ABSTRACT

The Interactions of Nicotine and Ethanol in the Brain: Can consuming alcohol cause nicotine cravings in people who have previously used both drugs together?

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Alcohol and nicotine use are both highly prevalent in the United States. It is estimated that over 19% of the population over the age of 18 smokes cigarettes, while over 51% of people over the age of 18 are current, regular drinkers (CDC, 2011; CDC, 2012). Epidemiological evidence appears to show a correlation between the usage of each drug: A longitudinal study of college students has shown that nicotine dependence and alcohol abuse each predict each other over time **(**Sher et. al., 1996**).** Additionally, alcoholic smokers have been found to smoke nearly double the number of cigarettes per day when compared to non-alcoholic smokers (Dawson, 2000). MRI studies on alcoholics have found that alcohol cue presentation increases the urge to drink and smoke (Cooney et al., 2003). Such evidence suggests that there may be a common underlying neural mechanism mediating the association between these two drugs.

The leading candidate for this association is the mesolimbic dopamine pathway. This pathway, projecting from the ventral tegmental area (VTA) to the nucleus accumbens (NAcc) has been found to mediate drug craving and reward for almost every major drug of abuse (Wise, 1998**).** In this paper, I will first look at the epidemiological evidence for this association. Then I will look at some important preliminary studies on ethanol and nicotine's effects on the mesolimbic dopamine pathway. After I have constructed a diagram of the major synaptic connections between these neurotransmitter systems and discussed their roles, I will then explore a large body of literature researching the effects both nicotine and ethanol on the two other major neurotransmitter systems which influence this pathway: GABA and glutamate. After discussing the direct effects of ethanol on nicotinic receptors, I will then summarize the evidence regarding the effects of prolonged exposure to nicotine, ethanol, or both drugs. Finally, I will discuss the current neuroanatomical model for nicotine addiction, and the ability of nicotine to elicit long-term potentiation at certain synapses in the VTA – a change which may both readily sensitize the mesolimbic system and provide a means of associating ethanol intoxication with nicotine consumption.

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CHAPTER I.

Psychological and Epidemiological Evidence

Introduction

In the United States it is estimated that over 19% – or nearly 45.3 million people over the age of 18 – smoke cigarettes (CDC, 2011). Smoking is the leading cause of preventable death and accounts for at least 443,000 deaths each year - nearly 1 out of 5 total deaths in the United States annually (CDC, 2002; CDC, 2008; Murphy, Xu, & Kochanek, 2012). Regular alcohol consumption, on the other hand, is even more prevalent in the United States: A 2012 survey found that nearly 51% of adults over the age of 18 are considered current, regular drinkers (CDC, 2012). Alcohol abuse affects a considerable percentage of American adults, with approximately 4.65% or 9,668,000 citizens having been found to abuse alcohol on a regular basis (Grant, Dawson, Stinson, Chou, Dufour, & Pickering, 2004). In light of these high rates of prevalence, it seems entirely possible that concomitant use would prevail among certain subpopulations.

In fact, this phenomenon has been overwhelmingly documented in the literature. During the early 1990's, an extensive amount of research was done on this association between alcohol consumption and tobacco usage. This literature is well-summarized by Deborah A. Dawson in the introduction to her study titled "Drinking as a risk factor for sustained smoking" (Dawson, 2000). The highlights include the following facts:

- "…persons with alcohol abuse and dependence are more likely to smoke and are heavier smokers than are members of the general population (as cited in Dawson, 2000, p. 235).

- "In a longitudinal study of a small sample of college students, tobacco dependence and alcohol use disorders (i.e. abuse and dependence) each predicted the other over time (Sher et al., 1996)."

Dawson goes on to summarize the mechanisms proposed to account for this phenomenon, and then document her own findings.

In her experiment, Dawson used multivariate analysis of data collected in a 1992 National Longitudinal Alcohol Epidemiology Survey (NLAES) designed by the National Institute on Alcohol Abuse and Alcoholism (NIAAA). The survey's sample size was 42,862 adults over the age of 18, and for her analysis Dawson defined lifetime smokers as those who reported smoking 100 cigarettes over the duration of their lifetime (n = 21,239) and past-year smokers as those who reported any cigarette smoking in the last year before the survey (n = 12,586). Alcohol users were defined as heavy, moderate, and light drinkers according to ethanol intake estimates that used separate conversion rates for beer, wine, and liquor and ethanol intake was measured for the past year and the period of heaviest drinking.

The results of her analysis are compelling: 53% of heavy drinkers smoked in the past year, while only 40% of moderate drinkers, 31% of light drinkers, and 23% of lifetime abstainers reported smoking in the last year. Lifetime smoking only prevailed in 28% of lifetime alcohol abstainers, but this rate more than doubled among past-year drinkers and former drinkers (60% and 63%, respectively). Finally, 75-80% of those defined by DSM-IV criteria as alcoholics were found to be devout smokers – smoking approximately 2 packs of cigarettes per day – while nonalcoholic smokers were only found to smoke around 1 pack per day.

In light of these statistics, it appears there is a high probability that these two drugs interact with each other or at least influence the same neurological systems. Physiological evidence of cross-tolerance has been documented in addition to these abuse correlations. A study by Burch, Fiebre, Marks, & Collins (1988) found that mice exposed to chronic nicotine were less sensitive to both the heart rate-decreasing and body temperature-decreasing effects of an ethanol challenge. Meanwhile, mice exposed to chronic ethanol were more sensitive to nicotine's effect on respiration rate and less affected by sharp, loud noises in measures of acoustic startle response. These cross-tolerance trials were conducted 6 hours after withdrawing each groups' chronic drug and assurance of full metabolic clearance of the drug, ruling out the possibility of direct physiological interaction between the drugs during cross-tolerance trials. Also, these cross-tolerance effects were *not* fully explained by changes in either nicotinic acetylcholine receptor (nAChR) number or affinity, suggesting the interaction of multiple neurotransmitters, receptors, and neural circuits.

Given that there is a high correlation between smoking and drinking (Dawson, 2000) and that these two drugs interact in a manner which may produce cross-tolerance not fully explained by changes in nAChRs, could other factors such as cue reactivity play a role? The answer appears to be yes. In a study of 40 DSM-IV diagnosed alcoholics who had smoked more than 10 cigarettes a day for at least one year, alcohol cue presentation was found to increase urges to both drink *and smoke* (Cooney, Cooney, Pilkey, Kranzler, & Oncken, 2003). Interestingly, nicotine deprivation led only to urges to smoke but not drink – suggesting a one-way correlation between usage of the drugs. Essentially, nicotine appears to have the strongest craving association, and this craving can be

induced by consumption or presentation of a paired pharmacological substance such as alcohol.

This assertion – that nicotine craving provides the compelling physiological response which can be conditioned to alcohol – is further supported by a cue-reactivity meta-analysis performed by Carter & Tiffany (1999). The study found that while the average effect size for craving of alcohol, cocaine, nicotine, and opiates together was +0.92, the craving effect size for nicotine was +1.18. Even more interestingly, craving effect size for alcohol was the lowest at +0.53. Given these data, it seems reasonable to conclude that cravings are much stronger for nicotine, and therefore more easily elicited by paired pharmacological substances such as ethanol.

A final piece of support for this assertion comes from an experiment performed by Rose, Brauer, Behm, Cramblett, Calkins, & Lawhon (2002) on heavy concomitant drinkers and smokers between the ages of 21-55 years old. In this experiment, participants swallowed either the non-specific nAChR antagonist mecamylamine or a placebo, ate, consumed 0.5 g/kg ethanol in 3 drinks over 40 minutes, then smoked one controlled delivery cigarette followed by a 2 hour ad-lib smoking session. Their blood was drawn both before and after the experiment and subsequent blood alcohol (BAC) and nicotine content analyzed. The experiment's alcohol administration only achieved an average BAC of 0.03 – a level typically associated only with the initial stages of alcohol consumption – however it was found that this low level of alcohol enhanced the reduction of nicotine craving associated with with nicotinic cigarette consumption. Although this study has flaws, it does make two things apparent: 1) There is a pharmacological interaction between initial doses of alcohol and subsequent nicotine consumption, and 2)

a source of motivation for nicotine consumption could come from the reward-potentiating effects of initial alcohol consumption. These principles should especially be kept in mind when we later look at diagrams of both drugs' effects on the mesolimbic dopamine reward system.

Environmental Influences on Drug-Seeking Behavior

We have already seen that alcohol cue presentation can increase the urge to smoke (Cooney et al., 2003). In light of this fact, a question remains: Can other environmental cues – even those completely nonpharmacological in nature – elicit the urge to smoke? Could the image of a cigarette, alcoholic beverage, or even the inside of a bar or large crowd at a party activate cortical circuitry that may modify limbic craving circuits? The answer to this, again, appears to be yes.

An fMRI study in 2001 found that alcoholics had increased activity in both the dorsolateral prefrontal cortex (PFC) and anterior thalamus when viewing alcohol cues as opposed to nonalcoholic cues when compared to controls (George et al., 2001). In the experiment, 10 alcoholics and 10 healthy controls who only drank socially were given a sip of alcohol and then presented alcoholic, non-alcoholic, and visual control pictures (blurred images) – all while undergoing an fMRI scan. The fact that alcoholics had greater dorsolateral PFC activation in response to alcohol cues suggests that simple environmental presentation of alcohol can cause firing of complex associative circuits which may prime limbic system reward circuits – a principle we will look at in great detail later in this paper.

In further support of this cue-induced cortical circuit activation, a recent meta-analysis by Engelmann et al. (2012) found that smoking cues evoke larger fMRI

responses in both the dorsal and medial PFC than neutral cues. Given that nicotine has a larger craving effect size than alcohol (Carter & Tiffany, 1999), it is entirely possible that ethanol's activation of PFC circuitry could trigger or "prime" activation of nicotine-related PFC circuitry – however this is a very complicated subject which should be reserved for discussion elsewhere.

While both the Engelmann et al. (2012) and George et al. (2001) papers are compelling, they don't address whether a completely nonpharmacological cue can be conditioned to consumption or craving of a drug such as nicotine. A set of operant conditioning experiments performed on rats by Cagguila et al. (2001) set out to answer just this question. With catheters implanted into their right jugular vein, rats were first trained to lever-press to food, and then to 1 second infusions of nicotine bitartrate. The nicotine infusions, however, were coupled with a 1 sec cue light and initiation of a 1 minute timeout period in which the chamber lights were turned off and no reinforcement could be acquired. After 20 days in which all rats were conditioned to nicotine+cues this way, they were split into 3 groups during a12 day extinction phase: saline+cues, nicotine+no cues, and saline+no cues. Finally, each extinction group was split further for a 5 day reacquisition phase; some groups had nicotine reintroduced, others cues reintroduced, etc.

Not surprisingly, rats which had both nicotine and cues removed displayed nearly complete extinction, or a near-100% drop in rate of infusion (ROI). The most interesting data however was that of the other two groups: The saline+cues group (nicotine removed) showed a 58% drop in ROI that remained stable for all 12 days of the extinction phase. This persistence suggests that the inert light cue became a conditioned reinforcer (CR)

with the nicotine, and that the presentation of these cues was sufficient to maintain lever pressing. Meanwhile, the nicotine+no cues group (light cues removed) showed a 63% drop in ROI that remained stable for all 12 days, suggesting that the light cues had just as powerful an effect on the rats' self-administration behavior as the nicotine reinforcement. Both of these groups' ROI remained significantly higher than the saline+no cues group for the entire extinction phase, and both groups almost immediately recovered their lever-pressing behavior when the absent reinforcer was reintroduced. The overwhelming conclusion that can be drawn from this study is that nonpharmacological cues can take on reinforcing properties that may be *as strong* as the pharmacological substance with which they were originally paired. It appears that the bar, the party environment, the noise – all of these may become CRs which may trigger the desire to smoke.

Incentive-Sensitization Theory and the Mesolimbic Dopamine Reward Pathway

Now that we've made it clear that the pharmacological effects of alcohol and nicotine - as well as environmental reinforcers both directly and indirectly associated with these drugs - can combine to increase craving for nicotine, some questions remain: What neuroanatomical changes orchestrate this craving? More importantly, where do these changes take place? In order to address the complex answers to these simple questions, I will start by taking a brief look at an addiction theory originally proposed by Robinson & Berridge in 1993 called Incentive-Sensitization Theory. Its four major tenets are as follows:

1. "Potentially addictive drugs share the ability to produce long-lasting changes in brain organization.
2. "The brain systems that are changed include those normally involved in the process of incentive motivation and reward.

3. "The critical neuroadaptations for addiction render these brain reward systems hypersensitive ('sensitized') to drugs and drug-associated stimuli.

4. "The brain systems that are sensitized do not mediate the pleasurable or euphoric effects of drugs, but instead they mediate a subcomponent of reward we have termed incentive salience or 'wanting.' We posit the psychological process of incentive salience to be specifically responsible for instrumental drug-seeking and drug-taking behavior (drug 'wanting')" (Robinson & Berridge, 2001).

