it appears that rewarding stimuli rely on both nAChR and mAChR; however, some rely selectively on one of the receptor types. For instance, brain stimulation reward and food reward appear to be mediated exclusively via mAChR.

Acetylcholine contribution to reward related learning

There is an emerging body of evidence linking acetylcholine neurotransmission in regions along the mesocorticolimbic DA pathway with reward-related learning, including the PPN, the source of acetylcholine innervations of the VTA. For instance, PPN lesions have been shown to prevent the acquisition of conditioned place preference to food, opiates, and amphetamine (Bechara and van der Kooy, 1989, 1992; Olmstead and Franklin, 1993, 1994), but failed to block the conditioned place preference effect following conditioning sessions (Bechara and van der Kooy, 1989, 1992; Bechara et al., 1992; Nader et al., 1994). Similarly, PPN lesions were found to disrupt the acquisition of a heroin maintained lever pressing task in some animals, but, following training, failed to disrupt heroin self-administration. However, these results remain inconclusive due to the observation that several PPN lesioned animals displayed minimal to no deficits during the acquisition phase of the heroin maintained lever-pressing task (Olmstead et al., 1998).

In accordance, PPN lesions have been shown to disrupt acquisition of a brain-stimulation maintained lever pressing task; however, responding for self-stimulation was also impaired in animals lesioned after acquisition (Lepore and Franklin, 1996). Although the above findings suggest a role of the PPN in reward-related learning, it remains undetermined whether the deficits observed were a result of the destruction of the cholinergic neurons as opposed to non-cholinergic neurons.

A role for cholinergic mediated synaptic plasticity has been demonstrated in the striatum. The primary source of cholinergic inputs to the NAcc arises from large aspiny interneurons, which constitute approximately 5% of the striatum s neural population (Graveland and DiFiglia, 1985). Behavioral pharmacological studies demonstrate that an intact cholinergic signal is necessary for the acquisition of a reward-maintained lever pressing task (Prado-Alcalá, 1985) and lesions of cholinergic neurons in the striatum impair reward-related learning (Kitabatake et al., 2003). Recently, the importance of mAChR, as oppose to nAChR, in the NAcc, has been implicated in reward-related learning. Pratt and Kelley (2004) demonstrated that injections of the muscarinic antagonist scopolamine, but not the nicotinic antagonist mecamylamine, into the NAcc impaired the learning and performance of a sucrose-reinforced lever pressing task.

Muscarinic receptors in the amygdala, a terminal region of the mesolimbic DA system, also appear to be critical for the establishment of conditioned rewards. It was found that when scopolamine is infused into the basolateral amygdala during a conditioning session when rats could learn an association between a stimulus and intravenous cocaine, it disrupts the ability of the cocaine-associated stimulus to later function as a conditioned reward (See et al., 2003) but when it was injected after the

conditioning had occurred it had no effect on the ability of the conditioned stimulus to function as a conditioned reward.

However, there is no evidence to date implicating muscarinic receptors in the VTA in reward-related learning. Based on the above findings, it is conceivable that like drug-related learning, food-related learning also involves synaptic modifications in the VTA. As an animal consumes a food reward, there is coincident activation of the food (US) signal (as food consumption is associated with increased levels of ACh in the VTA) and activation of the signal provided by environmental stimuli (presumably via activation of NMDA, AMPA/Kainate, or mGluR receptors. This concurrent activation in the VTA may allow the formation of associations between environmental stimuli and the food-rewarded task. Due to the concurrent activation of these receptors, the glutamate signal acquires the ability to activate the dopamine cells independently; thus, following the acquisition of the task, the food signal is no longer necessary for the performance of the task, as DA cells now respond to the glutamate signal. The purpose of this dissertation is to test the hypothesis that stimulation of mAChR, but not of nAChR, in the VTA is necessary for the acquisition, and neither are necessary for the expression, of food-related learning.

## Chapter 2: Overview of Specific Aims

Several theoretical models of reward and reward-related learning have been proposed all of which suggest that DA neurotransmission, primarily in regions along the mesocorticolimbic pathway, is crucial for mediating the rewarding effects of natural and drug rewards, and due to its mediation of reward, it has been suggested that DA functions as a key substrate in reward-related learning. During the learning process, it is believed that synaptic plasticity occurs, in which synaptic inputs from reward-predicting environmental stimuli are strengthened so that their future presentation will excite DA cells. Following learning, activation of these DA cells by conditioned environmental stimuli plays a role in goal-directed behaviors. However, the mechanisms responsible for learning-mediated synaptic alterations have not yet been specified.

Empirical findings to support portions of this model have been provided. For instance, DA neurotransmission along the mesocorticolimbic DA pathway has been shown to be critical for the rewarding effects of drugs of abuse (Koob, 1992; Wise, 1996; Woolverton and Johnson, 1992), food (de Wit and Wise, 1977; Wise et al., 1978), electrical brain stimulation (Bardo, 1998; Wise and Rompré, 1989), sexual contact (Blackburn et al., 1992), and conditioned rewards (Beninger and Ranaldi, 1994). Furthermore, DA appears to be involved in the acquisition of reward-related learning. DA cells fire prior to learning (in response to novel stimuli), during learning trials (in response to primary rewards and in response to reward-related stimuli), and upon presentation of conditioned environmental stimuli following learning (and not in response to the rewarding stimuli) (Ljungberg et al., 1992; Schultz et al., 1993; Mirenowicz and Schultz, 1994; Fiorillo et al., 2003; Takikawa et al., 2004; Miller et al., 1981; Kosobud et

al., 1994; Kiyatkin and Rebec, 2001; Hyland et al., 2002). Furthermore, pharmacological manipulations of the mesocorticolimbic DA system alter responding maintained by conditioned rewards (Taylor and Robbins, 1986; Wolterink et al., 1993; Taylor and Robbins, 1984), suggesting that the ability of conditioned environmental stimuli to elicit goal-directed behaviors relies on an intact DA system.

The question of how these DA cells become responsive to environmental stimuli remains unanswered. As previously mentioned, one possibility is that as environmental stimuli are repeatedly associated with rewarding stimuli, synaptic plasticity occurs, which allows reward-predicting stimuli to independently activate DA cells. The initially weak synaptic input signaling environmental stimuli is presumed to be a glutamatergic signal, and during the learning process, the NMDA glutamate receptors become active and serve as coincidence detectors. That is, activation of NMDA receptors signals the convergent activity of two independent synaptic inputs and thereby they strengthen the weaker synapse. Following learning, glutamatergic inputs onto DA cells are now sufficiently able to activate DA cells and produce goal-directed behaviors.

However, some neural substrate must be co-activated along with the glutamate signal in order for this synaptic plasticity to occur. This neural component should correspond to the signal provided by the rewarding stimulus (or US). Cholinergic innervations of DA cells are primary candidates for such a substrate in reward-related learning. For instance, increased acetylcholine neurotransmission is associated with nicotine administration (Blaha et al., 1996) feeding, drinking, and lateral hypothalamic self-stimulation (Rada et al., 2000). More specifically, it is likely that synaptic modulations occur as a result of cholinergic inputs to the VTA - the source of the

mesocorticolimbic DA pathway. The VTA appears to be a critical point for cholinergic modulation as pharmacological manipulations of cholinergic transmission in the VTA affect the reward effects of nicotine (Blaha et al., 1996) and brain stimulation (Redgrave and Horrell, 1976; Yeomans et al., 1985; Kofman and Yeomans, 1988).

Based on the findings mentioned above, it is highly probable that synaptic modifications occur at the level of the VTA as a result of reward-related learning and that acetylcholine neurotransmission is involved in the acquisition of such learning. The primary purpose of this dissertation is to investigate the precise role of acetylcholine in the VTA in food-related learning. Additionally, the role of VTA acetylcholine in food reward remains inconclusive although some evidence suggests that it may play a role. For instance, antagonism of mAChR in the VTA was found to reduce feeding (Rada et al., 2000; Ikemoto et al., 1996) and to attenuate approach-to-food behaviors (Ikemoto et al., 1996). However, there is no behavioral evidence directly relating mAChR activation to food reward *per se.* Therefore, this dissertation also addresses the question of whether the reinforcing value of food is a function of acetylcholine neurotransmission in the VTA. The following is a brief review of the specific hypotheses addressed in this dissertation.

Specific aim 1:

The first aim of this dissertation is to investigate the hypothesis that activation of mAChR, but not of nAChR, at the level of the VTA is necessary for the acquisition, but not the expression, of a food-rewarded lever-pressing task. The acquisition of an operant response requires several types of learning. For instance, during training, organisms are engaged in learning about (1) the appropriate motoric responses necessary to perform a particular goal-eliciting behavior; (2) stimulus-response associations, and (3)

environmental stimuli that are contingent upon and contiguous with the presentation of rewarding stimuli. Learning of environmental cues is perhaps the most important part of the learning process as this knowledge allows organisms to perform appropriate behaviors in the presence of appropriate environmental cues. That is, the attribution of incentive-motivational properties to environmental stimuli is critical for a behavioral response to be repeated in the future.

The first aim of this dissertation is to examine the role of acetylcholine neurotransmission in a food-maintained operant response. By presenting animals with a paradigm in which the role of acetylcholine is assessed during training and following training (see chapter 4), it is possible to distinguish between acquisition of an operant response and performance of the response following training. Furthermore, behavioral effects that follow pharmacological manipulations in either the acquisition phase or the expression phase, but not in both, eliminate the possibility of non-specific pharmacological effects, such as motoric impairment or drug-induced satiation. It is hypothesized that acetylcholine neurotransmission via mAChR, but not nAChR, is necessary for the acquisition of an operant response. That is, intra-VTA microinjections of scopolamine, a mAChR antagonist, but not of mecamylamine, a nAChR, will prevent the acquisition of the lever-press response. However, once the response has been acquired, activation of mAChR is no longer necessary for the behavioral response to be performed (i.e., for the acquired response to be expressed). That is, intra-VTA microinjections of either scopolamine or mecamylamine will fail to affect lever-pressing after the task has been acquired.

Specific aim 2

The second aim of this dissertation is to investigate the hypothesis that stimulation of VTA mAChR, but not of nAChR, at the level of the VTA is necessary for the acquisition, but not the expression, of a free-feeding task. Unlike a food-maintained lever pressing task, which requires acquisition of motoric responses along with formation of associations among various stimuli, a free-feeding task more specifically addresses associative learning, as the motoric behaviors involved in feeding have already been previously acquired. However, animals presented with novel food stimuli (45-mg Dustless Precision Pellets) must first learn that the food is palatable and must also attribute incentive-motivational properties to environmental stimuli for the appropriate feeding behaviors to be initiated in the presence of appropriate reward-predicting cues.

The second aim of this dissertation is to examine the role of acetylcholine neurotransmission in the VTA via mAChR and nAChR in a free-feeding task in which animals are presented with a novel food stimulus. By employing a paradigm in which the role of acetylcholine can be assessed during and following training (see chapter 5), it is possible to distinguish between acquisition of the feeding task and performance of the response following training. Furthermore, behavioral effects that follow pharmacological manipulations in either the acquisition phase or the expression phase, but not in both, eliminate the possibility of non-specific pharmacological effects, such as motoric impairment or drug-induced satiation. It is hypothesized that acetylcholine neurotransmission via mAChR, but not nAChR, is necessary for the acquisition of a feeding response. That is, intra-VTA microinjections of scopolamine, a mAChR antagonist, but not of mecamylamine, a nAChR, will prevent the acquisition of the free-feeding task. However, once the response has been acquired, activation of mAChR is no

longer necessary for the behavioral response to be performed (or for the acquired response to be expressed). That is, intra-VTA microinjections of either scopolamine or mecamylamine will fail to affect pellet-consumption after the task has been acquired.

Specific aim 3:

The third aim of this dissertation is to investigate the hypothesis that neither mAChR nor nAChR at the level of the VTA is necessary for the rewarding effectiveness of, and the motivation to obtain, a food reward once operant conditioning has been acquired. As it is hypothesized that neither mAChR nor nAChR is necessary for the expression of an operant or a free-feeding response, it is likely that these receptors are also unnecessary for an animal s motivation to engage in behaviors that result in the presentation of a food reward once an animal has learned how to obtain the reward. Previous studies have shown that once reward-related learning has occurred, DA cell activation is triggered by appropriate conditioned environmental stimuli rather than by the primary reward itself. Since DA function has been strongly related to the motivational aspects of behavior, it is hypothesized that once environmental stimuli become reward-predicting stimuli, cholinergic US inputs are no longer necessary to guide behavioral responses.

The third aim of this dissertation is to test the hypothesis that acetylcholine neurotransmission in the VTA is not necessary for the motivational aspects of a food-reward following acquisition of an operant response. That is, intra-VTA of scopolamine or mecamylamine will fail to affect lever-pressing under a progressive-ratio schedule of reinforcement in fully trained rats (see chapter 6).

## Chapter 3: General Methods

*Subjects*:

Subjects were 112 male Long Evans rats, facility-bred from males and females obtained from Charles River (Raleigh, NC) with initial free-feeding weights between 300-400g (at the time of surgery). The rats were individually housed and kept on a reversed 12:12 hour light:dark cycle (lights on at 1800 hrs). All experimental sessions were conducted during the dark phase with a brief exposure to light during transportation from their home cages to the experimental chambers. All experimental sessions were conducted during the dark phase so that rats were tested during their active phase.

One week following surgery, each rat s weight was reduced to 85% of its free-feeding value, to increase motivational state, and maintained there for the duration of the experiment through measured daily rations of Purina rat chow. To accomplish this, each rat was weighed daily prior to the experimental session and was fed (5-15g, depending on daily weight) several hours after the conclusion of each day s experimental session. Water was freely accessible at all times except during operant conditioning and free-feeding sessions.

*Surgery*

Each rat was anesthetized with sodium pentobarbital (65 mg/ml, intraperitoneally), and implanted with bilateral guide cannulae (20 gauge) using coordinates taken from the Paxinos and Watson (1986) stereotaxic atlas. The coordinates from cannula implantation directly into the VTA (N=90) were: 5.6 mm caudal to bregma, ±2.2 mm from the midline (angled at 10° toward the midline), and 7.8 mm below the surface of the skull. For rats in the dorsal control group (N=22), the coordinates for

cannula implantation were: 5.6 mm caudal to bregma, ±2.2 mm from the midline (angled at 10° toward the midline), and 5.8 mm below the surface of the skull. Obturators extending 1 mm beyond the tip of the guide cannulae were inserted and kept there at all times except during microinjections.

*Apparatus*

Operant conditioning sessions were conducted in operant conditioning chambers measuring 30 x 21 x 18 cm. Each chamber consisted of an aluminum top and three aluminum sides. The front side, which served as the door, was made of transparent plastic. The floor of each chamber consisted of aluminum rods. Each operant conditioning chamber was equipped with two levers, two white stimulus lights and a food trough, all on the right wall. Rewarded lever presses were accompanied by the illumination of both stimulus lights for 2 s. Each lever was positioned 2.5 cm away from the edge of the wall and extended 2 cm from the wall. Each white stimulus light was positioned 3 cm above a lever. The food trough measured 5 x 5 cm and was centered between the two levers at a height of 3 cm from the floor.

Free feeding sessions were conducted in experimental feeding chambers measuring 47 x 25.5 x 21.5 cm. Prior to each session, 300 Dustless Precision Pellets (45 mg, BioServ) were placed in a corner directly on the floor of each chamber, unrestrained by a container.

*Microinjections*

For each rat, the obturator was removed from one guide cannula and a stainless steel injector tube was inserted such that it extended 1 mm beyond the guide cannula. The injector tube was connected by polyethylene tubing to a 10 µl Hamilton syringe that

was preloaded with either scopolamine, mecamylamine or their vehicles. The test compound was injected over a 30-s period. The injector was kept in place for an additional 60 s after which it was removed and the obturator was replaced. This procedure was repeated for the contralateral side.

*Drugs and Doses*

The mAChR antagonist, scopolamine hydrobromide, and the nAChR antagonist, mecamylamine hydrochloride (both from Sigma-Aldrich, St. Louis, MO), were dissolved in distilled water. Drug solutions were mixed prior to the commencement of these experiments.

*Histology*

After a rat completed all tests, it was anesthetized with a lethal dose of sodium pentobarbital, perfused with saline followed by 10% formalin, and decapitated. The brain was removed and stored in 10% formalin for at least 7 days prior to being cut in 40-μm serial sections, stained with cresyl violet, and inspected for cannula implantation and injection sites. Histological verifications were performed while uninformed of behavioral data.

*Data Analysis*

For the fixed ratio data, mixed design factorial analyses of variance (ANOVAs) were conducted with dose as the between-subjects factor and day of testing as the within-subjects factor for both scopolamine and mecamylamine. Separate ANOVAs were conducted for Phases I, II, and III. Significant results ($p<.05$) were followed by tests of simple main effects and Bonferroni pairwise comparisons.

For the free-feeding paradigm, mixed design factorial ANOVAs were conducted with dose of scopolamine as the between-subjects factor and day of testing as the within-subjects factor. Such ANOVAs were conducted for Phases I and II. Significant results ($p<.05$) were followed by tests of simple main effects and Bonferroni pairwise comparisons. T-tests were performed to determine statistical differences between the two groups prior to continuation from phase to phase and to analyze Phase III data.

For the progressive ratio (PR) schedule of reinforcement, the initial response requirement was set to one for the first reward and was increased exponentially for each subsequent reward (according to the formula: $5 \times e^{(\text{reward\#} \times 0.22)} - 5$) until the ratio requirement ultimately becomes so high that rats cease to respond. The point at which rats stop responding is referred to as the break point (BP). BPs were operationally defined as the final number of ratios completed (which resulted in the delivery of a food reward) within 15 minutes of the previous one. For the PR data, the BP during the test session was expressed as a percentage of the average BP for the three sessions preceding the test session. The statistical analyses were conducted on these percentage of baseline values. Separate one-way ANOVAs with dose as a within-subjects factor were conducted on the scopolamine and mecamylamine data.

## Chapter 4: Blockade of Muscarinic, but Not of Nicotinic, Acetylcholine Receptors in the VTA Prevents Acquisition of Food-Reward Operant Responding

The neural mechanisms responsible for reward-maintained operant responding have not yet been fully identified. Thus far, evidence suggests that operant responding is maintained by an intact DA system in various brain regions, which is necessary for an organism to experience reward and to carry out motoric behaviors necessary to obtain a reward. Rewards can elevate extracellular DA concentrations in mesocorticolimbic and mesostriatal systems (Hernandez and Hoebel, 1988; Bradberry and Roth, 1989; Pettit and Justice, Jr., 1989; Chen and Reith, 1994; Ranaldi et al., 1999) and DA neurotransmission in these pathways appears critical for their rewarding effects; reductions in DA neurotransmission in the NAcc, mPFC, VTA, striatum or SN reduce responding maintained by food (Beninger et al., 1993; Beninger and Ranaldi, 1993; Aberman et al., 1998; Smith-Roe and Kelley, 2000; Baldwin et al., 2002; Sharf et al., 2005), cocaine (McGregor and Roberts, 1993; Ranaldi and Wise, 2001; Quinlan et al., 2004) or VTA brain stimulation (Kurumiya and Nakajima, 1988). Furthermore, DA has been implicated in reward-related learning (Wise and Rompré, 1989; Wise and Bozarth, 1987; Berridge, 1995; Wise, 2004) and is thought to mediate the incentive motivational properties of reinforcers (Berridge and Robinson, 1998; Ikemoto and Panksepp, 1999; Wise, 2004) However, the precise neurotransmitter systems that are involved in the *acquisition* of an operant response remain unknown.

Reward-related learning is a function of the acquired ability of conditioned environmental stimuli to successfully activate those DA cells that are responsible for the rewarding effects of, and motor responses to obtain, rewarding stimuli. Evidence for the

role of the DA system is provided by demonstrations that VTA DA cells increase firing (Schultz, 1997) and release DA (Blackburn and Phillips, 1989; Gratton and Wise, 1994) in response to presentations of reward-associated stimuli. Behavioral evidence supporting this hypothesis include studies in which activation of mesolimbic DA reinstated extinguished reward-related responding (Stewart, 1984; Ranaldi et al., 1999) and increases in mesocorticolimbic DA occur immediately prior to reward-related lever presses (Gratton and Wise, 1994; Kiyatkin and Gratton, 1994; Richardson and Gratton, 1996). Thus, reward-associated environmental stimuli are capable of independently activating DA cells in the mesocorticolimbic system, suggestion that at least some of the neural modifications that occur during the learning process involve the strengthening of synaptic inputs to DA cells from environmental signals.

According to the Hebbian model of synaptic alterations, a weak synaptic input becomes strengthened as a result of conjoint activity of the weak input with a strong synaptic input. As DA cells are activated in response to primary rewards prior to learning, it is plausible that the primary US signal that activates these DA cells is co-activated with weaker environmental signals, leading to a strengthening of the environmental signals. A possible source of the US signal is a cholinergic innervation of the VTA.

The VTA is innervated by axons of acetylcholine-secreting neurons in the pedunculopontine (PPN) and laterodorsal tegmental (LDT) nuclei. These cholinergic neurons form monosynaptic connections with DA neurons (Oakman et al., 1995; Clarke and Pert, 1985; Smith and Bolam, 1991; Lacey et al., 1990). It appears that acetylcholine-secreting neurons in the PPN (Blaha et al, 1996) and LDT (Omelchenko

and Sesack, 2005) project to the VTA and excite DA cells via stimulation of either mAChR or nAChR. Stimulation of both receptor subtypes produces burst firing in DA neurons (Gronier and Rasmussen, 1998) as well as DA release in the NAcc and frontal cortex (Nisell et al., 1994). These findings suggest that acetylcholine neurotransmission in the VTA controls the activity of DA cells.

It has been demonstrated that acetylcholine neurotransmission in the VTA is directly associated with reward-related behavior. For instance, feeding, drinking, and hypothalamic brain stimulation reward are associated with increased extracellular concentrations of acetylcholine in the VTA (Rada et al., 2000). Pharmacological manipulations of cholinergic neurotransmission in the VTA via infusions of mAChR agonists enhance the rewarding effect of brain stimulation (Redgrave and Horrell, 1976) while similar infusions of antagonists reduce it (Yeomans et al., 1985; Kofman and Yeomans, 1988; Yeomans et al., 1993; Yeomans and Baptista, 1997). In addition, mAChR antagonists in the VTA were shown to reduce eating in one study (Rada et al., 2000) and approach and consummatory responses for food in another (Ikemoto et al., 1996). These findings suggest that acetylcholine in the VTA, primarily through its actions at mAChR, mediates the incentive motivational properties of reinforcers.

Thus far, there is some evidence supporting the role of mAChR in other brain regions in reward-related learning. For instance, blockade of mAChR, and not of nAChR, in the NAcc disrupts the acquisition of a sucrose-reinforced operant response (Pratt and Kelley, 2004) and blockade of mAChR in the amygdala prevents the acquisition of the ability of a cocaine-associated stimulus to function as a conditioned reward (See et al., 2003).

Because of acetylcholine s ability to control the activity of DA neurons and its implications in reward-related behaviors, it is hypothesized that stimulation of mAChR in the VTA is necessary for the acquisition of reward-related operant learning. The following experiments address the hypothesis posed by Specific Aim 1 - activation of mAChR, but not of nAChR, in the VTA is necessary for the acquisition, but not the expression, of a food-rewarded lever-pressing task.

## Materials and Methods

### *Subjects and Materials*

Please refer to Chapter 3 (General Methodologies) for a full description of the subjects, surgery, apparatus, microinjection procedure, drugs and doses, histology procedure, and data analysis.

### *Experiment 1: Operant responding under a fixed ratio schedule*

One week after surgery, rats (N=54) were introduced to a three-phase protocol in which they were trained to press a lever under a fixed ratio 1 (FR1) schedule of food (one 45-mg pellet) reinforcement. Experimental sessions consisted of 10 consecutive 60-minute sessions, held one per day. Phase I consisted of four consecutive daily sessions in which the rats received bilateral microinjections of 0 (N=13), 2.5 (N=6), or 5 (N=8) µg/0.5 µl scopolamine or 0 (N=13), 5 (N=7), or 10 (N=7) µg/0.5 µl mecamylamine into the VTA immediately prior to being placed in the operant conditioning chambers. Phase II consisted of five consecutive sessions, held one per day, in which rats were placed in the chambers without drug treatment. Phase III consisted of a single test session in which the rats that previously received microinjections of either the 2.5 or 5 µg doses of scopolamine or the 5 or 10 µg doses of mecamylamine received vehicle microinjections

and those rats that previously received vehicle microinjections now received the 2.5 or the 5 μg doses of scopolamine or the 5 or 10 μg doses of mecamylamine.

*Experiment 2: Anatomical Analysis*

To ascertain that the behavioral effects of scopolamine were localized to the VTA, a separate group of rats was tested with scopolamine injections just dorsal to the VTA. Thus, one week following surgery, rats with cannula placements in which the injector was positioned 2 mm dorsal to the VTA were exposed to Phase I of the FR responding paradigm (N=10). Rats received microinjections of 0 (N=5) or 5 (N=5) μg/0.5 μl of scopolamine prior to each test session.

## Results

*Experiment 1: Fixed-Ratio Responding*

*Phase I: Drug-treatment*

Rats that received the 0 μg dose of scopolamine displayed a significant daily increase in lever pressing from the first to the fourth operant conditioning sessions while rats treated with the 2.5 or 5 μg doses of scopolamine failed to show this increase. Rather, scopolamine-treated rats maintained the same low levels of responding throughout the four sessions of Phase I (see Fig. 1a). A two-way factorial ANOVA with dose of scopolamine and day of injection as factors revealed a significant dose by day interaction ($F_{(6, 69)} = 4.115$, $P<.003$). Tests of simple main effect of day at each dose revealed a significant day effect for vehicle-treated ($F_{(3, 69)} = 17.07$, $P< .0001$), but not for scopolamine-treated, rats. Bonferroni tests on the data from the vehicle controls showed that, while lever pressing in the first and second test sessions did not differ significantly

from one another, lever pressing in the two subsequent test sessions differed significantly from the first two test sessions ($P<.05$) as well as from each other ($P<.05$).

From session 1 to session 4, rats maintained the same level of responding on the inactive lever regardless of scopolamine treatment (see Fig 2a). A two-way factorial ANOVA with dose of scopolamine and day of injection as factors revealed no significant day, dose, or a day by dose interaction effect.