The first tenet needs the least explanation. If a person develops an addiction, it seems inherent that changes in neural circuitry must have taken place to produce the addiction. The second tenet specifies where this change must take place: Systems involved in motivation and reward. Robinson and Berridge provide much evidence for this in their 2001 paper titled, "Incentive-sensitization and addiction." They cite many studies on behavioral sensitization in rats and mice, as well as studies regarding changes in systems involving the nucleus accumbens (NAcc). Specifically, they cite studies on the sensitization of D_1Rs (dopamine receptors) and desensitization of responses to glutamate in the NAcc. In fact, we will address many of the specific studies involving ethanol and nicotine's effects on the NAcc and the major system which projects to it – the mesolimbic dopamine system – later in this paper.

The third tenet further specifies the types of changes which must take place - namely sensitization. While sensitization of the mesolimbic dopamine system as a whole is a key factor in addiction, our investigation into the specific changes elicited by nicotine and ethanol will show that *differential desensitization* of *nicotinic* receptor subtypes (nAChRs) plays one of the most crucial roles in mediating these two drugs' interactions.

The fourth tenet is perhaps the most difficult to explain, but is nonetheless based on scientific findings such as addicts' seeming "bewilderment" at their inexplicable desire for their drug of addiction. Robinson & Berridge termed this "incentive salience attribution," in which a person transforms the sensory features of ordinary stimuli into especially salient stimuli – such as the nonpharmacological light cue we saw conditioned so strongly to nicotine in the experiment by Cagguila et al. (2001). This same transformation could easily occur with, say, a bar, a party environment, or even more easily a direct pharmacological cue such as an alcoholic beverage, to the point where perceiving any of them would especially stimulate nicotine craving. In this light, it seems that whether or not the actual *reward* of drug consumption changes is no longer important. Rather, the most important change is how our brain becomes attenuated, or sensitized to "wanting" the stimulation of this reward circuitry – i.e. the mesolimbic dopamine pathway.

All of this brings us to our primary question: Can consuming alcohol cause nicotine cravings in individuals who have previously consumed both at the same time? As we have already seen, epidemiological studies have shown a strong correlation between drinking and smoking (Dawson, 2000). Cue reactivity studies have further shown a strong correlation between alcohol cues and nicotine cravings (Cooney et al., 2003). Even nonpharmacological cues can be conditioned to have reinforcing properties as strong as nicotine (Cagguila et al., 2001). Given this evidence, it seems that alcohol as well as directly- and indirectly-related alcohol cues can be conditioned to elicit nicotine craving and consumption.

In this paper, I will first review the mesolimbic dopamine pathway and the effects of both ethanol and nicotine on its function. Then, I will use the findings from numerous research papers to construct a diagram of the major synaptic connections, as well as pre- and post-synaptic receptor locations in this pathway. I will continue to use this diagram as a reference for the rest of the paper as we look at these important topics: First, the effect of each drug on glutamatergic and GABAergic transmission in the mesolimbic dopamine pathway; Second, how nicotinic receptors (nAChRs) respond to and mediate the effects of ethanol in the mesolimbic pathway; Third, changes in the mesolimbic dopamine pathway in response to nicotine, ethanol, or both drugs; And lastly, the interactions in this pathway which can lead to the pairing of nicotine craving with ethanol consumption.

CHAPTER II.

Neuroanatomical Correlates of Craving and Reward

The Mesolimbic Dopamine Reward Pathway is Activated by both Nicotine and Ethanol

The mesolimbic dopamine pathway is comprised of a bundle of dopaminergic (DAergic) neurons which project from the ventral tegmental area (VTA) in the midbrain to the nucleus accumbens (NAcc) (See Fig. 1). This system has been observed to fire in response to self-administration of various drugs in rat studies, as well as in response to intracranial self-stimulation of the DAergic cell bodies in the VTA (Wise, 1998). Stimulation of this pathway results in dopamine (DA) efflux at synapses in the NAcc, and this efflux has been correlated with reward and the obtaining of goals, including natural behavior such as eating and drinking (Yoshida et al., 1992; Wise, 1998). Both nicotine and ethanol, among other drugs, act in this pathway, resulting in "reward," or facilitated dopamine effects on the NAcc.

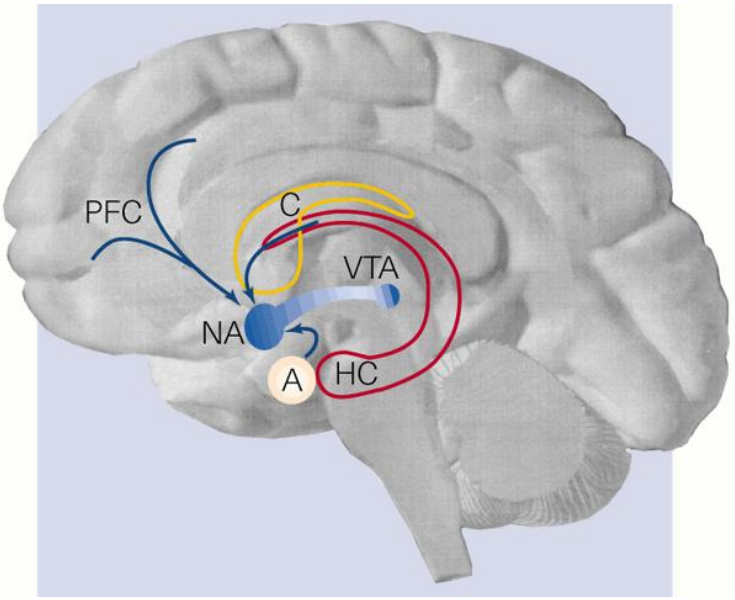

Figure 1. The Mesolimbic Dopamine Pathway.

1.) Nicotine Activates the Mesolimbic System

A microdialysis study of the mesolimbic dopamine pathway by Nisell, Nomikos, & Svensson (1994) serves as a great starting point to demonstrate how nicotine directly stimulates this reward pathway as well as a preview of how crucial glutamate

neurotransmission is to this system. In the study, nicotine or NMDA was directly perfused for 80 min or 60 min, respectively, into either the VTA or NAcc of awake male rats. Nicotine perfusion into the VTA increased NAcc DA levels by 23.5% for 60 minutes post-infusion, demonstrating that nicotine stimulates the dopamine neurons of the mesolimbic pathway to fire. Nicotine perfusion into the NAcc itself also increased NAcc DA efflux by 29.5%, however this only lasted for 20 minutes post-infusion before DA levels returned to baseline. The stimulation of DA efflux by direct NAcc nicotine administration suggests that nicotine receptors exist presynaptically on the terminals of mesolimbic DA neuron in the NAcc – a fact we will revisit when looking at our mesolimbic system diagram later in this paper. More importantly, the duration of the dopamine elevation resulting from nicotine perfusion into the VTA suggests that stimulation of nicotinic receptors on the somatodendritic region of mesolimbic DA neurons has the most pronounced effect on DA transmission. Finally, NMDA perfusion into the VTA increased NAcc DA concentrations by 44%, while direct perfusion into the NAcc increased DA concentrations by 90%. This fact suggests that glutamate transmission is highly intertwined with DA transmission in the mesolimbic system, and that NMDA receptors are also located presynaptically in the NAcc.

Another study performed two years prior by Corrigall, Franklin, Coen, & Clarke (1992) serves as a primary example of how crucial the mesolimbic DA system is to nicotine reinforcement. In this study, a group of rats which had been trained to self-administer nicotine in an operant chamber on a 1minute timeout FR5 schedule had the nicotine antagonist chlorisondamine infused into the cerebral ventricles 2 minutes prior to an operant session. When compared to controls, it was found that chlorisondamine

infusion produced a consistent, sustained reduction in nicotine self-administration. When this nicotine antagonist was infused into rats trained to respond for cocaine reinforcement, no reduction in self-administration was observed – suggesting that nicotine reinforcement is mediated by nAChRs.

Upon further experimentation, Corrigall et al. (1992) found that bilateral lesioning of the NAcc via microinfusion of the dopaminergic neurotoxin 6-OHDA caused a marked reduction in nicotine consumption over the full 3 weeks of the experiment. Rats which underwent this lesioning had extremely reduced DA levels in the NAcc (93% reduction), as well as 87% DA depletion in the olfactory tubercle and 23% in the striatum. This study provides strong behavioral and pharmacological evidence that the dopaminergic pathways of the mesolimbic system are involved in the reinforcement of nicotine consumption, and that nicotinic receptors play a predominant role in this reinforcement.

Microdialysis studies have also confirmed the effects of nicotine on the mesolimbic pathway and provided evidence that dopaminergic systems sensitize in response to chronic nicotine dosing (Marshall, Redfern, & Wonnacott, 1997). In the study dialysate samples were taken from the striatum, NAcc, and cortex after both subcutaneous administration and direct infusion of nicotine in order to determine nicotine's effect on dopaminergic transmission in these areas. The most interesting finding of this study was as follows: Seven days of chronic, discrete subcutaneous nicotine injections increased DA levels in response to a direct infusion of nicotine on the 8^{th} day in all 3 areas, reaching significance in the striatum and near-significance in the NAcc. However, administration of the same daily dose for 7 days via constant

subcutaneous infusion did not produce any increase in response to a direct infusion on the 8th day.

The specificity of this sensitizing effect to daily injections has two large implications: First, it confirms the third tenet of Robinson and Berridge's Incentive-Sensitization Theory (Robinson & Berridge, 1993). The systems associated with motivation and reward here did in fact become "hypersensitive" to discrete stimulation by nicotine. Second, it provides evidence that the sensitizing effect of nicotine on reward systems in the brain may be dependent on the dosing regimen of an addict. The smoking of a cigarette immediately spikes blood nicotine levels (Henningfield, Stapleton, Benowitz, Grayson, & London, 1993), mimicking the spikes in blood nicotine content which caused sensitization in Marshall et al.'s (1997) cohort of rats. Additionally, the average nonalcoholic smoker smokes approximately 1 pack of cigarettes per day (Dawson, 2000), meaning they will experience as many as 20 of these discrete sensitizing trials each day! Taken together with the results of the experiments by Corrigall et al. (1992) and Nisell et al. (1994), it is readily apparent that the mesolimbic dopamine pathway is activated by nicotine.

2.) Ethanol Activates the Mesolimbic System

Nicotine-induced stimulation of the mesolimbic dopamine system seems to be primarily mediated by nicotinic receptor binding and contributions from glutamate systems; Ethanol however is not so easy to understand. Because ethanol does not have a specific receptor which mediates its effects on the brain and body, we have for a long time had to assume that its effects on neural circuits such as the mesolimbic DA pathway are mediated by direct and indirect effects on many different neurotransmitter systems in

the brain (Carmichael & Israel, 1975). Some prominent researchers have suggested that it affects neurotransmission by changing the membrane characteristics surrounding receptors (Collins, 1990). By causing physical changes such as modification of the width of a receptor channel, the shape of a subunit, etc., alcohol does in fact have a large, diffuse affect on many different receptor types. We will discuss alcohol's varying effects on different nicotinic receptor types in extensive detail later in this paper, but for now we will highlight some foundational articles which demonstrate ethanol's stimulatory effect on dopamine transmission in the mesolimbic system.

An electrophysiological slice preparation experiment on dopaminergic VTA neurons performed in 1999 directly demonstrated that ethanol causes significant, dose-dependent increases in the firing rates of the mesolimbic dopamine system (Brodie, Pesold, & Appel, 1999). In this experiment VTA DA neurons were dissected, mounted on coverslips, and bathed in artificial cerebrospinal fluid (aCSF) while electrophysiological recordings were made. All cells used were confirmed to be tyrosine hydroxylase positive (TH+) by immunohistochemistry as well as by basal electrophysiological characteristics. Brodie and his colleagues found that ethanol excited all 44 of the dissociated VTA DA neurons tested in a dose-dependent fashion. The neurons showed an increase in firing rate of 19.3% over basal at 20 mM ethanol, 28.6% at 40 mM, 45% at 80 mM, and 64% at 120 mM ethanol. This clear dose-dependent increase in firing rate shows that ethanol directly stimulates the dopaminergic neurons of the mesolimbic dopamine pathway. While this experiment's evidence is compelling, it is also important to look at evidence from *in vivo* experiments to confirm that these

stimulatory effects take place in the dynamic environment of the intact central nervous system.

An important study by Ericson, Blomqvist, Engel, & Soderpalm (1998) observed the effect of alternating days of *in vivo* microdialysis of saline or mecamylamine – a nonselective nAChR antagonist – directly into the VTA of freely moving Wistar rats that were raised according to an ethanol preference model. Rats observed to consume 60% or more of their daily fluid intake from a bottle of 6% ethanol were selected during a screening process and their ethanol intake and NAcc dopamine levels were measured in response to either saline or mecamylamine. The study found that on days when mecamylamine was administered into the VTA ethanol intake decreased and accumbal dopamine levels stayed approximately the same. Alternatively, on days when the saline vehicle was administered NAcc dopamine levels still rose to 130% of the recorded pre-mecamylamine treatment baseline.