During session 1, rats emitted the first lever-press within 15 minutes of the session, regardless of scopolamine treatment (see Fig. 3a). However, one rat from the vehicle group and two rats from the 2.5 μg group did not emit any lever-presses during the first session. A Kruskal-Wallis test comparing latency to respond during session 1 revealed no significant difference among the scopolamine doses.

Rats that were treated with any mecamylamine dose (0, 5 or 10 μg) demonstrated a significant daily increase in lever pressing (Fig. 1b). A two-way factorial ANOVA with dose of mecamylamine and day of injection as factors revealed a significant day effect ($F_{(6, 72)} = 17.53$, $P<.0001$), but no day by dose interaction. Bonferroni tests on the data from rats that received the vehicle, 5 or 10 μg doses showed that, at all dose levels, lever pressing increased significantly from the first session to the second ($P<.05$) and from the third session to the fourth ($P<.05$). Lever pressing in the fourth session was significantly different from the first ($P<.001$).

From session 1 to session 4, rats maintained the same level of responding on the inactive lever regardless of mecamylamine treatment (see Fig 2b). A two-way factorial ANOVA with dose of mecamylamine and day of injection as factors revealed no significant day, dose, or a day by dose interaction effect.

During session 1, rats emitted the first lever-press within 25 minutes of the session, regardless of mecamylamine treatment (see Fig. 3b). However, three rats from the vehicle group, one rat from the 5 μg group, and one rat from the 10 μg group did not emit any lever-presses during the first session. A Kruskal-Wallis test comparing latency to respond during session 1 revealed no significant difference among the mecamylamine doses.

*Phase II: No drug-treatment*

In Phase II, rats that had previously received the vehicle dose of scopolamine maintained a high rate of lever pressing in each session. Rats that had previously received scopolamine microinjections demonstrated daily increases in lever pressing. Those rats that had previously received the 2.5 μg dose of scopolamine reached maximal lever pressing levels, and appeared similar to the vehicle controls, by the third session of this phase whereas the rats that previously received the 5 μg dose failed to reach the same maximal level as vehicle control rats even by the fifth session of Phase II (see Fig. 1a). A two-way factorial ANOVA comparing dose and day (the last day of Phase I and the first day of Phase II) revealed a significant dose effect ($F_{(2, 23)} = 19.42$, $P<.0001$), but no day effect nor day by dose interaction. However, an ANOVA on the data from all five operant conditioning sessions of Phase II revealed a dose by day interaction ($F_{(8, 92)} = 5.91$, $P<.0001$). Tests of simple main effect of day at each dose revealed a significant day effect in rats that previously received microinjections of 2.5 μg scopolamine ($F_{(4, 92)} = 10.74$, $P< .001$), and those that previously received microinjections of 5 μg scopolamine ($F_{(4, 92)} = 4.02$, $P< .01$), but not in rats that previously received the vehicle dose. Bonferroni tests on the 2.5 and 5 μg dose data showed that lever pressing on the first and

second sessions of Phase II differed significantly from the three subsequent sessions ($P<.05$), while the last two sessions of Phase II did not differ from each other. On the fifth, and final, session of Phase II, there was no significant difference in lever pressing between those rats that had previously received the 0 or the 2.5 µg doses of scopolamine, but those rats that had received the 5 µg dose failed to reach the same maximal levels reached by vehicle controls and differed significantly from those rats that had received a lower dose. A one-way ANOVA comparing day as a factor (the last day of phase II) revealed a significant day effect ($F_{(2, 26)} = 5.228$, $P< .013$). A Tukey Honestly Significantly Difference test on the data from all three dose groups revealed a significant difference between those rats that had received the 5 µg dose and those that had received the 2.5 µg ($P<.014$); however, the vehicle controls did not differ from either scopolamine dose group.

Rats that had previously received any dose of scopolamine in Phase I maintained the same level of responding on the inactive lever from the first to the last session of Phase II (see Fig 2a). A two-way factorial ANOVA comparing dose and day (the last day of Phase I and the first day of Phase II) revealed neither a dose effect nor a dose by day interaction. Similarly, an ANOVA on the data from all five operant conditioning sessions of Phase II revealed no significant day, dose, or day by dose interaction effect. On the fifth, and final, session of Phase II, there were no significant differences in lever pressing on the inactive lever among the three dose groups.

In Phase II, rats that previously received vehicle or a dose of mecamylamine showed an increase in lever pressing from the first to the second session of this phase; however, all rats maintained lever pressing rates from the second to the fifth operant

conditioning session (see Fig. 1b). A two-way factorial ANOVA comparing dose and day (the last day of Phase I and the first day of Phase II) revealed neither a dose effect nor a dose by day interaction. However, an ANOVA on the data from all five operant conditioning sessions of Phase II revealed a day effect ($F_{(4, 92)} = 7.157$, $P<.001$), but no dose by day interaction. Bonferroni tests on the data from all three dose groups showed that lever pressing on the first session differed significantly from all 4 subsequent sessions ($P<.05$). On the fifth, and final, session of Phase II, there were no significant differences in lever pressing among the three dose groups.

Rats that previously received vehicle or the 5 μg dose of mecamylamine in Phase I maintained the same level of responding on the inactive lever from the last session of Phase I to the last session of Phase II. Those rats that previously received the 10 μg dose of mecamylamine showed a reduction in inactive lever pressing from the last session of Phase I to the first session of Phase II, however responding on the inactive lever was maintained from the first to the last session of Phase II (see Fig. 2b). A two-way factorial ANOVA comparing dose and day (the last day of Phase I and the first day of Phase II) revealed a significant day by dose interaction ($F_{(2, 24)} = 4.684$, $P<.02$), but no day or dose effects. Tests of simple main effect of day at each dose revealed a significant day effect for rats treated with the 10 μg dose ($F_{(1, 24)} = 9.55$, $P< .001$), but not for vehicle or 2.5-mecamylamine-treated, rats. An ANOVA on the data from all five operant conditioning sessions of Phase II revealed no significant dose, day, and nor a dose by day interaction. On the fifth, and final, session of Phase II, there were no significant differences in lever pressing on the inactive lever among the three dose groups.

*Phase III: Dose reversal*

In Phase III, when rats that first received the 2.5 or the 5 μg doses of scopolamine in Phase I now received the 0 μg dose and rats that previously received the vehicle dose now received either the 2.5 or the 5 μg doses, there were no significant differences in levels of lever pressing among the groups (see Fig. 1a). Similarly, there were no significant differences in levels of lever pressing on the inactive lever (see Fig. 2a).

In Phase III, when rats that previously received the 5 or the 10 μg doses of mecamylamine in Phase I now received the 0 μg dose and rats that previously received the vehicle dose now received either the 5 or the 10 μg doses, there were no significant differences in levels of lever pressing among the groups (see Fig. 1b). Similarly, there were no significant differences in levels of lever pressing on the inactive lever (see Fig. 2b)

*Experiment 2: Anatomical Analysis*

There were no differences in lever pressing between rats that received the 0 or the 5 μg doses of scopolamine in a site 1.5-2.0 mm dorsal to the VTA in each of the four sessions in Phase I (see Fig. 4). Furthermore, both groups exhibited daily increases in lever pressing.

*Histology*:

Most of the VTA microinjection sites were localized in the ventromedial portions of the VTA with some injections occurring in the ventrolateral portions (see Fig. 5 and 6). Microinjections aimed at the anatomical control sites were approximately 1.5 to 2.0 mm dorsal to the VTA injection sites (see Fig. 5).

***Figure 1:*** Mean number of active lever presses for rats receiving bilateral microinjections of (a) scopolamine (0, 2.5 or 5 μg/0.5 μl) or (b) mecamylamine (0, 5, or 10 μg/0.5 μl) into the VTA. In Phase I, immediately before sessions 1 to 4, rats were treated with their respective doses of either compound. In Phase II, before sessions 5 to 9 rats received no drug treatments. In Phase III, before session 10 rats that previously received an active dose of either compound now received the vehicle dose and vice versa. Vertical lines represent the standard error of the mean (SEM).

***Figure 2:*** Mean number of inactive lever presses for rats receiving bilateral microinjections of (a) scopolamine (0, 2.5, or 5 μg/0.5 μl) or (b) mecamylamine (0, 5 or 10 μg/0.5 μl) into the VTA. In Phase I, immediately before sessions 1 to 4, rats were treated with their respective doses of either compound. In Phase II, before sessions 5 to 9 rats received no drug treatments. In Phase III, before session 10 rats that previously received an active dose of either compound now received the vehicle dose and vice versa. Vertical lines represent the standard error of the mean (SEM).

***Figure 3***: Mean 5-minute bin in which the first lever-press occurred in rats receiving bilateral microinjections of (a) scopolamine (0, 2.5, or 5 μg/0.5 μl) or (b) mecamylamine (0, 5, or 10 μg/0.5 μl) during session 1 of Phase I of the fixed-ratio operant responding paradigm. Vertical lines represent the standard error of the mean (SEM).

***Figure 4:*** Mean number of lever-presses for rats receiving bilateral microinjections of scopolamine (0 or 5 μg/0.5 μl) into a site 1.5 to 2 mm dorsal to the VTA. Vertical lines represent the standard error of the mean (SEM)

***Figure 5:*** Histological reconstruction of injection sites adapted from Paxinos and Watson (1986). Black circles for injections in the VTA group; grey circles for injections in the dorsal control group. The numbers to the right of each section indicate the distance posterior to bregma.

***Figure 6:*** A representative photomicrograph depicting the cannula tips in the ventrolateral portion of the VTA.

**Figure 1a:**

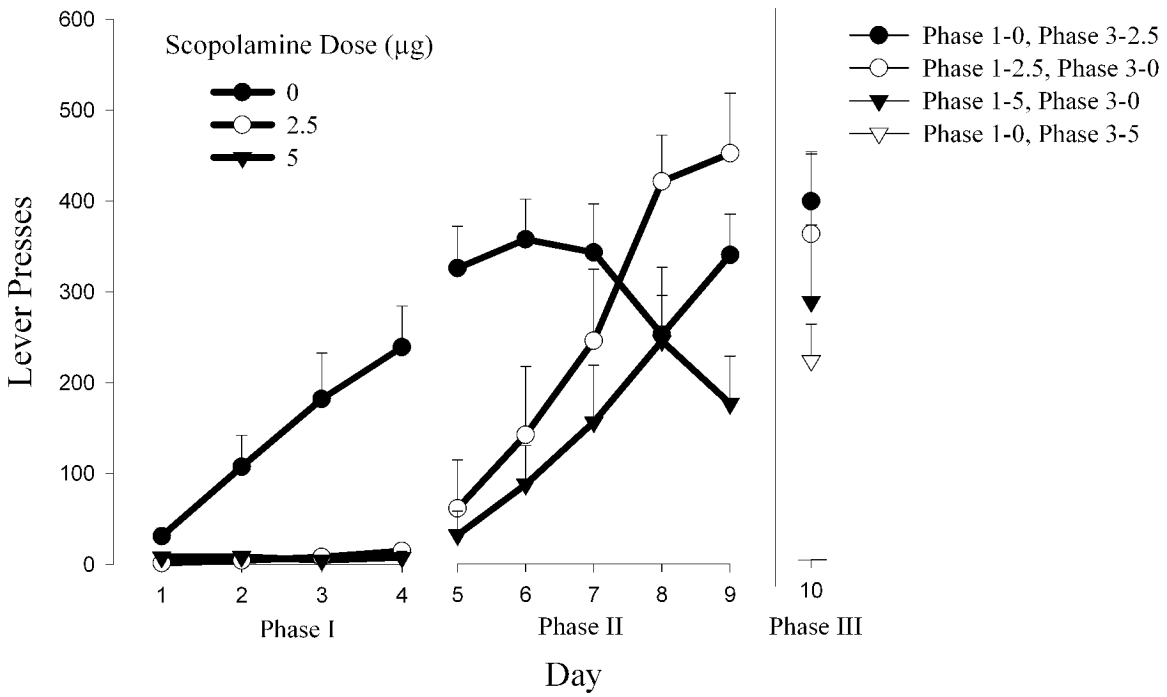
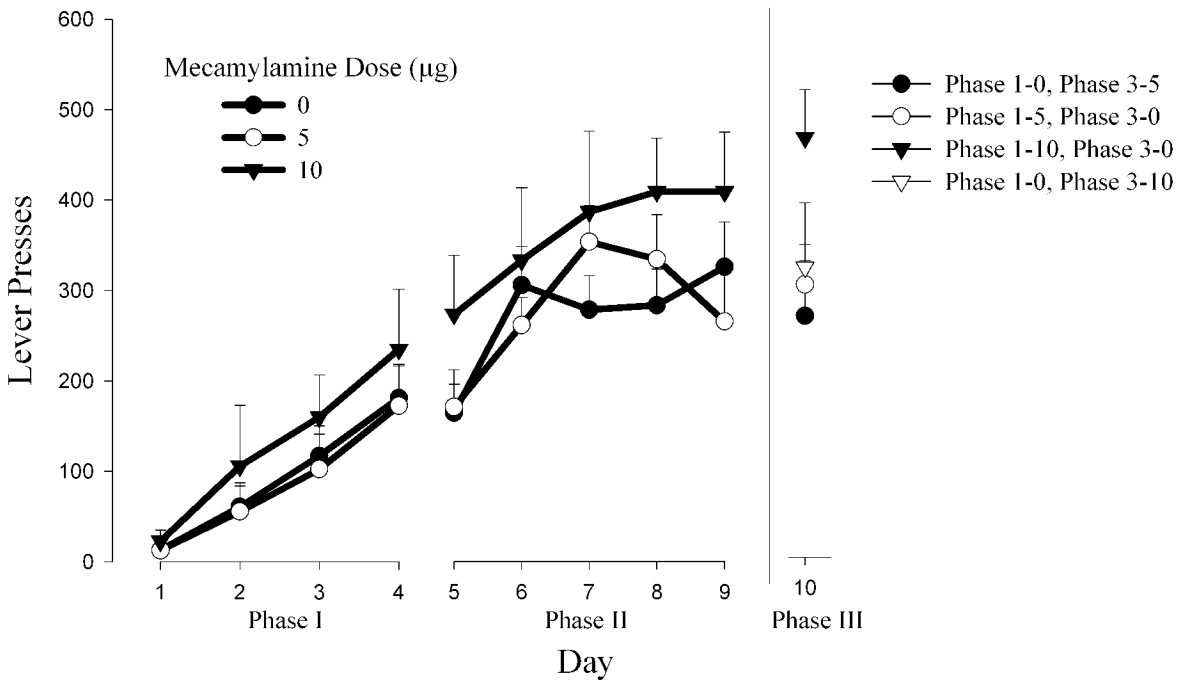



**Figure 1b:**



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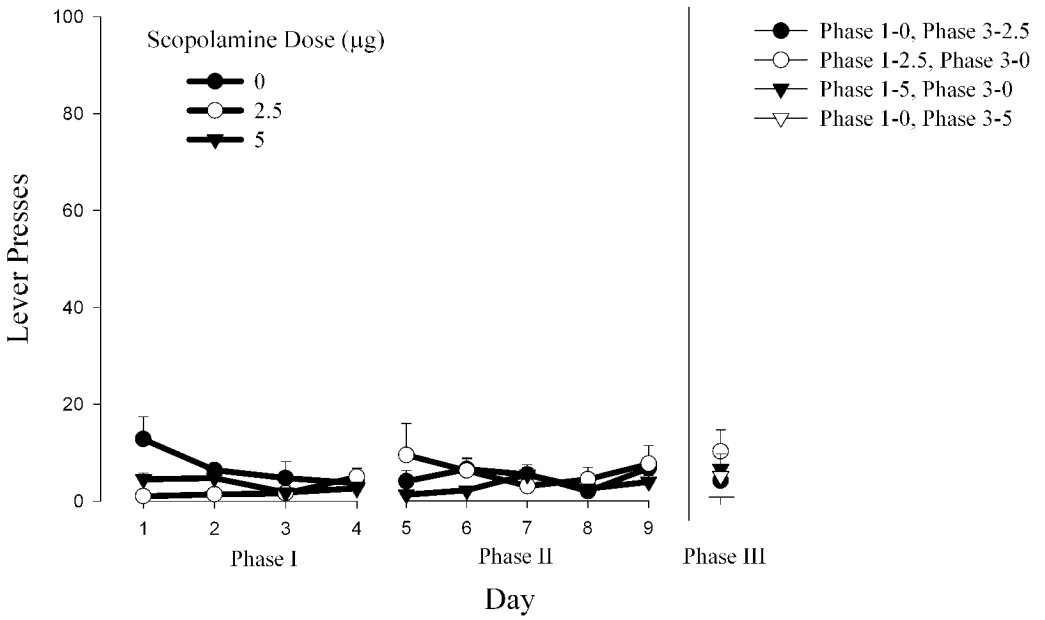


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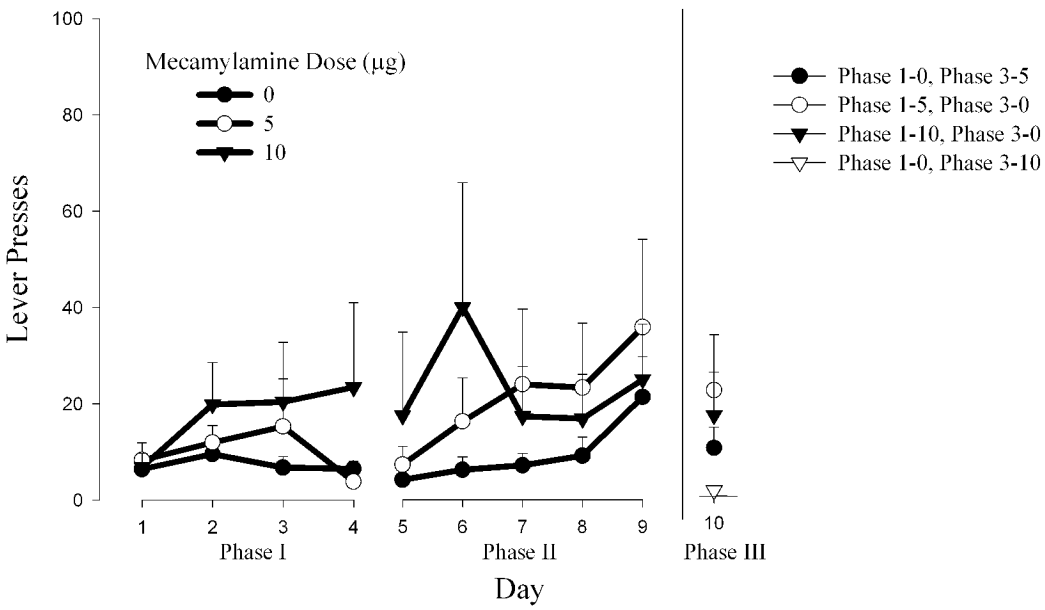

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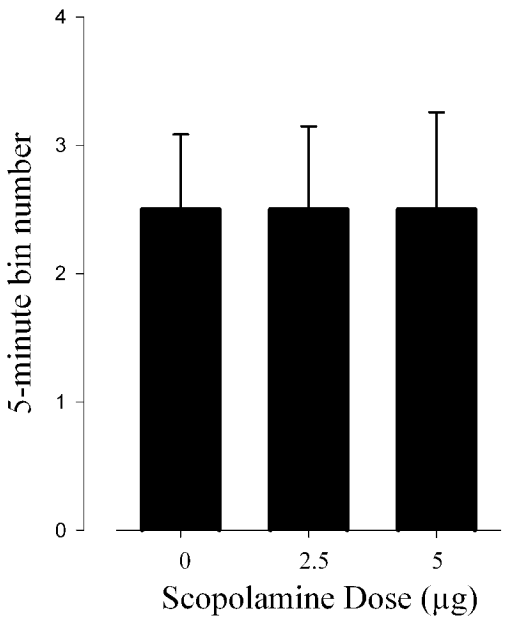



**Figure 3b:**

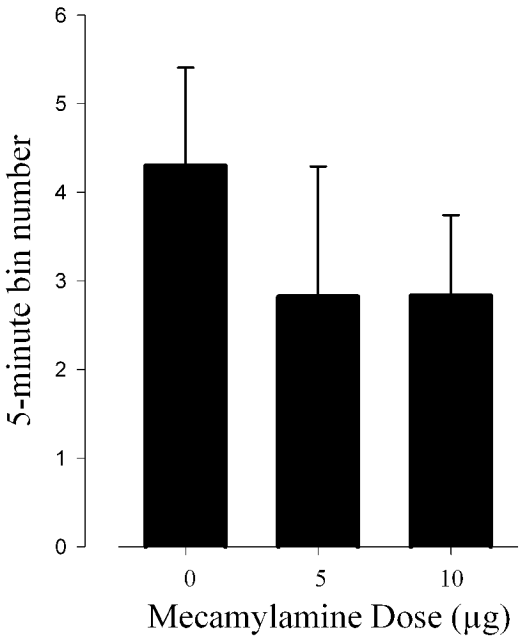


**Figure 4:**

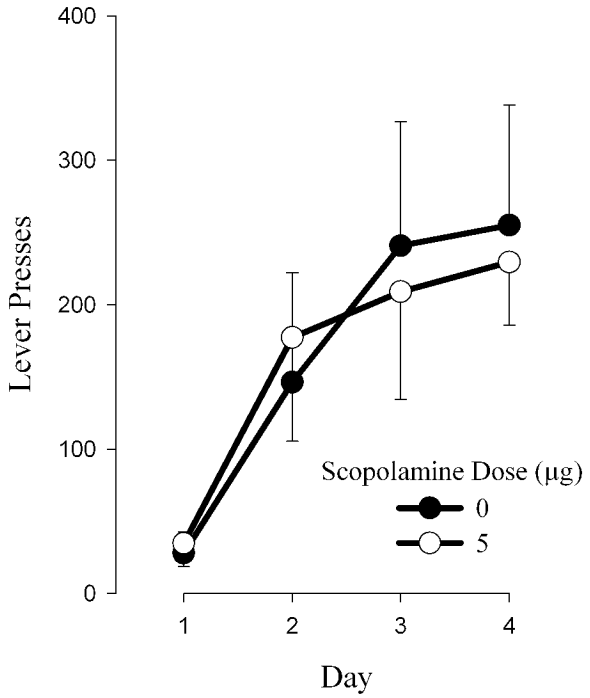



**Figure 5:**

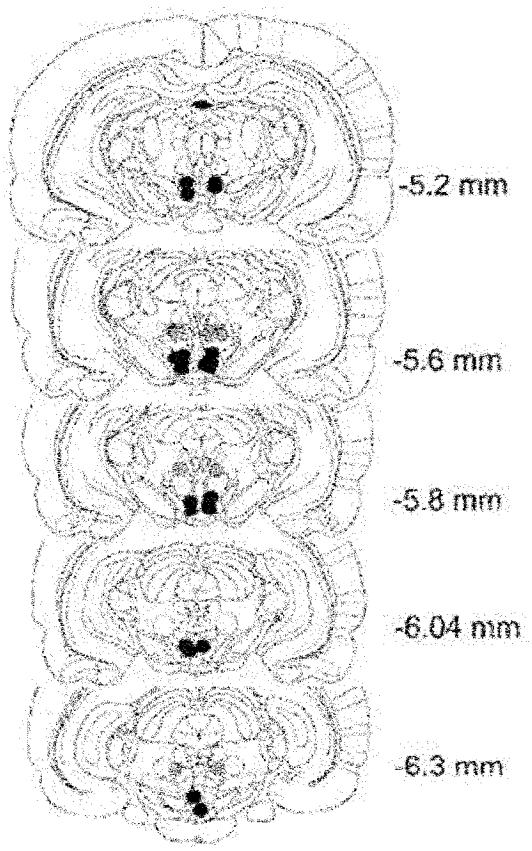
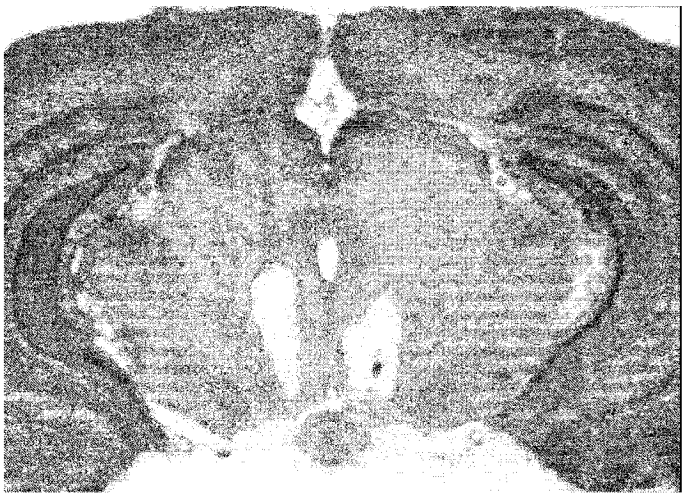


***Figure 6:***



## Discussion

The present study tested the hypothesis that VTA ACh neurotransmission is necessary for the acquisition of reward-related operant learning. It was found that in animals presented with the opportunity to acquire a food-rewarded operant response, intra-VTA treatment with scopolamine, a mAChR antagonist, prevented the acquisition of the operant response but not the expression of the same response after it was learned. On the other hand, intra-VTA mecamylamine, a nAChR antagonist, impaired neither the acquisition nor the expression of the operant response. These data suggest that VTA acetylcholine stimulation of mAChR, but not of nAChR, is necessary for the acquisition of a food-rewarded operant response and that neither is necessary for the expression of the learned operant.

In the present study, scopolamine blocked *acquisition* of FR responding but not *expression* of FR responding. One possible explanation is that VTA mAChR stimulation constitutes part of a food reward signal and that this reward signal is present and necessary for the acquisition of operant responding and still present but *no longer necessary* for the expression of an already acquired operant response. This hypothesis is expanded upon further below.

To address the possibility that effects of intra-VTA injections of scopolamine on the acquisition of operant responding were due to the drug diffusing to and acting at distal sites, the effects of scopolamine were tested in a site 1.5 to 2.0 mm dorsal to the VTA. Microinjections into the brain are associated with hydraulic pressure that can drive the substance up the cannula tracks toward the pressure sinks of the ventricles (Wise and Hoffman, 1992). Once in the ventricular system, the substance can be distributed

throughout the brain rapidly and cause behavioral effects through actions at receptors located in distal sites. However, microinjections in the site dorsal to the VTA failed to produce behavioral reductions similar to those seen with microinjections directly in the VTA, ruling out the possibility of dorsal diffusion to a distal site of action and supporting the conclusion that the behavioral effects of the intra-VTA microinjections of scopolamine were local.