While the ability of a nAChR antagonist to block ethanol-induced stimulation of the mesolimbic DA system is very interesting, I won't look at this in detail until later in the paper - as previously promised. The important fact to take away at this time is that ethanol self-administration raised NAcc dopamine levels – a measure correlated with obtaining drug "reward" – to 30% above normal! This study clearly demonstrates that oral ethanol ingestion by self-administering rats can stimulate the mesolimbic dopamine pathway, suggesting that the same mesolimbic stimulation can occur in humans drinking alcohol.

Interestingly, another study investigating the efficacy of the opiate antagonist naltrexone at blocking ethanol-induced stimulation of this reward pathway found that

ethanol self-administration in saline control rats resulted in elevation of dopamine levels in the NAcc to 130% of baseline (Gonzales & Weiss, 1998). They determined that this effect wasn't due to a decrease in ethanol consumption by the rats, but that naltrexone in fact reduced the efficacy of ethanol's induction of dopamine efflux. These lowered NAcc dopamine levels were correlated with decreased ethanol intake, suggesting that opiate antagonists such as naltrexone suppress ethanol administration by interfering with the dopamine aspect of ethanol reinforcement. This experiment makes it clear that ethanol stimulates the mesolimbic dopamine system and even shows that interference with the system can reduce the reinforcing aspects of drugs which stimulate it.

In summary, we know that direct perfusion of nicotine into either the VTA or NAcc produces elevated DA levels in the NAcc that remain elevated for significantly longer when the VTA is stimulated (Nisell et al., 1994). Lesioning of the NAcc using 6-OHDA reduces the dopamine levels of the NAcc by 93% and additionally reduces the reinforcing value of nicotine as demonstrated by self-administration studies (Corrigall et al., 1992). Most importantly, chronic exposure to daily doses of nicotine produces sensitization of the mesolimbic system while continuous infusion does not (Marshall et al., 1997). Ethanol stimulates the mesolimbic dopamine system too, as demonstrated by slice preparation and electrophysiological recording (Brodie et al., 1999). Additionally, oral ingestion of alcohol stimulates this system as well, raising NAcc dopamine levels to 130% of basal, and interference with this dopaminergic system reduces the reinforcement value of ethanol (Ericson et al., 1998; Gonzales and Weiss 1998). Taken together, these studies show that both nicotine and ethanol stimulate the mesolimbic dopamine pathway – providing a definitive site for further exploration in search of the synergistic

interactions between these drugs that could result in the conditioning of nicotine consumption with alcohol.

The Locations and Types of Nicotinic Acetylcholine, Dopamine, and Glutamate Neurons and Receptors in the Ventral Tegmental Area and Nucleus Accumbens:

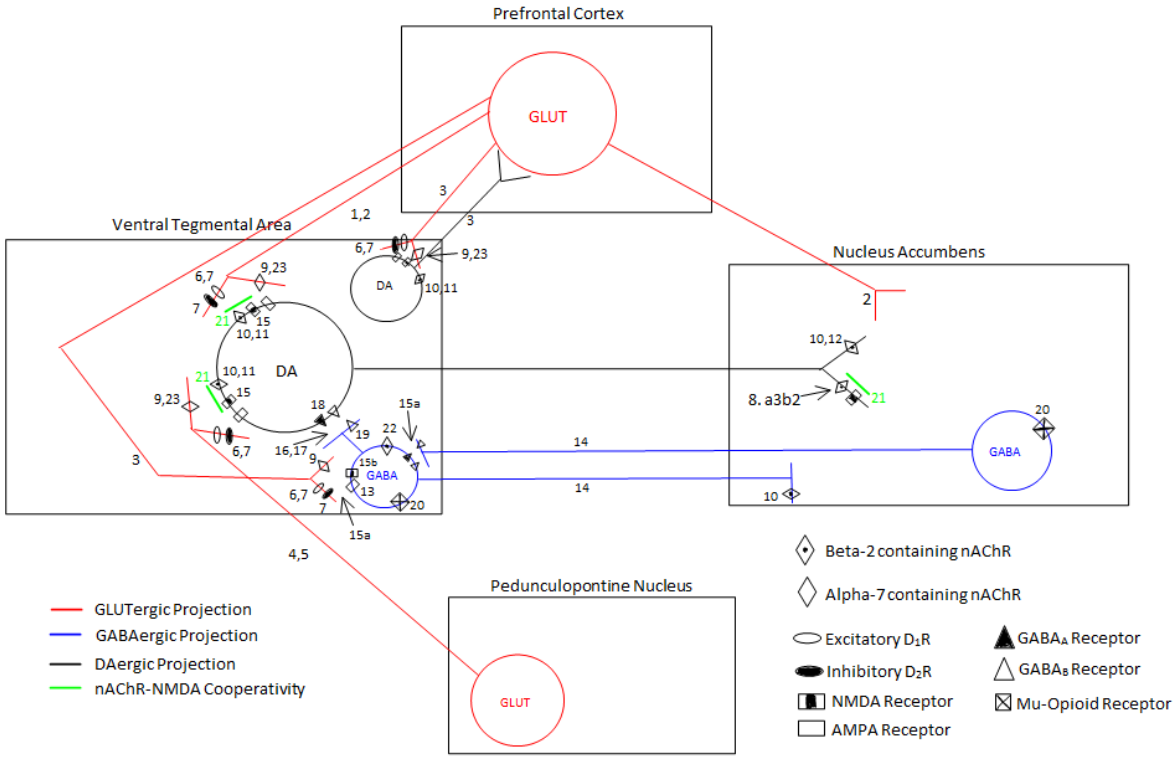

Figure 2. Diagram of Pertinent Synapses Involved in Nicotine-Ethanol Interactions in the Mesolimbic Dopamine System. (See Appendix for larger version).

Now that it is clear where to begin neuroanatomical exploration, I will briefly summarize important neuroscience literature from studies on the midbrain, ventral tegmental area (VTA), and nucleus accumbens (NAcc) which pertain to the interactions of our two drugs of interest – ethanol and nicotine. Since we already know that firing of dopaminergic neurons of the mesolimbic system is correlated with reinforcement of nicotine and ethanol (Corrigall et al., 1992; Gonzales & Weiss, 1998) and stimulation of the somatodendritic regions of these neurons in the VTA produces the longest-lasting elevation of dopamine efflux in the NAcc (Nisell et al., 1994), we will first look at

excitatory glutamatergic inputs to these neurons in the VTA as these inputs can have a profound effect on the firing rate of mesolimbic dopamine neurons.

1.) Glutamate Input to the Ventral Tegmental Area

One major source of glutamatergic input to the VTA is the prefrontal cortex. Since this portion of the brain is responsible for our higher-level stimulus associations and recognition of related environmental variables, it is especially important to determine whether or not this portion of the brain contributes excitatory input to the mesolimbic system. According to the research of Sesack & Pickel (1992), it appears that it does. Using both lesion-induced anterograde degeneration and immunoreactivity labeling tracing methods on male Sprague-Dawley rat brains, Sesack and Pickel determined that PFC efferents synapse on neuronal spines in the NAcc in close apposition to terminals which were positive for tyrosine hydroxylase (TH+) – likely dopaminergic terminals. Furthermore, PFC terminals synapse on both dopaminergic and unlabeled dendrites – likely GABA (Carr & Sesack, 2000) – in the VTA. This experiment confirms that the VTA as well as NAcc receive monosynaptic excitatory input from the PFC, which we see included in Figure 2 and denoted by the number 1.

Four years later, electrode-stimulated cell recording experiments confirmed that stimulation of the PFC can cause burst firing in dopaminergic VTA neurons (Tong, Overton, & Clark, 1996). In this experiment, 40 live, anaesthetized male rats underwent cell recordings of both the VTA and substantia nigra pars compacta (SNc). Stimulation of the PFC at 0.25 mA and 1.00 mA produced responses in 84.2% and 94.2% of VTA DA neurons, respectively. The authors noted that the burst patterns exhibited by these neurons resembled natural events, lending further support to the findings of Sesack and

Pickel (1992) and confirming the functionality of these synaptic contacts between glutamatergic PFC efferents and DAergic VTA cell bodies (denoted by the number 2 in Figure 2).

Later, further anterograde and retrograde tracing work was published which shed light upon two corticolimbic pathways linking the PFC and VTA (Carr & Sesack, 2000). Carr and Sesack combined anterograde tracing using biotinylated dextran amine (BDA) with retrograde tracing using FluoroGold immunogold labeling for TH and GABA, as well as immunocytochemistry and electron microscopy techniques to examine both the projections and neurochemical phenotypes of VTA neurons receiving input from the PFC. Their findings boil down to the discovery of two corticolimbic pathways:

PFC ----glut---->VTA ----gaba---->NAcc

PFC----glut----->VTA-----dopamine--->PFC

While they were not able to confirm a PFC----glut---->VTA---dopamine--->NAcc pathway as they hoped, they did note a considerable number of DA neurons targeted by PFC efferents which projected to unknown sites. It is therefore entirely possible that this pathway may still exist, or exist in a longer, more indirect fashion via other pathways in the brain. Furthermore, the PFC ----glut---->VTA ----gaba---->NAcc pathway may in fact play a large part in ethanol-induced nicotine craving, as I will discuss later in this paper. These findings are represented and denoted in Figure 2 by the number 3.

While the PFC is an important source of glutamatergic (GLUTergic) input to the VTA, many other regions have GLUTergic input to this area as well – such as the

pedunculopontine tegmental nucleus (PPT). Investigated in the same laboratory with the same tracing techniques as Tong et al. (1996), researchers found that stimulation of the PPT results in firing of midbrain dopamine neurons in a manner which resembled natural events (Lokwan, Overton, Berry, & Clark, 1999). These findings confirmed the functionality of GLUTergic PPT→VTA pathways which had previously been discovered by Charara, Smith, and Parent (1996). Using anterograde transport, pre-embedding immunohistochemistry for TH and calbindin D-28k, and post-embedding immunocytochemistry for glutamate and GABA, they found that 60% of the terminals projecting from the PPT to the VTA were GLUTergic, and that some synapsed on DA neurons. This PPT---glutamate--->VTA---dopamine--->NAcc pathway is represented in Figure 2 and denoted by the findings of these experiments with the numbers 4 and 5, respectively.

Given all of this excitatory input to the VTA, shouldn't there be glutamate receptors present on the cell bodies of these mesolimbic dopamine neurons? In fact, single-unit electrophysiological recordings of VTA DA neurons have shown that NMDA, AMPA, and Kainic Acid all produce dose-dependent increases in the firing rates of the DA neurons, suggesting that NMDA, AMPA, and kainate receptors all are present on mesolimbic DA neurons (Wang & French, 1993). Furthermore, it has been found that long-term potentiation (LTP) of these glutamatergic synapses in the VTA is dependent on NMDA receptors (Mansvelder & McGehee, 2000), providing further evidence of their presence. The location of these glutamate receptors is not limited only to the somatodendritic region: Synaptosome studies have found that NMDA receptors are present on the terminals of these dopamine neurons in the striatum of rats (Cheramy,

Godeheu, L'Hirondel, & Glowinski, 1996). As we will see, the combination of postsynaptic glutamate receptors on mesolimbic DA neurons, combined with the presence of both nicotine and dopamine receptors on presynaptic glutamate terminals will make GLUT→DA synapses a critical part of our research on the interactions of nicotine and ethanol in the mesolimbic DA pathway.

2.) Dopamine Receptors at VTA Synapses

Now that we have highlighted some of the major excitatory inputs to the ventral tegmental area, it is important to look at the various types of receptors which have been discovered to be present on both the presynaptic terminals of GLUTergic and DAergic projections and the postsynaptic membranes of the dendrites and cell bodies of VTA dopamine neurons. Excitatory D_1 and inhibitory D_2 dopamine receptors, various nicotinic cholinergic receptor (nAChR) subtypes, as well as NMDA and AMPA glutamate receptors all play major roles in mediating both ethanol and nicotine's effect on the mesolimbic dopamine pathway.

Let's first look at dopamine receptors. While we would expect these receptors to be present post-synaptically to dopaminergic terminals – and they most certainly are in numerous places in the brain – their role in mediating alcohol and nicotine interactions in the VTA is based around their function as terminal autoreceptors. That is, they are located on the presynaptic glutamate terminals – such as those from the PFC and PPT – and respond to retrograde transmission of dopamine from the somatodendritic region of VTA DA neurons (Deng, Ke-Yong, Zhou, & Ye, 2009; Xiao et al., 2009). These dopamine receptors seem to be involved primarily in mediating the initial effects of

ethanol, responding to ethanol-induced retrograde dopamine transmission from the somatodendritic region of VTA DA neurons.