It is unlikely that the present findings were due to scopolamine-induced satiation or non-specific (e.g., motoric) effects instead of motivational or learning deficits. During Phase III, when animals received scopolamine or mecamylamine, responding was maintained at the same level as in the previous session, when they did not receive microinjections. Thus, once the task was acquired, all animals demonstrated the capacity to consume the same number of pellets and to press the lever the same number of times under scopolamine treatment as when not. Additionally, increases in responding for all scopolamine-treated rats were selective for the reward-associated lever as responding on the inactive lever remained stable from the first to the last operant conditioning sessions regardless of scopolamine treatment. If scopolamine s effects were due to an impairment of motor ability, it would have reduced lever-pressing on both active and inactive levers; however, this was not the case. Furthermore, if we assume that time to emit the first lever press represents a measure of exploratory behavior, then scopolamine treatment did not decreases exploratory behavior as all rats, regardless of treatment dose, demonstrated similar latency to begin responding. Therefore, satiation and motoric effects can be ruled out.

That antagonism of VTA mAChR stimulation appears to block only acquisition of behavior, and not its performance, strongly suggests that VTA mAChR stimulation is a necessary signal during acquisition of operant learning but an unnecessary signal after acquisition. This suggests further that, during acquisition of operant learning, the relevant function served by VTA mAChR stimulation is acquired by another neural mechanism. One possibility is that the ability to activate DA cells and cause DA release, a mAChR function which presumably constitutes the mechanism of action for the role of VTA ACh in reward, is acquired by another pathway.

As stated above, DA neurotransmission in terminal regions of the mesocorticolimbic system is implicated in reward. DA concentrations in terminal regions of this system are enhanced by primary reward (Hernandez and Hoebel, 1988; Pettit and Justice, Jr., 1989; Ranaldi et al., 1999) and blockade of DA neurotransmission in these regions attenuates the rewarding effects of reinforcers (Wise, 2004), including food (Beninger et al., 1993; Beninger and Ranaldi, 1993; Aberman et al., 1998; Smith-Roe and Kelley, 2000; Baldwin et al., 2002; Sharf et al., 2005). In fact, DA released in the terminal regions of this system is implicated in reward-related operant learning; blockade of NAcc (Smith-Roe and Kelley, 2000) or medial prefrontal cortex (Baldwin et al., 2002) DA neurotransmission at D1 receptors, in conjunction with blockade of glutamate neurotransmission at NMDA receptors in these same regions, attenuates the rate of acquisition of a food-rewarded operant response. This suggests that a DA signal is critical for operant learning. VTA acetylcholine is implicated in facilitating this DA signal during food reward related behavior and, therefore, may play a role in the acquisition of operant learning through this mechanism. Food consumption elevates

acetylcholine concentrations in the VTA; acetylcholine stimulation of muscarinic receptors in VTA activates DA cells and causes DA release in NAcc; blockade of muscarinic receptors in VTA attenuates NAcc DA release and eating (Rada et al., 2000). It seems that VTA mAChR stimulation, by causing DA release in terminal regions, may be important for food reward. Thus, it is possible that blockade of VTA mAChR stimulation attenuated or eliminated the food-associated DA signal and thereby prevented the acquisition of operant learning.

In the present study, blockade of mAChR failed to disrupt operant responding *after* the operant response was acquired. Thus, the function served by VTA mAChR stimulation that is critical for acquisition is not critical for expression of operant learning. This implies that another neural pathway acquires the ability to perform this function. As stated above, it is possible that this critical function is the ability to activate DA cells. It is hypothesized that during the acquisition of food-related operant learning, conditioned stimuli acquire the ability to activate VTA DA cells. By activating these cells, they could function similarly to food reward itself - that is, they could elicit and reinforce approach (i.e., lever pressing) behavior.

The VTA DA cells receive glutamate afferents from the mPFC (Sesack and Pickel, 1992; Smith et al., 1996) the amygdala and the bed nucleus of the stria terminalis (Hopkins and Holstege, 1978; Pillipson, 1979) and the PPN (Charara et al., 1996), which could carry information about conditioned stimuli. The acetylcholine signal at mAChR might function as a signal for unconditioned stimuli. So, it is possible that VTA DA cells associate CS and US signals during operant learning. This hypothesis is supported by studies showing that glutamatergic synaptic activity in the VTA is associated with

induction of LTP of the DA neurons (Bonci and Malenka, 1999; Overton et al., 1999) and that the VTA is a critical site for synaptic modifications involved in the conditioning of environmental stimuli with drug rewards (Harris et al., 2004).

The current findings suggest that stimulation of mAChR in VTA is necessary for the formation of associations between environmental stimuli and a food-rewarded operant response and, once these associations are formed, stimulation of VTA mAChR is not necessary for the expression of the response.

## Chapter 5: Blockade of muscarinic, but not of nicotinic, acetylcholine receptors in the ventral tegmental area disrupts acquisition of a free-feeding task

As previously mentioned, acetylcholine neurotransmission in the VTA is implicated in reward-related behavior. Acetylcholine levels increase in the VTA following eating, drinking and self-stimulation of the lateral hypothalamus (Rada et al., 2000). A functional role for VTA neurotransmission at muscarinic acetylcholine (mACh) receptors in brain stimulation reward has been demonstrated by studies showing that muscarinic acetylcholine agonists administrated directly into the VTA enhance lever-pressing for brain stimulation reward (Redgrave and Horrell, 1976) while muscarinic acetylcholine antagonists reduce it (Yeomans et al., 1985; Kofman and Yeomans, 1988; Yeomans et al., 1993; Yeomans and Baptista, 1997). A role for VTA mAChR stimulation in food reward has also been suggested, although interpretations of supporting data remain inconclusive. Infusion of a high dose of a mACh receptor antagonist into the VTA attenuated approach and consummatory responses for a sucrose solution in a runway alley, but it also attenuated general activity making it impossible to conclude that its effects were related to food reward *per se* (Ikemoto and Panksepp, 1996).

Given that stimulation of VTA mACh receptors is implicated in eating and in mediating the rewarding effects of at least some primary rewards, it was hypothesized that VTA mACh receptor stimulation might be involved in feeding-related learning. Thus, the present experiments were aimed at investigating whether blockade of mACh receptor stimulation in the VTA, via microinjections of scopolamine, would attenuate eating, food reward and food-related learning. Thus, these experiments address the

hypothesis posed by specific aim 2 stimulation of VTA mAChR, but not of nAChR, at the level of the VTA is necessary for the acquisition, but not the expression, of a free-feeding task.

## Methods

*Subjects and Materials*

Please refer to Chapter 3 (General Methodologies) for a full description of the subjects, surgery, apparatus, microinjection procedure, drugs and doses, histology procedure, and data analysis.

*Experiment 3: Free-Feeding*

One week following surgery, rats (N=22) were exposed to a feeding paradigm consisting of three distinct phases. During each phase, rats were placed in feeding chambers containing 300 food pellets and kept there for 30 minutes. The number of pellets remaining in the chamber at the end of the 30 minute session was recorded. Phase I consisted of four consecutive sessions held one per day, in which rats received bilateral microinjections of either 0 (N=5) or 5 (N=7) μg/0.5 μl of scopolamine or either 0 (N=5) or 10 (N=5) μg/0.5 μl of mecamylamine directly into the VTA immediately prior to being placed in the feeding chambers. Phase II consisted of five consecutive sessions held one per day, in which rats were placed in the feeding chambers without drug pretreatment. Phase III consisted of a single test session in which those rats that received the 0 μg dose of scopolamine in Phase I received the 5μg dose of scopolamine, and vice versa. Those rats that received the 0 μg dose of mecamylamine in Phase I received the 10 μg dose in Phase III, and vice versa.

*Experiment 4: Acquisition of Conditioned Reward*

One week following surgery, rats (N=15) were exposed to a conditioned reward paradigm consisting of 2 distinct phases referred to as pre-exposure and conditioning phases.

During the pre-exposure phase, rats were placed in the operant conditioning chambers for five 40-minute sessions held one per day on five consecutive days. During this phase, pressing on one lever produced a lights-on stimulus for 3 seconds while pressing on the other lever produced a tone stimulus for 3 seconds. The lever associated with the lights-on stimulus was on the right side for half of the chambers and on the left side for the other half. The number of responses made on each lever during each pre-exposure session was recorded.

The conditioning phase consisted of four consecutive 60-minute sessions, held one per day on four consecutive days beginning two days after the last pre-exposure session. For each conditioning session, both levers were removed from the chambers. During each conditioning session, rats were exposed to 81 presentations of the 3-s lights-on stimulus according to a random time 45-s schedule. A randomly selected one-third of these presentations (27 presentations) were paired with the delivery of two 45 mg food pellets. Food presentations were partially, rather than continuously, paired with the lights-on stimulus presentations because Knott and Clayton (1966) demonstrated a greater magnitude of conditioned reward following partial pairing.

Rats were injected with either 0 or 5 μg/0.5 μl (Ns = 7 and 8, respectively) of scopolamine in the VTA prior to each conditioning session in order to assess the effects of scopolamine on the acquisition of the conditioned reward effect.

*Experiment 5: Anatomical Analysis*

One week following surgery, rats with cannula placements in which the injector was positioned 2 mm dorsal to the VTA were exposed to Phase I of the free feeding paradigm (N=10). Rats received microinjections of 0 (N=5) or 5 (N=5) μg/0.5 μl of scopolamine prior to each test session.

## Results

*Experiment 3: Free-Feeding*

*Phase I: Drug-Pretreatment*

In the first session of Phase I, rats that received the 0 μg dose of scopolamine and those that received the 5 μg dose of scopolamine ate less than one third of the total number of food pellets available and there was no significant difference between them in total food pellets consumed. In sessions 2 through 4, vehicle controls increased and maintained total food consumption to near maximal levels (263 ±22.35 pellets) while scopolamine pretreated rats did not show this increase (see Fig. 7a). A two-way factorial ANOVA with dose of scopolamine and day of injection as factors revealed a significant dose by day interaction ($F_{(3, 30)} = 33.03$, $P<.0001$). Tests of simple main effect of day at each dose revealed a significant day effect for vehicle controls ($F_{(3, 30)} = 38.00$, $P< .0001$), but not for scopolamine treated rats. Bonferroni tests on the data from the vehicle controls showed that consumption in the first test session differed significantly from that in the three subsequent test sessions ($P<.007$), while the subsequent test sessions did not differ from one another.

In sessions 1 through 4 of Phase I, rats that were pretreated with mecamylamine (0 or 10 μg) demonstrated a significant daily increase in total food consumption (see Fig. 7b). A two-way factorial ANOVA with dose of mecamylamine and day of injection as

factors revealed a significant day effect ($F_{(3, 24)} = 20.66$, $P<.0001$), but no day by dose interaction. Bonferroni tests on the data from rats that received the 0 or 10 μg doses showed that at both dose levels food consumption increased significantly from the first to the fourth feeding session ($P<.05$). Rats that had received a mecamylamine dose were slightly younger and weighed less than those rats that had received a scopolamine dose, which may account for the lower level of pellet consumption.

*Phase II: No drug-pretreatment*

In Phase II, rats that had previously received the vehicle dose of scopolamine continued to eat near maximal levels of food pellets in each session. Rats that had previously received scopolamine microinjections ate increasingly greater numbers of food pellets during each session and by the 4th and 5th session of this phase ate near maximal amounts of food and appeared similar to the vehicle controls (see Fig. 7a). A two-way factorial ANOVA comparing dose and day (the last day of Phase I and the first day of Phase II) revealed a significant dose effect ($F_{(1, 10)} = 37.12$, $P<.0001$), but no day effect nor day by dose interaction. However, an ANOVA on the data from all five feeding sessions of Phase II revealed a dose by day interaction ($F_{(4, 40)} = 9.26$, $P<.0001$). Tests of simple main effect of day at each dose revealed a significant day effect in rats that had previously received scopolamine microinjections ($F_{(4, 40)} = 24.65$, $P< .0001$), but not in rats that had previously received the vehicle dose. Bonferroni tests on the 5 μg dose data showed that consumption on the first and second feeding sessions differed significantly from the three subsequent feeding sessions ($P<.05$), while the last two sessions of Phase II did not differ from each other. On the fifth, and final, feeding

session of Phase II, there was no significant difference in the number of pellets consumed between the groups.

In Phase II, rats that previously received the vehicle or the 10 μg doses of mecamylamine maintained the same level of pellet consumption throughout all five feeding sessions (see Fig. 7b). A two-way factorial ANOVA comparing dose and day (the last day of Phase I and the first day of Phase II) revealed neither a dose effect nor a dose by day interaction. Similarly, an ANOVA on the data from all five free-feeding sessions of Phase II revealed neither a day effect nor a dose by day interaction.

*Phase III: Dose reversal*

In Phase III, when rats that had previously received the 0 μg dose of scopolamine in Phase I now received the 5 μg dose of scopolamine, there was a decrease in the number of pellets consumed. However, an independent samples t-test comparing pellet consumption between rats that received the 0 and the 5 μg doses during phase III revealed that the difference between the groups was not significant (see Fig. 7a). Although there was a large variability in the amount of feeding by those rats that received the 5 μg dose during Phase III, all rats consumed at least 100 food pellets, a robust amount of feeding much greater than that seen during Phase I.

In Phase III, when rats that had previously received the 0 μg dose of mecamylamine in Phase I now received the 10 μg dose, and vice versa, pellet consumption remained at maximal levels regardless of drug treatment (see Fig. 7b).

*Experiment 4: Acquisition of Conditioned Reward*

Microinjections of scopolamine into the VTA prior to each of the four conditioning sessions resulted in reduced food consumption during sessions 2 to 4 (see

Fig. 8). Rats that received the 5, compared to the 0, μg dose of scopolamine consumed a significantly smaller number of pellets on all but the first day of injection. The rats that received the 0 μg dose of scopolamine maintained the same (maximal) level of food consumption throughout all four test sessions, while rats that received the 5 μg dose of scopolamine reduced their food consumption from their initial 47 ±4.32 pellets on the first day of testing to 16.87 ±5.60 on the second and approximated this reduced level in the subsequent sessions. A two-way ANOVA with dose and day of injection as factors revealed a significant dose by day interaction ($F_{(3, 36)} = 3.425$, $P<.02$). Tests of simple main effect of day at each dose revealed a significant day effect in the 5 μg dose of scopolamine group [$F_{(3,36)} = 5.29$, $P<.02$]. The number of pellets consumed on the first day of injection was not significantly different between the groups.

*Experiment 5: Anatomical Analysis*

There were no differences in the number of pellets consumed between rats that received the 0 or the 5 μg doses of scopolamine in a site 1.5-2.0 mm dorsal to the VTA in each of the four sessions in Phase I (see Fig. 9). Furthermore, both groups exhibited daily increases in pellet consumption.

*Histology*:

Most of the VTA microinjection sites were localized in the ventromedial portion of the VTA with some injections occurring in the ventrolateral portion (see Fig. 10 and 11). Microinjections aimed at the anatomical control sites were approximately 1.5 to 2.0 mm dorsal to the VTA injection sites (see Fig. 10).

***Figure 7*:** Mean pellet consumption for rats receiving bilateral microinjections of (a) scopolamine (0 or 5 μg/0.5 μl) or (b) mecamylamine (0 or 10 μg/0.5 μl) during 3 phases of a free-feeding task. Immediately before sessions 1 to 4, rats were pretreated with their respective doses of either compound, before sessions 5 to 9 rats received no drug pretreatments, and before session 10 rats received the opposite drug pretreatment. Vertical lines represent the standard error of the mean (SEM).

***Figure 8:*** Mean pellet consumption for rats receiving bilateral microinjections of scopolamine (0 or 5 μg/0.5 μl) during the conditioning phase of the conditioned reward paradigm in which a lights-on stimulus was paired with the delivery of food. Vertical lines represent the standard error of the mean (SEM).

***Figure 9*:** Mean pellet consumption for rats receiving bilateral microinjections of scopolamine (0 or 5 μg/0.5) into the VTA or a site 2 mm dorsal to the VTA. Vertical lines represent the standard error of the mean (SEM).

***Figure 10:*** Histological reconstruction of injection sites adapted from Paxinos and Watson (1986). Black circles for injections in the VTA group; grey circles for injections in the dorsal control group. The numbers to the right of each section indicate the distance posterior to bregma.

***Figure 11****:* A representative photomicrograph depicting the cannula tips in the ventrolateral portion of the VTA.

**Figure 7a:**

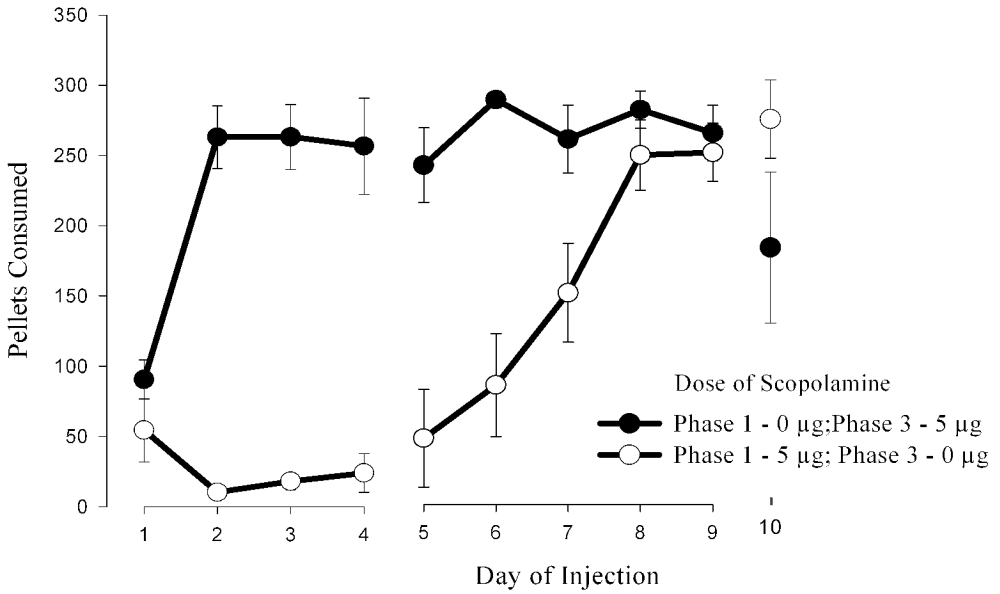



**Figure 7b:**

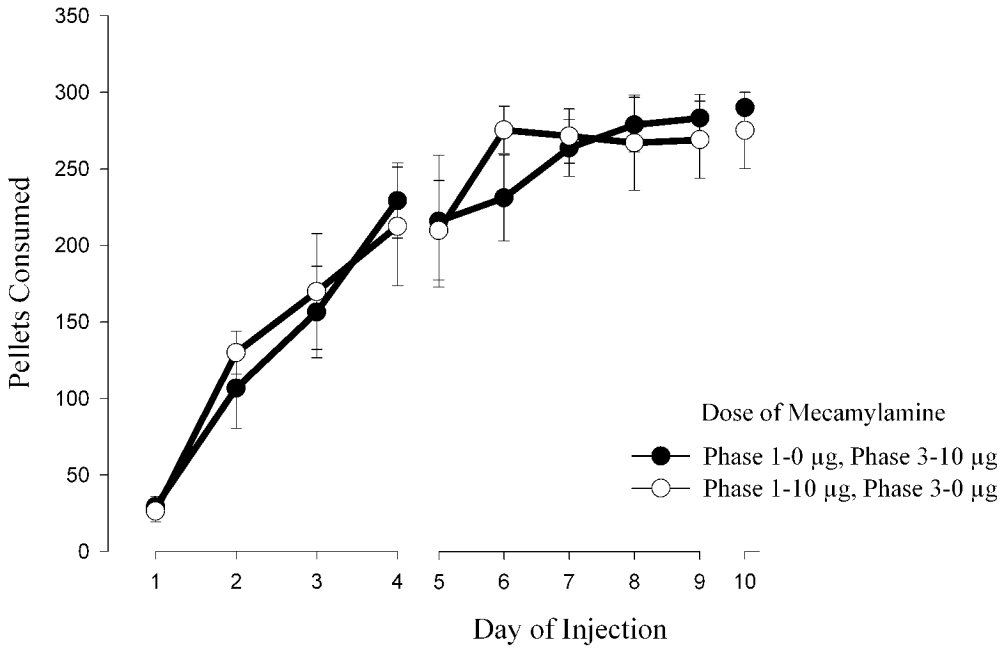


**Figure 8:**

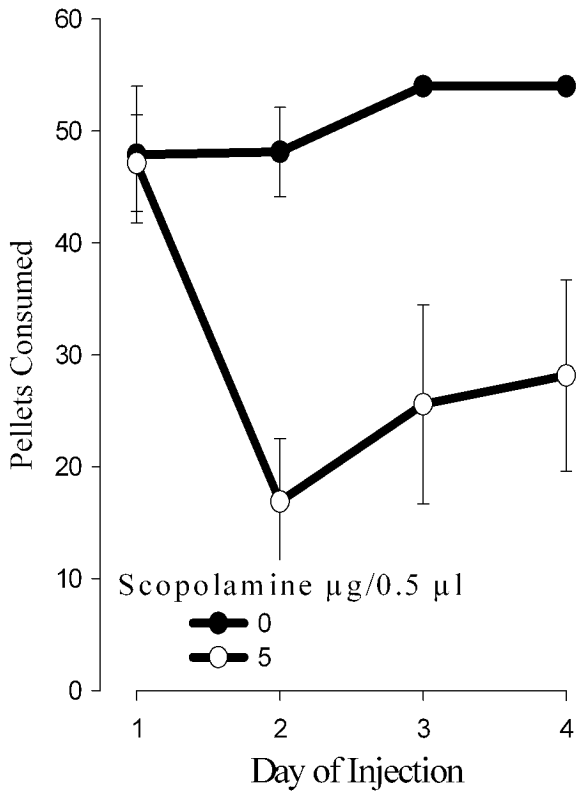



**Figure 9:**

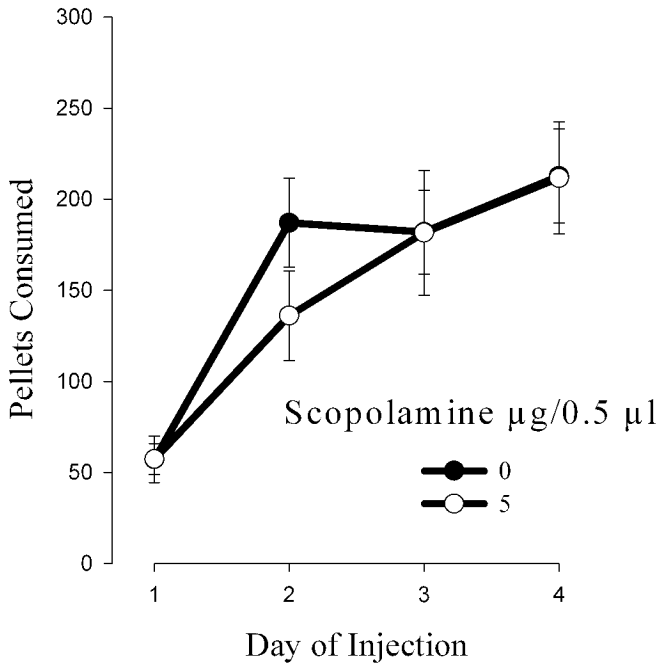


**Figure 10:**

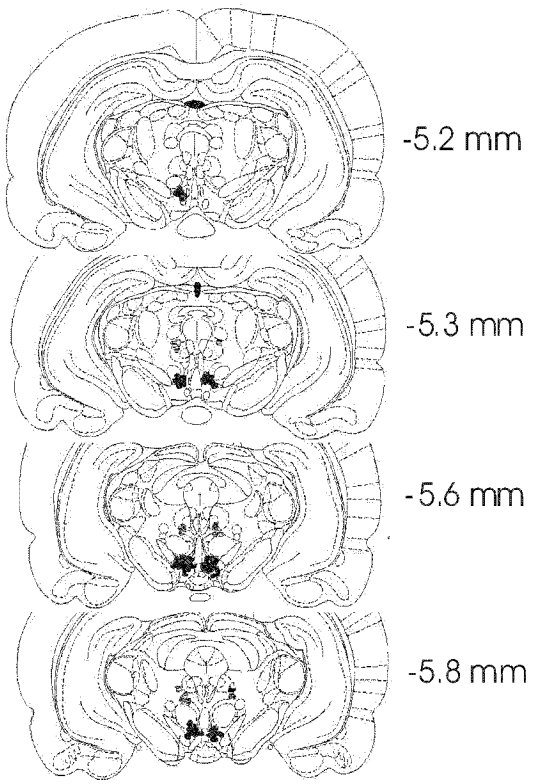
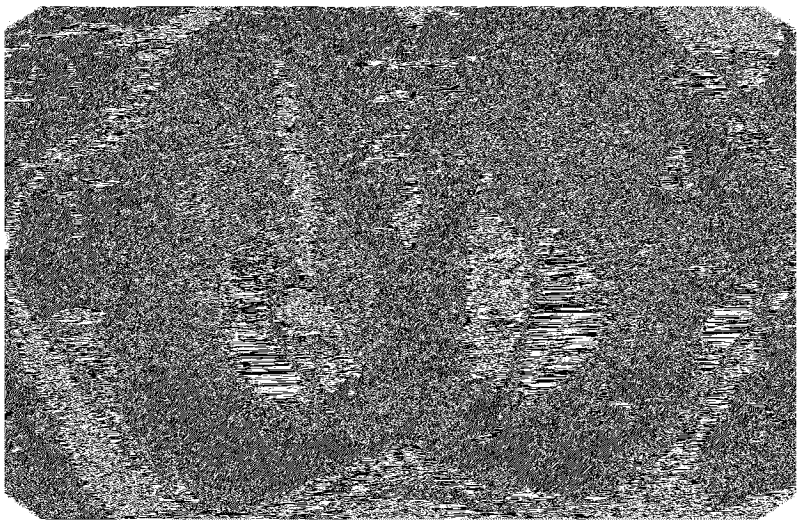



***Figure 11:***



## Discussion

The role of acetylcholine neurotransmission at mACh receptors in the VTA in feeding-related learning was investigated. Microinjections of scopolamine during Phase I where animals were presented with the opportunity to learn a new task eating a novel food (pellets) in a novel environment (experimental cage) resulted in the animals eating significantly less food than vehicle-treated control animals presented with the identical learning task. When scopolamine treatment was terminated, animals ate greater amounts of food daily until their food intake resembled that of the vehicle control group. It is not likely that the eating deficit in the scopolamine-treated rats resulted from non-specific motoric effects of the treatment as intra-VTA scopolamine injections prior to the already learned eating task in the initially vehicle-treated rats did not significantly reduce eating, suggesting no scopolamine-induced performance deficits of this task. Rather, it appears that scopolamine interfered with the acquisition of this novel eating task. On the other hand, intra-VTA mecamylamine, a nAChR antagonist, impaired neither the acquisition nor the expression of the free-feeding response. Altogether, these data suggest that VTA acetylcholine stimulation of mAChR, but not of nAChR, is necessary for the acquisition of a free-feeding response and that neither is necessary for the expression of the learned response.