A study by Deng et al. (2009) sought to confirm this hypothesis: Ethanol causes release of dopamine from the somata and dendrites of VTA DA neurons which acts in a retrograde fashion on excitatory dopamine D_1 receptors (D_1Rs) located on glutamate terminals, facilitating glutamate release and subsequent firing of the VTA DA neurons in a positive feedback loop. In order to investigate this hypothesis, Deng and his colleagues mechanically separated midbrain DA neurons to isolate this synapse, perfused GABA antagonists to prevent interference, and then measured the small excitatory postsynaptic currents (sEPSCs) elicited by AMPA receptors on the postsynaptic membrane in response to ethanol and various other substances such as the AMPA antagonist DNQX.

The results of the experiment confirmed the authors' hypothesis. DNQX application abolished these receptor-mediated sEPSCs, confirming AMPA receptor participation and measurement. A concentration of 40 mM ethanol dramatically increased sEPSC frequency in a dose-dependent and reversible manner, confirming its stimulation of VTA DA

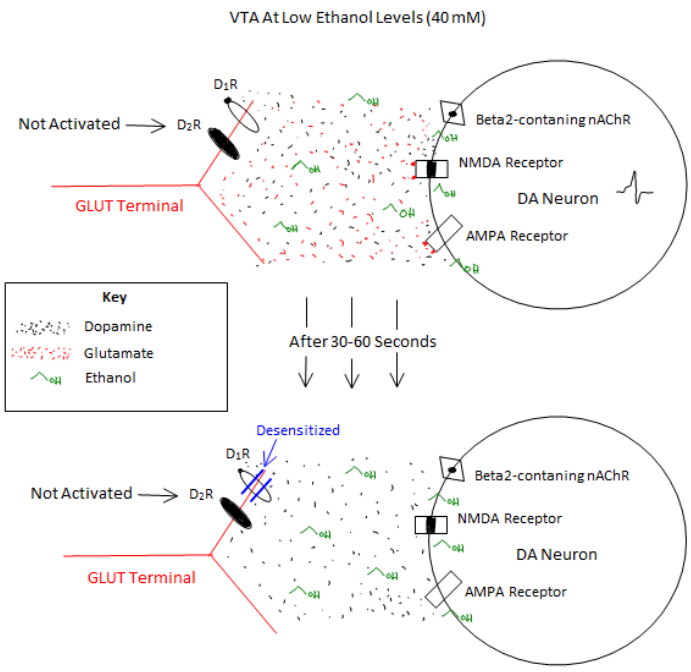

Figure 3. Short-term DA-GLUT Positive Feedback Loop in VTA at Low Ethanol Levels. (See Appendix for larger version).

neurons. Additionally, 10 μM of the D_1R antagonist SKF83566 suppressed this stimulating effect of ethanol but had no effect when applied alone, suggesting that these presynaptic D_1Rs are critical for ethanol-induced facilitation of glutamate transmission. The D_1R agonist SKF38393 (10 μM) significantly increased sEPSC frequency - further confirming their hypothesis - but most importantly: D_1Rs were found to *desensitize rapidly* (30-60 sec). Dopamine depletion using reserpine resulted in significantly lower ethanol-induced sEPSC frequencies, indicating its necessity for ethanol's effect. Furthermore, chelation of postsynaptic calcium completely eradicated ethanol's stimulatory effect, confirming that calcium-dependent retrograde dopamine release from the somatodendritic region is necessary for ethanol-induced facilitation of glutamatergic transmission.

Taken together, the exhaustive set of experiments performed by Deng and his colleagues provides overwhelming evidence that this positive feedback loop can jump-start mesolimbic dopamine cell firing (see Figure 3, previous). The rapid desensitization of these D_1Rs, however, suggests that this positive feedback loop only occurs at the onset of alcohol consumption. Still, this "jump-start" could serve as a neuroanatomical event which could become conditioned to the desire for nicotine. While this seems like a reasonable conclusion regarding dopamine receptors, this is not where our exploration of their role ends. Experiments from the same lab have shown that at higher levels of ethanol, inhibitory D_2 receptors may become activated – causing active inhibition of glutamatergic transmission at this synapse (Xiao et al., 2009).

These experiments by Xiao et al. (2009) used the same AMPA-mediated sEPSC measurement at the same synapses but this time in slice preparations from the VTA. The

use of slice preparations allows for measurement of cell behavior in a more "synaptically intact" environment. They ran all of the same experiments as Deng et al. (2009), and found nearly identical results. There was, however, one result which they decided to explore further: 40 mM ethanol had a greater potentiating effect on sEPSC frequency, increasing sEPSC frequency by over 60%, than 80 mM ethanol – which only increased sEPSC frequency by 40%. Xiao and colleagues hypothesized that this was a result of inhibitory dopamine D_2 receptor (D_2R) activation at higher ethanol concentrations.

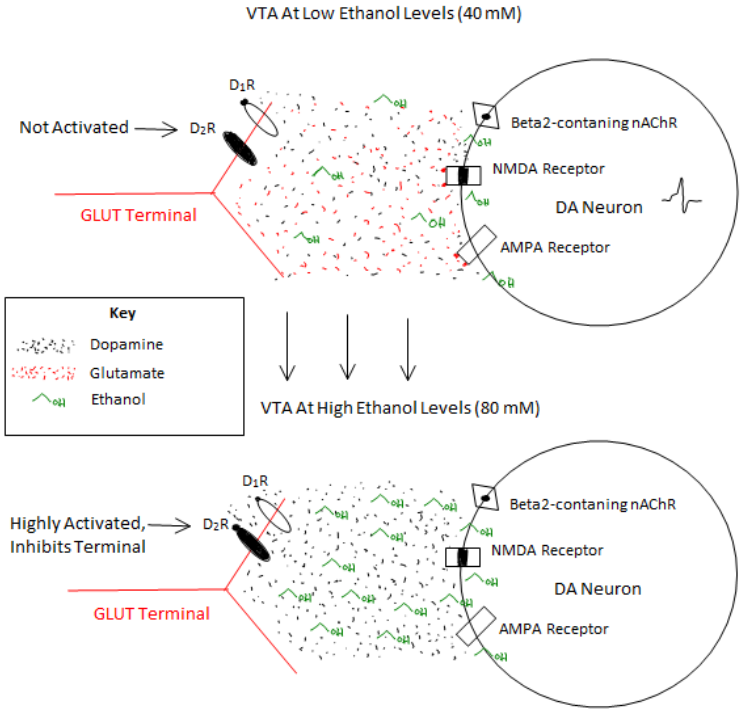

Figure 4. D_2R Activation Occurs at High Ethanol Levels, Inhibiting GLUT Transmission. (See Appendix for larger version).

To investigate, they treated slices with 20 mM or 80 mM ethanol, with or without raclopride – a D_2R antagonist. With only ethanol, each dose increased sEPSC frequency approximately 38% over basal. When raclopride was administered with 20 mM ethanol, sEPSC frequency only increased by 43% over basal - a nonsignificant increase from the ethanol-only trial – however when raclopride was administered with 80 mM ethanol, sEPSC frequency increased to 173% over basal! This was a significant increase from the 38% over basal seen with 80 mM ethanol only, and provides overwhelming evidence that D_2R activation occurs at high doses of ethanol, inhibiting glutamate transmission at these

glutamate terminal-dopamine neuron synapses in the ventral tegmental area (see Figure 4, above). The findings of Deng et al. (2009) and Xiao et al. (2009) are represented in Figure 2 by the numbers 6 and 7, respectively.

With this dynamic feedback loop in mind, we can now begin to see how alcohol and nicotine interact. If nicotine were to potentiate the firing of these excitatory synapses – and consequentially the firing of the mesolimbic dopamine pathway – then consumption of nicotine while intoxicated could be seen as a compensatory response to the slumping of firing frequencies at high levels of ethanol. For someone who had previously experienced the high firing rates or "reward" of using both drugs at the same time, the experience of a slump in mesolimbic firing after the initial burst could trigger a response to seek out a cigarette to prevent this decrease in reward circuitry firing. As we will see, nicotinic receptors are indeed present presynaptically on glutamatergic and dopaminergic terminals (Marchi, Risso, Viola, Cavazzani, & Raiteri, 2002; Kaiser, Soliakov, Harvey, Leutje, & Wonnacott, 1998), as well as postsynaptically on the dendrites and cell bodies of mesolimbic dopamine neurons (Maskos et al., 2005). These receptors can therefore potentiate the firing of these excitatory glutamatergic synapses in the VTA, firing of mesolimbic DA neurons by stimulation of their cell bodies, and firing of dopaminergic terminals in the NAcc.

3.) Nicotinic Receptors in the VTA and NAcc

Now that we have begun to understand the complex interactions of glutamatergic nerve terminals, presynaptic receptors, and dopamine efflux, we will look at a receptor found both pre- and post-synaptically throughout the mesolimbic dopamine system – the nicotinic receptor. This pentameric, ionotropic receptor is typically heteromeric and

composed of 2 alpha subunits and 3 beta subunits in the central nervous system. There are nine known alpha subunits – α_2 - α_{10} – and three known beta subunits – β_2 - β_4 (Lukas et al., 1999). Given that any of these alpha and beta subunits can combine in a 2:3 ratio, it is readily apparent that different combinations of nicotinic receptor subunits can result in nicotinic receptors with vastly differing physical, pharmacological, and electrochemical properties. There is one known nicotinic homoreceptor present in the CNS – the α_7 nAChR – which will prove to be very important to our exploration of this system later in this section. For now, let's look at an experiment which can explain Nisell et al.'s 1994 finding that nicotine perfusion into the NAcc increased NAcc DA efflux by 29.5%.

An experiment by Kaiser et al. (1998) used both synaptosomes and striatal slices from rats to explore which nicotinic receptors are located presynaptically on dopamine terminals. Synaptosomes are essentially isolated nerve terminals, providing an excellent way to study presynaptic receptors, while slices provide a more intact preparation of CNS tissue upon which to experiment. Using the $\alpha_3\beta_2$ nAChR-specific antagonist α-conotoxin MII (aCTX-MII) and the nonselective nAChR antagonist mecamylamine (MEC), these researchers tested how well these antagonists inhibited radioactive dopamine (rDA) release from striatal synaptosomes and slices induced by perfusion of the nAChR agonist anatoxin-a. A concentration of 1 μM anatoxin-a evoked a significant peak of rDA release – one significantly higher than basal in both slices and synaptosomes – demonstrating that stimulation of presynaptic nAChRs located on DAergic nerve terminals can cause dopamine release. This observation provides one explanation for Nisell et al. (1994): Nicotine perfusion into the NAcc activated presynaptic nAChRs on DAergic nerve terminals, causing dopamine efflux.

Further experimentation on the synaptosomes and slices found that MEC inhibited rDA release by 77% in synaptosomes and 88% in slices, while aCTX-MII inhibited rDA release by 56% in synaptosomes and only 28% in slices. The fact that MEC showed greater antagonization than aCTX-MII in synaptosomes proves that nAChR subtypes other than just $\alpha_3\beta_2$ nAChRs are present on DAergic nerve terminals. This greater antagonization is expected as MEC antagonizes a wide variety of nAChR subtypes and aCTX-MII only antagonizes the $\alpha_3\beta_2$ subtype. The reduced efficacy of aCTX-MII in slices vs. synaptosomes is also expected as there are more intact synaptic connections which could influence rDA efflux, however MEC's increased efficacy in inhibiting rDA efflux in slices vs. synaptosomes confounds this assertion.