The failure of scopolamine microinjections in a site 1.5-2.0 mm dorsal to the VTA to reduce consumption rules out the possibility that reductions observed following intra-VTA injections were due to diffusion of the drug to a dorsal site (for explanation, see Chapter 4), supporting the conclusion that the behavioral effects of the intra-VTA microinjections of scopolamine were local.

It is unlikely that the present findings were due to scopolamine-induced satiation effects instead of motivational or learning deficits. During the free-feeding task, the average number of pellets consumed by rats that received the 5 μg dose of scopolamine on days two through four was 17.33 (±3.97) whereas rats receiving the same dose of scopolamine during Phase III consumed an average of 170 (±51.39), approximately ten times the amount consumed by scopolamine-treated rats in Phase I. Thus, satiation cannot account for the reduction in food consumption.

Rats that received scopolamine during Phase I of the free feeding experiment did not show maximal pellet consumption until the last days of Phase II with a gradual acceleration of pellet consumption; that is, these animals demonstrated feeding patterns that resembled a typical learning curve. It is not likely that the low food consumption in the initial sessions of Phase II was maintained by possible long-lasting effects of scopolamine carried over from the previous injections. Carry-over scopolamine effects should have produced cumulatively larger daily effects on pellet consumption in the free-feeding task, and this was not observed. Therefore, the gradual increase in pellet consumption after scopolamine-treatment was stopped reflects some other, likely behavioral, mechanism.

Rats that received scopolamine prior to conditioning sessions in which a lights-on stimulus was paired with a food stimulus showed the same pattern of low levels of food consumption as did their counterpart rats in the free-feeding paradigm. It can be deduced that the reduced eating is not a consequence of food availability. Rats in the conditioning sessions received two pellets per discrete trial while rats in the free-feeding sessions had 300 pellets available simultaneously, yet both groups demonstrated similar inter-session

patterns of consumption. Furthermore, the lights-on stimulus in the conditioning sessions, which presumably predicted the delivery of the food pellets, did not affect this inter-session pattern. Thus, blockade of mACh receptor stimulation in the VTA reduced food consumption regardless of whether the food was available in its entirety or delivered progressively and regardless of whether a predictive CS was present or absent.

It is conceivable that scopolamine reduced feeding in Phase I because it produced neophobia-related or other anxiogenic effects that interfered with eating. However, two observations argue against this possibility. First, if scopolamine produced anxiogenic effects that interfered with eating then rats treated with scopolamine should have demonstrated lower consumption during the first session of phase I than rats not receiving scopolamine who, presumably, would not be experiencing anxiety. However, both groups of rats consumed the same amount of food on the first session of Phase I. Second, the pattern of food consumption in rats that received scopolamine in the conditioning phase of the conditioned reward paradigm was similar to that of rats that did not receive scopolamine. The fact that the scopolamine-treated rats were already habituated to the conditioning chambers by the time feeding occurred and still demonstrated reduced food consumption argues against the possibility that some novelty-related behavioral effect of the environment itself was a factor in reducing feeding.

Scopolamine in the VTA may have blocked learning in the present study. The gradual increase in consumption after scopolamine-treatment was stopped suggests that animals learned new environment-response associations that is, they learned associations between feeding chamber stimuli and feeding. The fact that this gradual increase in feeding did not occur when animals received daily scopolamine

microinjections suggests that scopolamine prevented the formation of associations between the environment in which feeding could occur and the behavioral requirements of the feeding task. Thus, it is possible that the novel environment that could become associated with feeding remained an environment not associated with feeding on each occasion in which the rats received scopolamine and only when scopolamine treatment was stopped were the animals able to form associations between the feeding environment and feeding. The fact that the rate of learning following cessation of scopolamine treatment was slower than the rate of learning for those rats who were not treated with scopolamine can be explained in terms of latent inhibition (Lubow and Moore, 1959), in which rats may have learned that either the environment is irrelevant to the feeding task or that consumption of the food does not result in reward. The observed rate difference supports the notion that blockade of mACh receptor stimulation in the VTA prevented acquisition of a feeding task.

The current findings suggest that intra-VTA microinjections of scopolamine reduced free-feeding and impaired the acquisition, but not the expression, of the feeding task. Therefore, stimulation of mACh receptors in the VTA appears to play a role in the learning of associations between the feeding environment and feeding, but not in the performance of this learning.

## Chapter 6: Blockade of acetylcholine receptors in the ventral tegmental area fails to reduce the motivational effects of food.

Thus far, the literature suggests that acetylcholine neurotransmission in various brain regions is essential for the rewarding effectiveness of rewarding stimuli and the motivation to obtain such stimuli. For instance, acetylcholine receptors located on DA cells in the VTA appear to be crucial for brain stimulation reward as intra-VTA microinjections of the non-selective mAChR antagonists, atropine and scopolamine, raise lateral hypothalamus brain stimulation thresholds (Yeomans et al., 1985; Kofman & Yeomans, 1988). Furthermore, one of the most widely used and one of the most addictive drugs, nicotine, achieves its rewarding effects by activating the mesolimbic DA system via activation of nAChR in the VTA (Corrigall et al., 1994). Therefore, both receptors subtypes have been shown to contribute to reward and motivation.

Although it appears that VTA mACh receptor stimulation is involved in brain stimulation reward, a definitive role for its involvement in food reward remains non-conclusive. Infusion of a high dose of a mACh receptor antagonist into the VTA attenuated approach and consummatory responses for a sucrose solution in a runway alley, but it also attenuated general activity making it impossible to conclude that its effects were related to food reward *per se* (Ikemoto and Panksepp, 1996). Therefore, it remains uncertain whether the neural mechanisms underlying food reward are the same as those underlying nicotine and/or brain stimulation reward.

The findings presented in chapters 4 and 5 point to a role of mAChR in the VTA in food-related operant and free-feeding learning. Blockade of these receptors prevented the acquisition, but not the *expression*, of reward-related behavioral responses.

Therefore, it appears that mAChR-mediated DA cell excitation is critical for the rewarding effectiveness of food during the acquisition phase; however, during the acquisition phase, another neural network adopts the ability to excite these DA cells so that following the acquisition phase, DA cell excitation is mediated by some other mechanism, and is no longer dependent upon the mAChR signal. Therefore, it is highly probable that mAChR activation is necessary for neither the motivation to obtain a food reward nor the rewarding effects of the food stimulus in fully trained animals.

Although the findings presented in chapters 4 and 5 suggest that mAChR do not play a role in the motivation to obtain a food-reward in fully trained animals, these findings, by themselves, remain inconclusive. The experimental manipulations presented thus far have required a relatively low response-to-reward ratio (i.e., work requirement to obtain a reward). An emerging body of literature cautions against making interpretations of such data due to a possible interaction between behavioral effects and response requirements (Salamone et al., 1997; Salamone and Correa, 2002). For example, several DA antagonists, including SCH 23390 and SKF 83566, have been shown to reduce lever-pressing for food, but to increase *al libitum* feeding (Salamone et al., 1991, 1997; Koch et al., 2000), suggesting that drug-induced behavioral effects may be a function of the response-to-reward ratio rather than a reduction in motivation. Therefore, to fully address the question of mAChR in motivation, it is necessary to investigate scopolamine-induced behavioral effects in a paradigm with a high response-to-reward ratio, such as lever-pressing under a progressive ratio schedule of reinforcement.

The following experiment addresses the hypothesis posed by Specific Aim 3 neither mAChR nor nAChR at the level of the VTA is necessary for the rewarding effectiveness of, and the motivation to obtain, a food reward. In this experiment, animals are tested with scopolamine and mecamylamine under a progressive ratio schedule of food reinforcement, which will supplement the findings discussed in chapters 4 and 5.

## Methods

*Subjects and Materials*

Please refer to Chapter 3 (General Methodologies) for a full description of the subjects, surgery, apparatus, microinjection procedure, drugs and doses, histology procedure, and data analysis.

*Experiment 6: Operant responding under a progressive ratio schedule*

One week after surgery, rats (N=14) were trained to press a lever reinforced by food on a fixed ratio 1 (FR1) schedule of reinforcement (i.e., each lever press resulted in the delivery of a single food pellet). When animals demonstrated acquisition of the lever press response, operationally defined as 3 consecutive sessions where the total number of rewards per session was greater than 200, a progressive ratio (PR) schedule of reinforcement was introduced. Rats demonstrated acquisition on the FR1 schedule of reinforcement within the first 5 sessions. The reward magnitude was maintained at 1 food pellet. Under the PR schedule, the response requirement was set to 1 for the first reward and increased exponentially for each subsequent reward according to the formula: $5 \times e^{(\text{reward\#} \times 0.22)} - 5$. The ratios generated by this formula are 1, 3, 5, 7, 10, 14, 18, 24, 31, 40, 51, 65, 82, 104, 131 and so on. Under this schedule, the ratio requirement ultimately becomes so high that rats cease to respond. The point at which rats stop responding is

referred to as the break point (BP). BPs were operationally defined as the final number of ratios completed (which resulted in the delivery of a food reward) within 15 minutes of the previous one. Stable BPs were defined as three consecutive sessions during which BPs did not vary by more than ±10% of the mean BP of the three sessions. Rats demonstrated stable responding within the first five days of training under this schedule.

After BPs stabilized, the rats were tested with bilateral microinjections of either scopolamine (0, 2.5 or 5 μg/0.5 μl) or mecamylamine (0, 5 or 10 μg/0.5 μl) into the VTA prior to the test sessions. Each rat was tested with as many of the doses as possible; due to illness and/or out-of-target range weight fluctuations, 4 rats did not complete all 4 test sessions. Each test occurred on a different day and stable BPs were re-established between pairs of test sessions. At least 3 non-test sessions were conducted between one test session and the next.

## Results

*Experiment 6: Progressive Ratio Responding*

Microinjections of the 2.5 or 5 μg doses of scopolamine directly into the VTA failed to reduce BPs and resulted in BPs that approximated baseline performance (see Fig. 12a). A one-way ANOVA with dose of scopolamine as a factor revealed no significant dose effect. Similarly, microinjections of the 5 and 10 μg doses of mecamylamine into the VTA also failed to reduce BPs (see Fig. 12b). A one-way ANOVA with dose of mecamylamine as a factor revealed no significant differences among the three doses.

*Histology*:

Most of the VTA microinjection sites were localized in the ventromedial portion of the VTA with some injections occurring in the ventrolateral portion (see Fig. 5 and 6 in Chapter 4). Microinjections aimed at the anatomical control sites were approximately 1.5 to 2.0 mm dorsal to the VTA injection sites (see Fig. 5 in Chapter 4).

***Figure 12*:** Mean percentage of baseline BPs in rats receiving bilateral microinjections of (a) scopolamine (0, 2.5 or 5 μg/0.5 μl) or (b) mecamylamine (0, 5, or 10 μg/0.5 μl) into the VTA prior to each test session. Vertical lines represent the standard error of the mean (SEM).

**Figure 12a:**

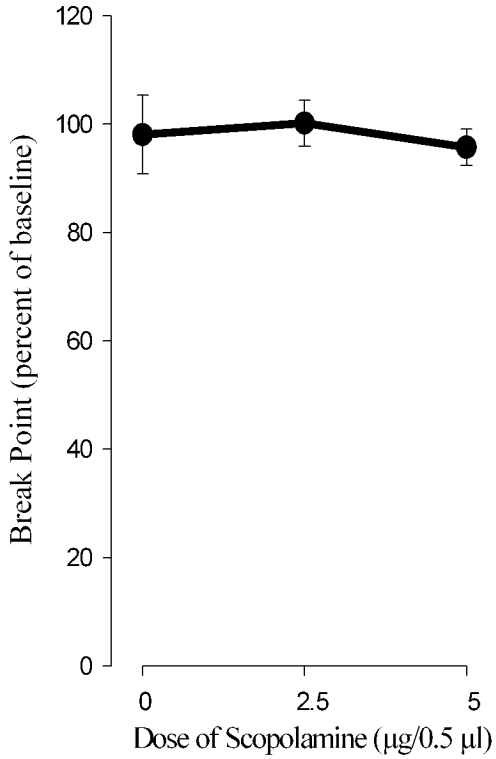
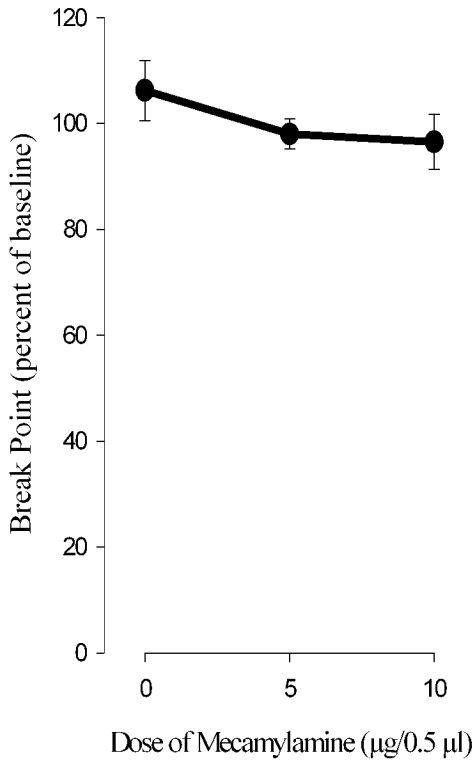



**Figure 12b:**



## Discussion

Blockade of acetylcholine neurotransmission at either muscarinic or nicotinic receptors did not affect BPs for food-rewarded responding under a PR schedule of reinforcement. These findings in combination with those presented in chapter 4 that neither scopolamine nor mecamylamine reduced lever pressing on an FR schedule of food reinforcement suggest that blockade of acetylcholine receptors in the VTA does not reduce the rewarding effectiveness of, or the motivation to obtain, a food reward. These findings are in opposition to previous findings in which mAChR antagonist attenuated feeding (Rada et al., 2000) and approach responses for a sucrose solution (Ikemoto and Panksepp, 1996).

In conjunction with the previous results presented within this dissertation, these results are not surprising. Given the findings that blockade of VTA nAChR failed to affect the acquisition or expression of responding for food under a FR schedule of reinforcement, it is not surprising that it had no effect on responding under a PR schedule. These findings suggest that stimulation of nAChR in the VTA is not involved in food reward. However, the finding that blockade of mAChR stimulation failed to reduce BPs  interpreted as a failure to reduce food reward  does not necessarily imply that VTA ACh action at muscarinic receptors is not involved in food reward. Rather, it suggests that mAChR do not play a role in food reward and/or motivation to obtain food once the food and/or environment is no longer novel. Altogether, the present data suggest that mAChR are involved in the *acquisition* of FR responding, but not *expression* of FR responding nor *expression* of PR responding.

As previously mentioned, one possible explanation is that VTA mAChR stimulation constitutes part of a food reward signal and that this reward signal is present and necessary for the acquisition of operant responding and still present but *no longer necessary* for the expression of an already acquired operant response. That is, during the acquisition phase, activation of mAChR is necessary for reward-paired environmental stimuli to adopt incentive-motivational effects. After training has occurred, reward-directed behaviors are initiated and/or maintained by environmental stimuli signals, eliminating the necessity of the acetylcholine signal.

## Chapter 7: General Discussion

The present set of experiments investigated the role of acetylcholine neurotransmission in the VTA in feeding, food-reward, and food-related learning. This was accomplished through pharmacological manipulations of the cholinergic system. To antagonize cholinergic actions in the VTA, intra-VTA microinjections of scopolamine, a non-selective mAChR antagonist, and mecamylamine, a non-selective nAChR antagonist, were administered. The effects of these cholinergic agents on food-reward and food-related learning were assessed in separate behavioral paradigms.

In the first set of experiments (see chapter 4), the effects of mAChR and nAChR blockade on the acquisition and expression of a food-maintained operant response were assessed. Pharmacological manipulations were made in rats during training on a fixed ratio (FR1) schedule of reinforcement or following training (i.e., once rats have demonstrated maximal performance). The results showed that blockade of mAChR prevents the acquisition of a lever-pressing for food operant response, whereas nAChR antagonists had no effect. Blockade of neither mAChR nor nAChR affected lever-pressing once animals were fully trained. Similar manipulations at a dorsal site failed to affect acquisition of the lever-press response, suggesting that the behavioral effects of scopolamine were local to the VTA.

In the second set of experiments (see chapter 5), the effects of mAChR and nAChR antagonists on free-feeding and in the acquisition and expression of a free-feeding task were assessed. Again, pharmacological manipulations were made in rats during the acquisition phase and following training once rats demonstrated maximal pellet consumption. The results showed that blockade of mAChR reduced feeding in rats

when a novel food is presented in a novel environment. Similarly, blockade of mAChR prevented the acquisition of a free-feeding task, whereas it had no effect on the expression of the task, once the task has been learned. Blockade of nAChR failed to affect feeding or acquisition and expression. Similar manipulations at a dorsal site failed to affect acquisition of the feeding response, suggesting that the behavioral effects of scopolamine were local to the VTA.

Finally, in the third set of experiments (see chapter 6), the effects of mAChR and nAChR on the rewarding and motivational effects of food were assessed. Here, pharmacological manipulations were made after rats were trained to lever-press for food under a progressive ratio (PR) schedule of reinforcement. The results showed that in animals fully trained to lever-press for food, blockade of mAChR or nAChR failed to affect BPs; hence, blockade of acetylcholine neurotransmission in the VTA failed to affect the motivation to obtain a food reward.

These findings suggest a key role for mAChR in the VTA in feeding and in the rewarding effects of a food stimulus in a novel feeding task. Blockade of mAChR attenuated lever-pressing for food and food consumption when animals were presented with a novel food stimulus and/or a novel feeding environment. The reduction of behavioral responding to obtain and/or consume a food reward suggests that mAChR are essential for the motivational effects of the food stimulus. The motivational effects of food, and other rewarding stimuli, are often attributed to an increase in DA cell firing and DA release in various terminal regions along the mesocorticolimbic pathway (see Chapter 1). It is highly probable that acetylcholine signal at mAChR is essential for the

motivational effects of a food reward in a novel feeding task since it is the primary signal that excites these DA cells.

These findings further suggest that acetylcholine signals via mAChR are crucial for reward-related learning. During the acquisition phase, synaptic plasticity is presumed to occur, in which another unknown neural mechanism adopts the capability to activate DA cells and cause DA release; thus, VTA mAChR stimulation is no longer the primary signal for initiation and or maintenance of responding. As an animal learns a food-related task, it forms associations between relevant environmental stimuli and the feeding and or operant responding task. It is presumed that synaptic connections between environmental signals and DA cells become strengthened as a result of paired presentations. As a result, following acquisition, DA cells come under the control of the environmental stimuli and mAChR activation is no longer necessary for their activation.

As an animal begins to consume a food-stimulus, an acetylcholine signal activates DA cells. At the same time, glutamate signals, presumably via NMDA receptors, are also activated in response to environmental stimuli. Because the DA cells are activated at the same time that the glutamate receptors are activated, it is likely that synaptic plasticity occurs, resulting in a strengthening of the synapse between the glutamate afferents and the VTA DA cell. Due to this synaptic enhancement, the glutamate signals acquire the ability to independently activate the DA cells. Therefore, following pairing, it is the conditioned environmental stimuli that are responsible for the motivational effects of the food; that is, the conditioned stimuli have adopted incentive motivational effects and are now capable of eliciting goal-directed behaviors, eliminating the need for the mAChR signal.

The present findings are the first to link activation of mAChR in the VTA to the *acquisition* of reward-related learning. Although mAChR in other brain regions have been previously implicated in the acquisition and expression of a lever-pressing for sucrose task (Pratt and Kelley, 2004) and in the acquisition of a cocaine-associated conditioned reward (See et al., 2003), this is the first demonstration of their role in the VTA in the acquisition of a food-maintained operant response. Additionally, these data suggest a dissociation between cholinergic functions in the VTA and NAcc. Microinjections of scopolamine into the NAcc were found to impair the acquisition *and* performance of a lever-pressing for sucrose task (Pratt and Kelley, 2004), whereas the present findings show that intra-VTA scopolamine does not affect lever-pressing in fully trained animals.

The present findings that blockade of mAChR in the VTA blocks feeding are in accordance with previous findings in which intra-VTA infusions of the muscarinic antagonist atropine partially inhibited feeding (Rada et al., 2000) and disrupted both consummatory and approach responses for sucrose (Ikemoto and Panksepp, 1996). Altogether, these findings suggest that the primary unconditioned food stimulus is signaled by a cholinergic input to the VTA and that this signal is necessary for the rewarding effectiveness of food. Interestingly, however, blockade of mAChR during the expression of the free-feeding task failed to affect pellet consumption, suggesting that mAChR play a role in feeding only when the food and/or the environment is novel.

Thus far, several neurotransmitter systems have been implicated in feeding behaviors, including DA, glutamate, GABA, and acetylcholine. In particular, it is the actions of these neurotransmitters in the NAcc that has been implicated in the mediation

of feeding and drinking behaviors (Hoebel, 1997; Salamone et al., 1997; Stratford and Kelley, 1997; Rada et al., 1998). Evidence for the role of DA comes from microdialysis and voltammetry studies in which feeding is associated with significant increases in DA levels in the NAcc (Pfaus et al., 1990; Wenkstern et al., 1993; Di Chiara, 1995; Wilson et al., 1995; Richardson and Gratton, 1996). Similarly, previous studies have demonstrated a role of glutamate and GABA neurotransmission in the NAcc in feeding. For instance, microinjections of non-NMDA receptor antagonists or GABA agonists into the NAcc induced feeding (Kelley and Swanson, 1997; Stratford and Kelley, 1997; Stratford et al, 1998). Thus, it is not surprising that blockade of mAChR alone does not prevent ingestive behaviors. Based on these findings, it is possible that following acquisition of a feeding task, other neurotransmitter systems play a larger role compared to acetylcholine or that compensatory mechanisms are applied to sustain the biologically important act of feeding. Altogether, previous findings, in combination with the present findings, support the widely accepted viewpoint that feeding is controlled by an intricate neural circuitry, in which several neurotransmitter systems are highly involved.

An interesting result of the present experiments is that blockade of nAChR failed to affect either food-reward or food-related learning. A role of nAChR in reward has been established by studies in which systemic administration of nicotine enhanced DA release, particularly in the NAcc (Imperato et al., 1986), and this enhancement is reduced following microinjections of mecamylamine into the VTA. Further evidence is provided by studies in which DHßE, a nicotinic blocker, infused into the VTA attenuated lever-pressing for nicotine, whereas atropine, a muscarinic antagonist, failed to affect it (Corrigall et al., 1994). Recently, it has also been discovered that activation of nAChR

contributes to the rewarding effects of hypothalamic brain stimulation reward, although mAChR appear to be much more important (Yeomans and Baptista, 1997). Thus, the current data, in conjunction with previous findings, suggest that nAChR contribute differentially to the rewarding effectiveness of various rewarding-stimuli. Since in the present studies blockade of nAChR failed to affect feeding or BPs for animals lever-pressing for food, it is not surprising that it also failed to affect the acquisition and expression of food-related learning.

During the training process, reward-associated environmental stimuli may have become *incentive motivational stimuli* that is, they may have acquired the ability to elicit approach or other behavioral responses similar to the primary reward. Previous findings suggest that mesocorticolimbic DA plays a role in the acquisition of incentive effects by neutral stimuli (Salamone, 1994; Kiyatkin, 1995; Schultz et al., 1997). During training, DA cells respond equally to the primary reward and to reward-associated environmental stimuli (Ljungberg et al., 1992; Schultz et al., 1993; Mirenowicz and Schultz, 1994; Fiorillo et al., 2003; Takikawa et al., 2004); however, following training, the same DA cells respond selectively to environmental cues rather than to the primary reward (Miller et al., 1981; Kosobud et al., 1994; Kiyatkin and Rebec, 2001; Hyland et al., 2002). Thus, reward-related learning appears to involve a shift of DA cell activation (which is essential for reward and reward-related motoric behaviors) from the primary reward to reward-predicting environmental stimuli.

The present findings that neither mAChR nor nAChR play a role in food-directed motivational responses are the first to conclusively demonstrate the role of acetylcholine in food reward. As previously mentioned, some evidence exists that mAChR play a role

in food reward and the motivational effects of food, including the studies in which atropine attenuated feeding (Rada et al., 2000) and approach responses for sucrose (Ikemoto and Panksepp, 1996). However, in the study in which atropine reduces approach behaviors, overall motoric responses were reduced as well, leaving it questionable as to whether atropine reduced motivation. The present data suggest that mAChR may play a role in food-reward when food and/or food-associated environmental stimuli are novel, but once the task is acquired, they no longer play a role in the motivation to lever-press for food nor to feed. Similarly, fully trained animals responding under a PR schedule of food reinforcement maintain responding even when pretreated with scopolamine suggesting that another neural mechanism is guiding motivation.

## Conclusions

The present experiments support the hypothesis that acetylcholine neurotransmission via mAChR, but not nAChR, is a critical component in the acquisition, but not the expression of reward-related learning. The results from chapter 4 provide evidence that intact functioning of mAChR is required for the acquisition of a food-maintained operant response, whereas neither mAChR nor nAChR are required for the expression of the task. The results from chapter 5 demonstrate that importance of mAChR in feeding when the food and/or the environment in which feeding occurs is novel, but no longer contributes following their habituation. Furthermore, activation of mAChR, but not of nAChR, is crucial for feeding-related learning to occur, but neither is necessary for it to be expressed. Finally, the results from chapter 6 indicate that in fully trained animals, neither mAChR nor nAChR are necessary for the motivation to lever-

press for food under a PR schedule of food-reinforcement. Altogether, these data suggest that an intricate neural circuitry, involving VTA mAChR, is involved in feeding, food-reward, and reward-related learning.

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