I personally believe that MEC's increased efficacy is likely due to antagonization of non- $\alpha_3\beta_2$ presynaptic nAChRs present on excitatory axoaxonal connections (see Figure 5, right). Inhibition of non-$\alpha_3\beta_2$ nAChRs on glutamatergic nerve terminals which synapse just prior to DAergic terminals would further antagonize rDA efflux in a manner which aCTX-MII could not. Glutamatergic PFC afferents have

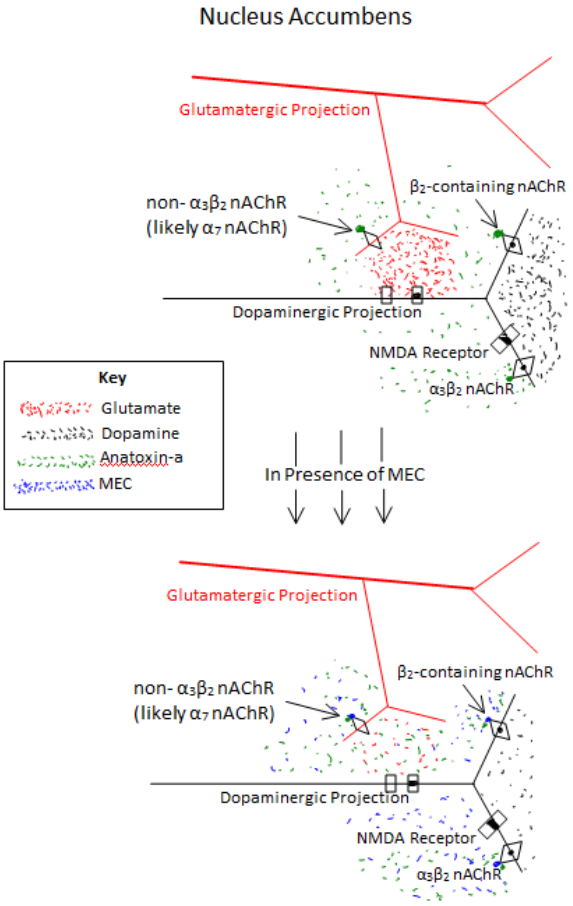

Figure 5. MEC inhibits non- $\alpha_3\beta_2$ nAChRs on Axoaxonal Glutamate Terminals. (See Appendix for larger version).

already been shown to synapse on the somatodendritic regions of mesolimbic dopamine neurons (Tong et al., 1996; Carr & Sesack, 2000), as well as on neuronal spines in the NAcc in close apposition to dopamine terminals (Sesack & Pickel, 1992). Overall, the findings of Kaiser et al. (1998) definitively show that $\alpha_3\beta_2$ nAChRs as well as other nAChR subtypes are present on presynaptic DA nerve terminals. These findings are represented in Figure 2 by the number 8.

Another synaptosome study provides definitive evidence that nAChRs are present on glutamatergic nerve terminals in both the striatum of rats and the cortex of humans (Marchi et al., 2002). In this study, synaptosomes were prepared using tissue samples from these areas and the release of radioactive glutamate and aspartate was measured in response to the nAChR agonists anatoxin-a, nicotine, ACh (administered with 0.1 µM atropine to prevent metabotropic acetylcholine receptor interference), and epibatidine. In the rat striatum, anatoxin-a significantly and dose-dependently increased radioactive aspartate (rAsp) release. Most importantly, this effect was abolished by mecamylamine (MEC), MLA, and alpha-bungarotoxin. Since alpha-bungarotoxin is a specific antagonist of the α_7 nAChR homoreceptor, this effectiveness confirms the presence of this α_7 nAChR on glutamatergic terminals in the striatum. This finding is represented in Figure 2 by the number 9.

So, if $\alpha_3\beta_2$ nAChRs are present on mesolimbic DA nerve terminals such as those projecting from the VTA to the NAcc and α_7 nAChR are present on GLUTergic nerve terminals such as those projecting to the VTA from the PFC and PPT, are these the only nicotine receptors responsible for increasing mesolimbic dopamine pathway firing? The answer appears to be a resounding, "No!" We have already seen that direct perfusion of

nicotine into the VTA has a much longer-lasting effect on increasing DA levels in the NAcc than direct perfusion of nicotine into the NAcc (Nisell et al., 1994). While we could posit that this effect was due entirely to stimulation of presynaptic α_7 nAChRs on glutamate terminals and subsequent activation of a GLUT terminal---glutamate---->mesolimbic DA Neuron---dopamine--->NAcc pathway and then continued activation via the positive feedback loop discussed earlier (Deng et al., 2009; Xiao et al., 2009), the rapid desensitization this feedback loop within 30-60 sec means it cannot be responsible for this 60 minute increase in NAcc DA levels found by Nisell et al. (1994). Additionally, a study of midbrain dopamine neurons found that 80-90% of these neurons expressed $\beta_{2\text{-}3}$ and $\alpha_{4\text{-}6}$ mRNAs (Azam, Winzer-Serhan, Chen, & Leslie, 2002), making it very likely that nAChRs exist on the dendrites and cell bodies of these mesolimbic dopamine neurons in the VTA.

In fact, a complex experiment using wildtype, β_2 nAChR subunit knockout mice, and lentiviral expression vectors to reintroduce the β_2 nAChR subunit into some of the knockout mice showed that β_2-containing nAChRs are present and active on the cell bodies of dopaminergic neurons in the VTA (Maskos et al., 2005). Briefly, wildtype mice (WT), β_2-knockout mice (KO), and β_2-knockout mice injected with the β_2-gene lentivector (VEC) were used to test both nicotine self-administration acquisition and the firing response of VTA DA neurons to nicotine with *in situ* electrophysiological recordings. An enhanced green fluorescent protein (eGFP) was injected with the lentivector in order to trace β_2-subunit re-expression. KO and WT mice were also injected with eGFP.

It was found that nicotine injection increased VTA DA neuron firing 1.5-fold for 10 minutes in WT mice, but had no effect on KO mice. Interestingly, VEC mice did show an initial increase in firing, but only for 2 minutes, suggesting that β_2-reexpression can restore some of the normal functional effects of nicotine on these DA neurons. These re-expressed β_2-containing nAChRs were present on DA cell bodies at the site of injection – the VTA – as well as on mesolimbic DA projections and DA terminals in the NAcc. Only WT and VEC mice showed nicotine-seeking behavior in a Y-maze test as well as decreasing self-administration latencies; however VEC mice showed a significant delay in the initial acquisition of this self-administration behavior. Together, these results definitively show that β_2-containing nAChRs are sufficient and necessary for sensitivity to nicotine reward in drug-naïve mice, and that β_2-containing nAChRs are present on the cell bodies of dopaminergic neurons in the VTA. This finding is represented in Figure 2 by the number 10.

Further support for both the presence and necessity of these β_2-containing nAChRs on dopaminergic cell bodies comes from an *in vivo* microdialysis experiment on wild-type (WT) and β_2 knockout (KO) mice by Picciotto et al. (1998). In this experiment, mesencephalic dopamine neurons in the ventral striatum and part of the dorsal striatum were monitored with a dialysis probe in anaesthetized mice, and dialysate DA levels were sampled in response to introperitoneal (IP) administrations of nicotine. In WT mice, an IP dose of 0.125 mg/kg increased DA levels by 50%, while a 0.500 mg/kg dose increased DA levels by 150%. In KO mice, dialysate DA levels did not significantly change in response to any dose of nicotine, suggesting that β_2-containing nAChRs on dopaminergic cell bodies are essential for this increase in dopamine efflux. In the second portion of the

experiment, patch-clamp recording techniques were used to monitor the response of dopaminergic neurons in slices of the substantia nigra (SN) and ventral tegmental area (VTA) to 10 μM nicotine. In WT mice neurons, nicotine increased discharge frequency in 10 of 15 WT mice dopamine neurons. This effect was blocked by administration of the β_2-containing nAChR antagonist DHβE, suggesting that β_2-containing nAChRs mediated this discharge behavior. No response was observed in 14 of the 15 KO mice DA neurons, further confirming the presence of necessity of β_2-containing nAChRs on the cell bodies of dopamine neurons in the VTA. Voltage-clamp experiments further confirmed this data, and additional experimentation found that there were no significant differences in DA metabolite levels, DA transporter quantity, D1 or D2 receptor levels, cAMP, DA-stimulated adenylyl cyclase activity, or tyrosine hydroxylase activity. Together with the findings of Maskos et al. (2005), this experiment confirms the presence and necessity of β_2-containing nAChRs on the somatic region of mesolimbic dopamine neurons. The findings of Picciotto et al. (1998) are represented in Figure 2 by the number 11.

Before moving on, let's recapitulate what we know about nicotinic receptors in the mesolimbic dopamine system: $\alpha_3\beta_2$ nAChRs as well as other β_2-containing nAChRs are located presynaptically on dopaminergic terminals in the striatum and cortex (Kaiser et al., 1998). Additionally, β_2-containing nAChRs are present on mesolimbic DA projections and DA terminals in the NAcc (Maskos et al., 2005). β_2-containing nAChRs have also been found on the cell bodies of dopamine neurons in the striatum and ventral tegmental area (VTA), and have been proven to be essential to nicotine-induced firing of mesolimbic DA neurons (Maskos et al., 2005; Picciotto et al. 1998). Furthermore, the homomeric α_7 nAChR has been found to be exist on glutamatergic terminals in both the

striatum and cortex (Marchi et al., 2002). Since glutamatergic input to the VTA and and NAcc has been shown to come from the PFC and PPT (Sesack & Pickel, 1992; Tong et al., 1996; Carr & Sesack, 2000; Charara et al., 1996; Lokwan et al., 1999), the presence of α_7 nAChR on these terminals provides another means by which nicotine can indirectly increase the firing of mesolimbic dopamine neurons.

An experiment by Wonnacott, Kaiser, Mogg, Soliakov, & Jones (2000) which utilized immunogold electron microscopy to visualize β_2-containing nAChRs in the striatum of rats summarizes the differences in nAChR subtypes on presynaptic terminals well. Using β_2-specific monoclonal antibody 270, they found gold particles present on dopaminergic terminals but not glutamatergic, indicating that β_2-containing nAChRs are present on dopaminergic terminals but not glutamatergic terminals. This finding is consistent with the findings of Kaiser et al. (1998) that $\alpha_3\beta_2$ nAChRs as well as other β_2-containing nAChRs are located presynaptically on dopaminergic terminals, as well as the finding that α_7 nAChRs are present on glutamatergic terminals (Marchi et al., 2002). Wonnacott and her colleagues additionally found that radioactive dopamine (rDA) release in response to nicotine or the nicotine agonist anatoxin-a was higher in slices than synaptosomes, and that this elevated DA efflux in slices was prevented by administration of glutamate antagonists. This finding provides extremely strong evidence for the claim that stimulation of nicotinic receptors on glutamate terminals in the VTA can elicit mesolimbic DA neuron firing via glutamatergic stimulation of these DA neurons. The findings of Wonnacott and her colleagues are represented with the number 12 in Figure 2.

4.) Reciprocal GABA Circuits are Present in the Mesolimbic System and Contain Mu-opioid Receptors

In truth, any discussion involving ethanol would not be complete without the inclusion of its effects on γ-Aminobutyric Acid (GABA) transmission. Ethanol has long been known to have potentiating effects on the ionotropic inhibitory $GABA_A$ receptor (Mehta & Ticku, 1988). In fact, many of ethanol intoxication's classic "impaired" symptoms – such as impaired judgment, or impaired motor coordination – have long been attributed to this potentiation of inhibitory transmission. However, upon close examination of the effects of ethanol on the mesolimbic system, it appears that this is far from the case. It appears that while ethanol potentiates *some* aspects of GABAergic transmission, the predominant effect of ethanol in the ventral tegmental area is inhibition of GABAergic interneurons; resulting in indirect potentiation of mesolimbic dopamine neurons via a "disinhibitory" mechanism (see review in Mansour, Fox, Akil, & Watson, 1995). While we will devote a great deal of discussion to the effects of ethanol on GABA transmission later in this paper, it is best to first construct a picture of this GABAergic circuitry. In this section, we will briefly cite autoradiographic, immunocytochemical, tracing, and other studies which have elucidated the layout of this neurotransmitter's circuitry in the mesolimbic system.

While interest in the VTA initially focused around its dopaminergic neurons and their implication in drug addiction and reward, voltage clamp studies soon found that other neurons were present in the VTA. An early voltage-clamp study by Johnson & North (1992b) on midbrain slices discovered the existence of a non-dopaminergic neuron originating in the VTA. They found that this neuron – later determined to be GABAergic

– as well as the dopamine neurons received both excitatory and inhibitory amino acid input. These inputs included excitatory amino acid input mediated by NMDA and AMPA receptors, GABA acting at $GABA_A$ and $GABA_B$ receptors, and dopamine acting at inhibitory D_2 receptors – represented by the number 13 in Figure 2. Does this sound familiar? It should, as we have already outlined the location and origin of many of these synapses in the mesolimbic system.

Needless to say this discovery sparked a flurry of research into "non-dopaminergic" neurons in the VTA. Older studies had already provided evidence that GABAergic neurons projected from the nucleus accumbens to the VTA (as cited in Van Bockstaele & Pickel, 1995, p. 251), making evident the possibility of an inhibitory feedback mechanism on DAergic transmission in the mesolimbic system. In light of the evidence from Johnson & North (1992b) and Walaas & Fonnum (1980), Van Bockstaele & Pickel (1995) decided to use fluorogold retrograde tracing in combination with immunocytochemistry and immunogold-silver labeling to both confirm this GABAergic NAcc→VTA and determine whether GABA neurons projected from the VTA to the NAcc as well. In fact, these researchers both confirmed the existence of this GABAergic NAcc→VTA pathway and discovered that in addition to GABAergic interneurons in the VTA, there exists a "subpopulation" of GABAergic neurons which project to the NAcc. These findings are denoted by the number 14 in Figure 2, and would set the stage for the discovery of new GABAergic circuitry which is profoundly affected by ethanol and has a crucial indirect effect on dopaminergic transmission in the mesolimbic system.

So, if reciprocal GABA projections exist in the mesolimbic system – which they do – then there remain even more important questions: Where do they synapse? How do

they interact with mesolimbic DA circuitry? Are these GABA projections connected in series with dopamine projections, or do they synapse on one another? Perhaps both? Fortunately, many of these questions have been answered by a thorough experiment performed by Steffensen, Svingos, Pickel, & Henriksen (1998). In their experiment, Steffensen and colleagues used microelectrodes to collect both intracellular (IC) and extracellular (EC) recordings from the VTA of live, anesthetized male Sprague-dawley rats placed in a stereotaxic apparatus. Their findings were substantial: Stimulation of the internal capsule evoked "both antidromic and orthodromic VTA non-DA spikes," or firing of what was later in the experiment determined to be GABAergic neurons. Most importantly, they found that electrode stimulation of the NAcc resulted in constant, tonic inhibition of these VTA GABA neurons! This datum confirmed the functionality of the GABAergic NAcc→VTA pathway originally discovered by Walaas & Fonnum (1980) and elucidates the existence of synaptic connectivity between GABA projection neurons from the NAcc and local GABA neurons in the VTA. As we will see later, these connections – represented by the number 15a in Figure 2 – will prove crucial in mediating the effects of ethanol on dopaminergic transmission in the VTA.

In further exploration, Steffensen et al. (1998) separately perfused the competitive NMDA antagonist APV and noncompetitive NMDA antagonist MK-801 into the VTA using microelectrophoresis. They found that both NMDA antagonists significantly reduced the firing of these VTA GABA neurons elicited by stimulation of the internal capsule, suggesting that these GABA neurons receive excitatory input from glutamatergic neurons whose fibers run through the internal capsule. Finally, the authors injected these tested neurons with neurobiotin before using immunogold labeling to identify which

neurons were GABA neurons. In fact, these neurobiotin-labeled neurons did sequester immunogold labeling for GABA. Furthermore, they received "asymmetric excitatory-type" synapses from unlabeled terminals – likely glutamate terminals – as well as "symmetric, inhibitory-type" synapses which sometimes contained immunogold labeling for GABA! In addition, *no* neurobiotin-labeled cells contained labeling for tyrosine hydroxylase, confirming that the VTA neurons tested were in fact GABAergic interneurons.

This experiment provides *very* strong evidence that local VTA GABA interneurons receive GABAergic input from the NAcc, excitatory input via the fornix and fimbria, as well as partially NMDA-mediated excitatory input from other locations in the brain (represented by the numbers 15a and 15b, respectively, in Figure 2). However, can we be sure that these local VTA GABA interneurons synapse on adjacent dopamine neurons in the VTA? While Steffensen & Colleagues (1998) did not outright confirm this, a previous experiment by Johnson & North (1992a) did: Using mu-opioid receptor activation to hyperpolarize midbrain GABA interneurons, these authors demonstrated that blocking local GABAergic interneurons in the VTA facilitates firing of VTA DA neurons via an indirect, disinhibitory mechanism. Furthermore, an experiment published a year after that of Steffensen et al. (1998) demonstrated that blocking GABAergic innervation causes burst firing of VTA DA neurons (Kitai, Shepard, Callaway, & Scroggs, 1999). These experiments by Johnson and North (1992a) and Kitai et al. (1999) - represented in Figure 2 by the numbers 16 and 17, respectively - confirm the existence of these local inhibitory synapses. Additionally, it is evident that this synaptic input to VTA DA neurons is mediated by both GABAa and GABAb receptors (Theile, Morikawa,

Gonzales, & Morrisett, 2008; Xiao & Ye, 2008) and that $GABA_B$ receptors also exist on these presynaptic GABA terminals (Ariwodola & Weiner, 2004). These data are represented by the numbers 18 and 19 in Figure 2. In light of these data, we can say with little hesitation that local VTA GABA neurons *do* inhibit nearby VTA DA neurons, and any kind of inhibition of these GABA neurons can indirectly facilitate firing of VTA DA neurons via disinhibition.

The adept reader may have just noted that we mentioned an author using mu-opioid receptor (MOR) activation to hyperpolarize midbrain GABA neurons (Johnson & North, 1992a). While this at first may seem just a convenient method for inhibiting GABA neurons, we will see that this is in fact a *crucial* facet of GABA neuron morphology in the midbrain. In fact, two different studies have demonstrated that MORs are distributed *only* on non-dopaminergic neurons in the VTA (Dilts & Kalivas, 1989; Garzon & Pickel, 2001). In the first experiment, Dilts & Kalivas (1989) used 6-OHDA – a dopamine-specific neurotoxin – to lesion only DA neurons in the VTA. They found that while lesioning DA neurons resulted in a 65% loss of neurotensin receptor binding, MOR distribution and density were completely unaffected. Such a result provides very strong evidence for the claim that MORs are only present on non-DAergic neurons – i.e. GABAergic neurons – in the VTA. Even further support for this claim comes from an electron microscopy and immunocytochemistry experiment performed by Garzon & Pickel (2001) which sought to further elucidate the physical distribution of these receptors in the VTA. In their experiment, Garzon and Pickel found that more than 50% of labeled MORs were located on the somatic or dendritic region of neurons in the VTA. Only 8% of the MORs were distributed on terminals, and furthermore only 10% of

MOR-containing dendrites stained positive for tyrosine hydroxylase. These data, taken together, demonstrate that MORs are only present on GABAergic neurons in the VTA, and are primarily located on the somata and dendrites of these VTA GABA interneurons. The findings of both Garzon & Pickel (2001) and Dilts & Kalivas (1989) are denoted by the number 20 in Figure 2.

5.) Other Important Findings Regarding Synaptic Interactions in the Mesolimbic System

Before moving on to a discussion of the many changes that occur in the mesolimbic system in response to use or abuse of alcohol, nicotine, or both substances, we'll take a short look at some of the more complex interactions that can arise from the interplay of cholinergic receptors – both nicotinic (nAChR) and muscarinic (mAChR) – with the glutamate receptor NMDA. In a study on rat striatal synaptosomes, Cheramy, Godeheu, L'Hirondel, and Glowinski (1996) sought to determine if activation of cholinergic receptors such as nAChRs and mAChRs could cause enough local membrane depolarization to remove the Mg^{2+} block from nearby NMDA receptors and cause the sudden, large depolarizations associated with NMDA activation. To do this, they used the endogenous agonist acetylcholine, the nAChR agonist nicotine, the mAChR agonist oxotremorine, and NMDA in various combinations in the presence or absence of Mg^{2+} and measured radioactive dopamine (rDA) in 5-minute fractions to determine these receptors' interactions. In the presence of Mg^{2+}, acetylcholine alone dose-dependently stimulated spontaneous release of rDA – up to 41% above baseline at the highest dose. Unsurprisingly, NMDA alone had no effect on rDA efflux due to the presence of the Mg^{2+} block. However, when this highest dose of acetylcholine was combined with NMDA, rDA release jumped to 70% above baseline and remained elevated at the 10- and

15-minute sample points. These observations suggest that cholinergic receptor activation can remove the Mg^{2+} block from NMDA receptors and cause large depolarization and subsequent dopamine efflux.

In order to discern the relative contributions of nAChRs and mAChRs to this Mg^{2+} removal, Cheramy and colleagues performed the same tests again but with nicotine and oxotremorine rather than acetylcholine. Nicotine stimulated rDA efflux to 50% above baseline for all 15 minutes of application, while oxotremorine evoked a large initial response – up to 90% above baseline – which decreased to only 20% above baseline after 15 minutes of application. When nicotine was applied with NMDA, rDA efflux spike to nearly 90% above baseline and decreased to 50% above baseline after 15 minutes of application. Oxotremorine with NMDA spiked rDA efflux to nearly 135% above baseline initially, but decreased to approximately 130% after 15 minutes of application. Cholinergic antagonists reduced these responses in a dose-dependent manner, and application of both nicotinic (mecamylamine) and muscarinic (atropine) cholinergic antagonists at the same time completely blocked rDA efflux.

This data clearly shows that both nAChRs and mAChRs can depolarize the presynaptic membrane of dopaminergic terminals enough to remove the Mg^{2+} block from NMDA receptors and cause a large efflux of dopamine – an effect denoted by the number 21 in Figure 2. It appears that nicotine receptors have a longer lasting effect on membrane depolarization and can elicit constant, elevated DA release for a longer period of time than muscarinic receptors – which appear to induce a larger, immediate efflux which decreases rapidly. This concomitant effect of nicotinic receptors and NMDA receptors is especially important to our discussion: PFC efferents synapse on neuronal spines in the

nucleus accumbens (NAcc) in close apposition to dopaminergic terminals (Sesack & Pickel, 1992). It is therefore entirely possible that – in the presence of nicotine – glutamate released from these PFC terminals could diffuse over to these dopamine terminals and cause opening of NMDA receptor channels that have been unblocked by nAChR stimulation, resulting in a large efflux of dopamine in the NAcc. In fact, NMDA caused a 90% increase in dopamine efflux when infused into the NAcc of awake, moving rats (Nisell et al., 1994), lending support to this theory of glutamate-nicotine receptor interaction in NAcc. Such an interaction could induce LTP at these dopaminergic synapses, or a host of other changes in synaptic plasticity which could predispose a nicotine user to future cravings. NMDA receptors have been shown to be involved in the dynamic plasticity of synaptic function at these synapses (Mansvelder, Keath, & McGehee, 2002; Wooltorton, Pidoplichko, Broide, & Dani, 2003).

The implications surrounding these findings in the accumbal portion of the mesolimbic dopamine pathway are fascinating, but even more fascinating are the implications of research on the ventral tegmental area that have come from the labs of Hubert Mansvelder and Daniel McGehee at the University of Chicago. Among other findings, Mansvelder and McGehee have found that GABA neurons synapsing on the somatodendritic region of mesolimbic dopamine neurons contain somatic β_2-containing nAChRs which desensitize at lower concentrations of nicotine than the α_7 nAChRs present on glutamatergic synapses (Mansvelder et al., 2002). This disinhibition of mesolimbic DA neurons occurs even amidst rising levels of nicotine which can continue to stimulate glutamate transmission, giving rise to a disinhibited, stimulated mesolimbic pathway. Furthermore, the same lab has found that nicotinic stimulation both pre- and

post-synaptically can induce NMDA receptor-dependent LTP at these glutamatergic synapses (Mansvelder & McGehee, 2000). These findings of Mansvelder et al. (2002) and Mansvelder & McGehee (2000) are denoted by the numbers 22 and 23, respectively, in Figure 2.

While I will fully summarize and discuss these findings later, we can begin to see now that the combination of presynaptic and postsynaptic receptors present on these excitatory and inhibitory synapses in the mesolimbic system create the perfect storm for dynamic changes that could predispose a user to addiction. Furthermore, when we later look at ethanol's effect on these nicotinic receptors we will directly observe how, despite initial priming at low ethanol levels, high ethanol levels can interfere with transmission at these synapses – an interaction which could lead to smoking as a compensatory reward mechanism under heavy intoxication. However, before we move on to these exciting conclusions it is best to first look at how both ethanol and nicotine affect this glutamatergic transmission in the VTA. We will then look at how ethanol directly affects nicotinic receptors and finally at the changes in the mesolimbic system that can result from exposure to one or both of these drugs.

Glutamatergic Transmission is affected by both Nicotine and Ethanol in the Mesolimbic Dopamine Pathway:

1.) Nicotine's Effects on Glutamatergic Transmission in the Mesolimbic Dopamine Pathway are Predominately Mediated by Cooperation of NMDA receptors with Nicotinic Receptors

Now that we have a firm understanding of the synapses and receptors critical in dynamic mesolimbic DA system function, we can move on to how nicotine and ethanol

affect excitatory glutamate transmission in this system. In order to best understand these pharmacological interactions, we will first look at nicotine's effects on glutamate receptor function in this system, and more specifically an experiment by Schilstrom, Nomikos, Nisell, Hertel, and Svensson (1998) which tested the direct effects of intra-VTA AMPA and NMDA administration on accumbal dopamine efflux before testing nicotine's effects on this system and the interactions of glutamate receptor antagonization with nicotine-induced accumbal DA efflux.

Both AMPA and NMDA receptor activation in the VTA have been found to elicit dopamine release in the NAcc, however only NMDA antagonization appears to inhibit nicotine-induced mesolimbic dopamine system activation (Schilstrom et al., 1998). In this experiment, Schilstrom and colleagues used a combination of microdialysis and subcutaneous administration of nicotine, as well as pre- and concomitant perfusion of AMPA and NMDA antagonists directly into the VTA in order to test DA release in the NAcc – a measure of mesolimbic dopamine system activation (Wise, 1998). Intra-VTA perfusion of NMDA and AMPA both produced significant increases in NAcc DA efflux, as well as increases in the primary metabolites of dopamine – DOPAC and HVA – confirming that these receptors are involved in activation of the mesolimbic DA system. Individual perfusion of both the AMPA antagonist CNQX and the NMDA antagonist AP-5 directly into the VTA failed to have any significant effect on DA concentrations in the NAcc, suggesting that these antagonists do not interfere with normal DA production or efflux.

Systemic administration of nicotine via subcutaneous (SC) injection significantly increased NAcc DA release, with a peak effect of 77% greater DA efflux over basal

measurements at 100 minutes post-injection; DOPAC and HVA were also significantly elevated. Interestingly, intra-VTA administration of CNQX had *no significant effect* on nicotine-induced DA release – nicotine still elicited near-identical levels of DA efflux in the NAcc. This result suggests that AMPA receptors do not play a significant role in the mediation of nicotine-induced mesolimbic DA system activation. However, intrategmental administration of AP-5 significantly reduced the systemic nicotine-induced NAcc DA overflow. While accumbal dopamine overflow was still increased, AP-5 significantly decreased both the peak DA efflux (34-44%) and the duration of the dopamine elevation (peak at 60 minutes). DOPAC and HVA were still elevated as observed in the nicotine-only condition. Intrategmental administration of nicotine significantly increased NAcc DA overflow (peak 35% at 40 minutes post-injection) as well as DA metabolite levels, suggesting that the VTA is a primary mediator of systemic nicotine-induced mesolimbic DA system activation.

The major implication of these results from Schilstrom et al. (1998) is as follows: A significant portion of nicotine's activation of the mesolimbic dopamine pathway is due to activation of the excitatory ionotropic glutamate NMDA receptor. While direct AMPA receptor activation does elicit DA release in the NAcc, it does not appear that this receptor plays a part in nicotine's activation of the mesolimbic DA system. Nicotine appears to primarily utilize NMDA receptor activation in the mesolimbic DA pathway. Nicotinic receptors have already been found to be able to depolarize the membrane of a neuron enough to remove the Mg^{2+} block from NMDA (Cheramy et al., 1996). Additionally, nicotinic receptors are present both pre- and post-synaptically at these glutamate-dopamine synapses, and NMDA receptors are present postsynaptically on the

cell bodies of these mesolimbic DA neurons (Marchi et al., 2002; Maskos et al., 2005; Picciotto et al. 1998; Wang & French, 1993). Therefore, in the VTA, it appears that administration of nicotine can activate presynaptic α_7 nAChRs, causing release of glutamate from glutamatergic terminals which activates postsynaptic NMDA receptors that have had their Mg^{2+} block removed by the membrane depolarization from nearby β_2-containing nAChRs (see Figure 6, below). Keeping this mechanism in mind, we now will look at another similar experiment which used the AMPA receptor antagonist CNQX and the NMDA receptor antagonist MK-801 to investigate NAcc DA overflow.

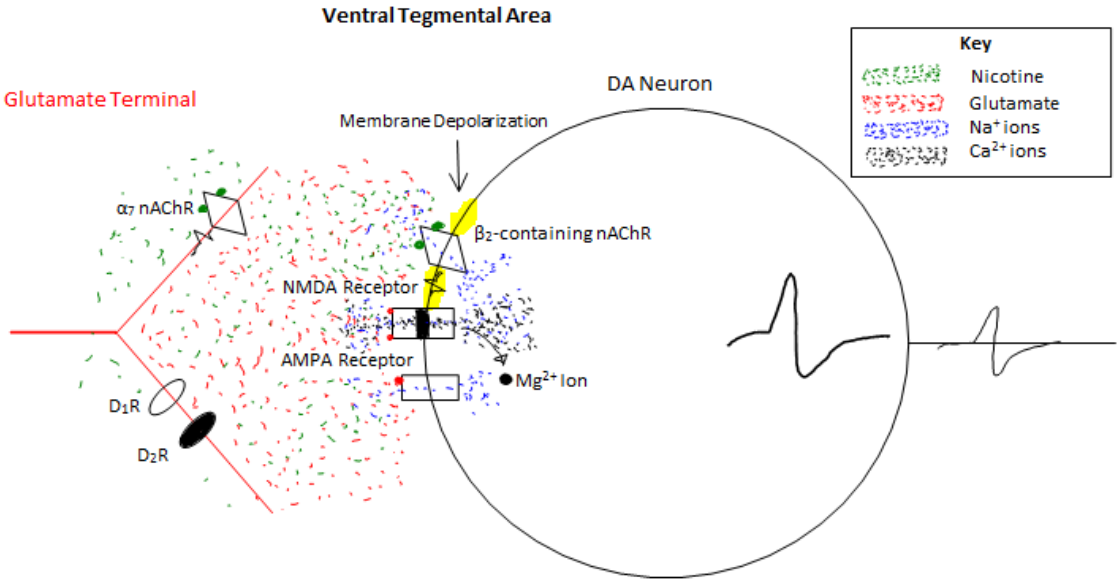

Figure 6. Cooperativity of nAChRs with NMDA Receptors in the Ventral Tegmental Area. (See Appendix for larger version).

In an experiment conducted in 2002, the ability of nicotine, cocaine, and amphetamine to elicit elevated levels of dopamine in the nucleus accumbens was explored using various receptor antagonists (Sziraki, Sershe, Hashim, & Lajtha, 2002). The drugs were individually administered intravenously (IV) and DA measurements were taken from the nucleus accumbens using microdialysis. Nicotine administration increased

NAcc dialysate DA levels by 50%, while cocaine caused a 100% increase, and amphetamine a 1000-3000% increase in NAcc dialysate DA levels. While the dosages used for cocaine and amphetamine were consistent with the range of use of typical human addicts, it should be noted that the dosage of nicotine used – 100 µg/kg – was approximately four times the average amount humans receive after smoking a single cigarette.

After these initial experiments, various receptor antagonists were administered directly into the VTA and their effects on drug-induced NAcc DA efflux were measured. Most importantly, mecamylamine – a general nAChR antagonist – antagonized nicotine-induced NAcc DA efflux but had no effect on either cocaine- or amphetamine-induced NAcc DA efflux. Atropine – a muscarinic acetylcholine receptor (mAChR) antagonist – antagonized both nicotine- and cocaine-induced NAcc DA efflux, but had no effect on amphetamine. However, it is important to note that VTA infusion of atropine alone caused a decrease in basal NAcc DA levels, suggesting an indirect action independent of the drug mechanisms being explored.

Consistent with the findings of Schilstrom et al. (1998), CNQX – an AMPA and Kainate receptor antagonist – had no effect on any drug-induced NAcc DA overflow, suggesting that ionotropic glutamate receptors play a minimal role in the mediation of these drugs' rewarding effects on the brain. Also consistent with previous findings, the NMDA antagonist MK-801 did inhibit nicotine-induced NAcc DA overflow by 66%, while not having any effect on cocaine- or amphetamine-induced NAcc DA overflow.

Such specificity of action suggests that NMDA receptors in the VTA play a specific role in the mediation of nicotine-induced drug reward in the mesolimbic DA

system, but not the actions of other drugs. The specificity of this NMDA receptor action to nicotine only further corroborates activation of a cooperative mechanism of action such as that discovered by Cheramy et al. (1996) in synaptosome membranes. In our case, cooperative interactions of stimulated nAChRs and NMDA receptors on the postsynaptic membrane of these glutamate terminal → dopamine neuron VTA synapses elicit large, excitatory depolarizations of mesolimbic DA neurons in the VTA which lead to increased firing of the mesolimbic system and "reward" stimulation – measured as dopamine efflux in the NAcc. Neither cocaine nor amphetamine stimulate nicotinic receptors, making it impossible for them to activate this cooperative receptor mechanism. Together with the findings of Schilstrom et al. (1998), this experiment by Sziraki and colleagues (2002) provides strong evidence for a cooperative postsynaptic nAChR-NMDA receptor interaction which depolarizes mesolimbic DA neuron cell bodies in response to concomitant glutamate and nicotine stimulation in the VTA.

In a paper published shortly after that of Sziraki et al. (2002), Hernandez, Segovia, & Mora (2003) studied the effects of activating AMPA and NMDA receptors on extracellular concentrations of dopamine, acetylcholine (ACh), and GABA in the striatum of awake, moving rats. NMDA and AMPA were perfused directly into the striatum via microinfusion in 2-4 month old Wistar rats and dialysate levels of DA and ACh were subsequently measured. Perfusion of 100 μM AMPA significantly increased dialysate DA levels, decreased ACh levels, and increased levels of GABA. Administration of 500 μM NMDA had the same effects - significantly increasing dialysate DA levels, decreasing ACh levels, and increasing levels of GABA. As

expected, simultaneous perfusion of AMPA or NMDA antagonists blocked their respective agonists' effects.

Attempting to explain the mechanisms behind all of these changes in neurotransmitter levels is not possible in this paper. However, the finding that NMDA and AMPA both increased DA levels in the striatum of awake, moving rats is important: It demonstrates that the findings we have gathered from *in vitro* experiments replicate real changes in striatal dopamine levels that occur during normal CNS function. Furthermore, the fact that ACh – our body's natural neurotransmitter for nAChR activation – decreases when AMPA or NMDA receptors are stimulated may represent a natural mechanism in our reward circuitry designed to counteract and prevent the overstimulation of reward pathways which appears to occur from simultaneous nAChR and NMDA receptor stimulation.

In another experiment using freely moving rats, Svensson, Mathe, Nomikos, & Schilstrom (1998) explored the role of glutamate and its receptors along with nicotine and nAChRs in mediating the dopaminergic cell firing patterns in the ventral tegmental area. The authors used the NMDA antagonist MK-801 and the nAChR agonist nicotine to explore these receptors. Systemic MK-801 was found to increase NAcc DA levels while simultaneous perfusion of 1mM of the AMPA antagonist CNQX into the VTA completely abolished this effect. Systemic nicotine also induced NAcc DA efflux and was antagonized by infusion of the NMDA receptor antagonist AP-5 but not affected by CNQX administration, a finding consistent with those of both Schilstrom et al. (1998) and Sziraki et al. (2002).

A question remains: How does systemic administration of MK-801 by itself promote NAcc DA efflux? The answer lies in the firing patterns of VTA neurons: NMDA receptor antagonization alone induced a continuous, high frequency pattern of DA neuron stimulation produced by the predominance of excitatory post-synaptic potentials (EPSPs) coming from ionotropic AMPA and kainate receptors. This predominance is also the reason why concomitant administration of AMPA/Kainate antagonist CNQX completely abolishes this elevated NAcc DA – these AMPA/kainate EPSPs are blocked. However, when nicotine is administered the bursting pattern of DA cell firing is augmented – the large depolarizations of the NMDA receptors predominate and are seen to occur more frequently due to the potentiating effect of nAChRs on NMDA receptors. As discussed previously, nAChRs have been shown to display a permissive effect on the firing of NMDA receptors (Cheramy et al., 1996). The fact that concomitant administration of CNQX with nicotine does not abolish or block this nicotine-induced DA efflux suggests that nicotine can stimulate the mesolimbic system through cooperative activation of NMDA receptors, providing even further evidence for cooperation between nAChRs and NMDA receptors in stimulating mesolimbic DA neurons.

In a recent article, Grilli, Pittaluga, Merlo-Pich, & Marchi (2009) used a novel approach to evaluate the effects of continuous blood levels of nicotine administered to live rats via osmotic minipump on NMDA-induced radioactive DA release in synaptosomes prepared from either the NAcc or prefrontal cortex (PFC). All rats were equipped with the pumps and administered either saline or .125 mg/kg/h of nicotine bitartrate for 14 days, after which they were decapitated and synaptosome preparations were made. All subsequent experiments performed on these preparations were done in

the absence of Mg^{2+} and with glycine – a cotransmitter required for NMDA activation. This is a crucial fact, as the subsequent results of experiments performed under these conditions reflects how the NMDA receptors would respond *after* freeing of the Mg^{2+} block from nearby membrane depolarization by nAChRs or AMPA receptors.

Synaptosomes prepared from the *PFC* of rats exposed to the continuous nicotine infusion showed an inhibitory effect: The maximum release of NMDA-induced radioactive DA release was 43% less than vehicle rats. This inhibitory effect dropped to just 15% below vehicle after 7 days post-withdrawal, and disappeared after 14 days. Meanwhile, synaptosomes prepared from the *NAcc* of rats exposed to continuous nicotine showed a potentiating effect: The max overflow of NMDA-induced radioactive DA release was 36% greater than vehicle rats. This sensitizing effect was completely abolished after 7 days post-withdrawal. This experiment provides strong evidence for the interaction of nicotine with NMDA receptors. It also supports the third tenet of Incentive Sensitization Theory: NMDA receptors in this reward area indeed appear to have become "hypersensitive" to stimulation (Robinson & Berridge, 2001). Given that nAChRs have been shown to display a permissive effect on the firing of NMDA receptors (Cheramy et al., 1996), it is reasonable to hypothesize that repeated activation of unblocked NMDA receptors by glutamate released from nearby synapses in the NAcc may sensitize these presynaptic NAcc NMDA receptors. In light of the evidence that NMDA, nicotine, and AMPA receptors are all present on mesolimbic dopaminergic cell bodies in the VTA (Wang & French, 1993; Maskos et al., 2005; Picciotto et al., 1998), it is entirely possible that this sensitization may occur in NMDA receptors present on these mesolimbic DA neuron cell bodies in the VTA as well.

Additional evidence supporting the role of nicotinic receptors in sensitizing and altering the function of glutamate receptors in the VTA has been provided very recently by Gao, Yang, Zhang, Lukas, & Wu (2010). In this experiment, a single intraperitoneal dose of nicotine given to Wistar rats was found to significantly alter the AMPA/NMDA receptor current ratio in the VTA as quickly as one hour later, and was seen to persist for up to 72 hours. The contribution of AMPA receptors was found to increase significantly – presumably due to mechanisms such as the activation of silent synapses and/or sensitization of pre-existing AMPA receptors in the VTA. In fact, 24 hours post-injection, new AMPA receptors were found to be contributing to the functional responses. Interestingly, 7 consecutive daily injections of the same dose of nicotine produced the same results, the only difference between the two being the duration of the receptor ratio increase: a single dose altered the ratio for 5 days, while 7 consecutive daily doses altered the ratio for 8-11 days after administration. Pre-administration of subunit-specific nAChR antagonists before nicotine dosage demonstrated that antagonizing either alpha7 (MLA) or beta2 (MEC) subunit nAChRs alone was not sufficient to abolish this effect. However, coadministration of both antagonists blocked this receptor ratio shift, suggesting that activation of either nAChR subunit type was sufficient for this ratio shift. MK-801 pre-administration completely blocked this shift, heavily implicating NMDA receptors in these changes, while DA antagonists did not block this shift – suggesting that they participate downstream from this nAChR-NMDA interaction, a logical place given the nature of the mesolimbic pathway. The firing rate of VTA DA neurons increased overall, consistent with sensitization and/or proliferation of AMPA receptors observed. This is expected, as Ca^{2+} and CAMKII-mediated proliferation

of AMPA receptors has long been known to result from large bursts of calcium entering a neuron through NMDA receptors (Perkinton, Sihra, & Williams, 1999).

It would be most intriguing to re-analyze the voltage-clamp data from this experiment and see how the pattern of VTA DA neuron firing compares with the firing patterns observed by Svensson et al. (1998) in response to nicotine and AP-5. It initially appears as if this firing pattern should mimic the continuous patterns observed by Svensson and his colleagues in response to AP-5 given the increase in AMPA receptors; however the bursting pattern they observed in response to nicotine is more likely, given that these AMPA depolarizations would potentiate the large depolarizations of NMDA receptor firing in an unblocked, unantagonized setting. NMDA receptors do appear to be the predominate glutamate receptors mediating nicotine reward signals in the nucleus accumbens (Sziraki et al., 2002). Regardless, the most important data gathered from this experiment is the fact that only antagonization of both α_7 homomeric and β_2 – containing nAChRs prevented this shift. This evidence suggests that LTP can occur at this synapse via activation of either presynaptic α_7 nAChRs or postsynaptic β_2–containing nAChRs, making this an extremely dynamic, high-plasticity synapse that could undergo long-term potentiation even if one of the nAChR subtypes were to become completely desensitized! The implications of this plasticity are vast, and we will surely revisit them once we have observed alcohol's effects on both glutamate and nicotinic receptors. For now, we will continue to look at more evidence concerning the "hypersensitization" of NMDA receptor function in response to nicotine consumption.

A separate, recent study by Kenny, Chartoff, Roberto, Carlezon Jr., & Markou (2009) sought to explore the effect of the NMDA antagonist LY235959 on intracranial

self-stimulation (ICSS) thresholds in both the VTA and the central amygdala (CeA). A primary goal of this study was to identify the brain regions in which NMDA receptors (NMDARs) regulate nicotine reinforcement using three methods: Assessment of NMDAR subunit expression in these regions following IV nicotine administration; *in vitro* intracranial recording in order to assess the excitatory effects of nicotine on NMDAR-mediated transmission in these regions; and the effect of direct infusion of LY235959 into these regions on nicotine self-administration. In the first experiment, a .25 mg/kg dose of nicotine was found to lower the ICSS threshold below baseline, while this threshold was elevated in rats pretreated with LY235959 before this dose. This evidence suggests that blocking NMDA receptors makes activating "reward" circuitry in the brain more difficult, impeding self-stimulation. Such evidence is completely in line with all of our previous findings and suggests that NMDAR cooperativity with nAChR stimulation occurs even in nicotine-naïve central nervous systems.

In a second experiment, rats which had been allowed to self-administer nicotine for 7 days were administered LY235959 prior to a self-administration session and observed. Pre-administration of LY235959 blocked the lowering of post-nicotine reward thresholds usually observed in these rats and even elevated the reward threshold at higher doses. Furthermore, LY235959 dose-dependently and significantly decreased nicotine self-administration during the session. The authors also administered LY235959 *after* nicotine self-administration sessions to see whether the blocking of nicotine-induced ICSS threshold-lowering observed in pre-treated rats was due to LY235959 inhibiting the action of nicotine on the reward threshold or its effect on NMDA receptors. All post-self administration doses (0.5, 1.0, and 2.5 mg/kg) of LY235959 reversed the nicotine-

induced lowering of the reward threshold. When these experiments were run with food as the reinforcer it was found that LY235959 only decreased food self admin at the highest doses used in any experiment – 2.5 and 5 mg/kg – suggesting that only these highest doses impair basic functionality of normal reward-seeking behavior. All of this data supports our current assertion that NMDAR cooperativity with stimulated nAChRs is highly involved in mediating nicotine-induced mesolimbic stimulation in the VTA.

Continuing this investigation, Kenny and colleagues (2010) proceeded to run 3 more experiments centered around the VTA, CeA, and PFC. Expression of NMDAR subunits NR2A, NR2B, and NR1 as well as AMPAR subunits GluR1 and GluR2 was found to be significantly increased in the CeA after nicotine self-administration. The VTA showed a significant increase in NR2A expression, while the NAcc showed a significant increase in GluR1 expression. Finally, the PFC showed a significant *decrease* in NR2A, NR2B, and GluR2 expression. It is important here to note that synaptosomes prepared from the PFC of rats exposed to continuous nicotine infusion have shown a 43% reduction in NMDA-induced radioactive DA release, corroborating this PFC result and providing more evidence that NMDARs in the PFC desensitize after continued nicotine exposure (Grilli et al., 2009). The contrary increase in subunit expression seen in the CeA, VTA, and NAcc suggests a sensitizing effect in response to repeated nicotine exposure, a finding which is again in line with those of Grilli et al. (2009) and the tenets of Incentive Sensitization Theory (Robinson & Berridge, 2001).

A follow up experiment using paired-pulse facilitation (PPF) on rat CeA slices showed that while nicotine had no effect on basic membrane properties such as input resistance or spike amplitude, it increased the transmission of NMDA-mediated

excitatory post-synaptic currents (EPSCs), overall glutamate EPSCs, and non-NMDA EPSCs. The increase in transmission was measured using the PPF ratio, represented as first pulse / second pulse; as the second pulse increases in size (signifying potentiation), the PPF ratio decreases, signaling the increase in transmission. While this experiment focused on the central amygdala and is not as applicable as the VTA experiments, it still shows evidence of cooperativity between nicotine and NMDA receptors.

In a final experiment, direct injection of LY235959 via bilateral cannulae into either the VTA or CeA dose-dependently decreased nicotine self-administration, suggesting that these sites play a large role in mediating the rewarding effects of nicotine. Altogether, this set of experiments provides a large amount of evidence for NMDA receptors playing a critical role in the mediation of nicotine reward in the mesolimbic dopamine system via their activation and sensitization in the VTA.

Before we conclude this section, let's take a brief look at a short experiment by Schilstrom, Fagerquist, Zhang, Hertel, Panagis, Nomikos, and Svensson (2000) which highlights the role presynaptic α_7 nAChRs play in the ventral tegmental area. In this experiment, microdialysis techniques were used to measure the extracellular concentrations of glutamate and aspartate in the VTA in response to subcutaneous injections of 0.5 mg/kg of nicotine. Additionally, the effect of direct microinfusion of the α_7 nAChR antagonist methyllycaconitine (MLA) into the VTA before nicotine dosage was explored in these pentobarbital-anaesthetized rats. Finally, the medial PFC was lesioned with ibotenic acid and the number of α_7 nAChR subtypes was measured to investigate whether presynaptic α_7 nAChRs on glutamatergic PFC inputs make up a significant portion of this excitatory input to the VTA.

As we would expect based on our previous findings, subcutaneous nicotine significantly increased extracellular glutamate and aspartate levels in the VTA, while pre-administration of MLA completely prevented this effect of nicotine. This suggests that presynaptic α_7 nAChRs are mediating this effect. In fact, synaptosome studies have shown presynaptic α_7 nAChRs to be present on glutamatergic synapses in the VTA (Marchi et al., 2002). Additionally, slice preparations have shown greater DA release than synaptosomes in response to nicotine, and this effect is blocked by glutamate antagonist administration (Wonnacott et al., 2000). Together, all of these facts suggest that nicotine stimulates presynaptic α_7 nAChRs, eliciting glutamate release at glutamate-dopamine synapses in the VTA that can increase the firing rate of mesolimbic dopamine neurons.

The most interesting findings of Schilstrom et al. (2000), however, came from the lesioning experiment: When measured with radioactive alpha-bungarotoxin binding, it was found that lesioning of medial PFC input decreased the number of α_7 nAChRs in the VTA by approximately 30%! This finding provides very strong evidence that nicotine can directly affect cortical inputs to the mesolimbic dopamine system by activating presynaptic α_7 nAChRs and altering glutamate transmission at these PFC inputs. With this evidence in mind, it doesn't seem far-fetched – but rather *likely* – that nicotine's interference with corticolimbic transmission in the VTA could be responsible for the pharmacological association of alcohol cues with nicotine craving (Cooney et al., 2003). With this in mind, let's take a closer look at how ethanol can effect excitatory glutamate transmission in the mesolimbic system and then how these two drugs could interact to elicit associations between alcohol consumption and nicotine craving.

2). Ethanol's Effects on Glutamatergic Transmission in the Mesolimbic Dopamine Pathway

We have already discussed and summarized two articles by Deng et al. (2009) and Xiao et al. (2009) which discovered the participation of presynaptic dopamine receptors on glutamate terminals in the PFC in a positive feedback loop (See "Dopamine Receptors at VTA Synapses"). Briefly, ethanol causes retrograde dopamine release from the somatodendritic region of VTA mesolimbic dopamine neurons which diffuses back and stimulates excitatory D_1Rs on the presynaptic glutamate terminals, causing dopamine release (Deng et al., 2009). These D_1Rs desensitize within 30-60 seconds, however, making this only a temporary positive feedback loop which occurs at the onset of alcohol intoxication. At higher ethanol levels, however, inhibitory D_2Rs are stimulated by the retrograde dopamine release, causing active inhibition of glutamate transmission at these VTA synapses and less glutamatergic excitation of mesolimbic dopamine neurons (Xiao et al., 2009).

Since we already know that α_7 nAChRs exist on these presynaptic glutamate terminals in the VTA and can stimulate firing of these terminals (Marchi et al., 2002; Schilstrom et al., 2000), it seems probable that someone who had previously experienced nicotine's ability to enhance and continue the firing of these glutamatergic synapses and thus the mesolimbic DA system would seek this drug in order to compensate for the loss of mesolimbic stimulation at high ethanol levels. The cooperative contribution of both pre- and postsynaptic nAChR stimulation along with nAChR-gated NMDA receptor stimulation can create large EPSPs which elevate mesolimbic DA system firing and NAcc dopamine efflux for a long period of time (Cheramy et al., 1996; Nisell et al.,

1994). While this compensatory mechanism theory is attractive and seems to explain many of the current findings to date, the truth about ethanol is that its widespread effects on different neurotransmitter systems make it difficult to study. As an example, let's take a closer look at the complex effects of ethanol administration in the mesolimbic system on NAcc dopamine levels before we move on to its effects on glutamatergic systems.

In a five part microdialysis study on male Wistar rats, Ericson, Molander, Lof, Engel, and Soderpalm (2003) sought to exhaustively test the effects of direct ethanol administration into the VTA or NAcc on dopamine levels in the NAcc. Interestingly, in their first experiment direct administration of any dose of ethanol into the VTA did not have any effect on NAcc dopamine levels. While this at first seems confounding, after taking into account that ethanol has no direct way of stimulating mesolimbic neurons this seems less concerning. Additionally, the temporary positive feedback loop documented by both Deng et al. (2009) and Xiao et al. (2009) has been shown to desensitize within 30-60 seconds, and therefore cannot be expected to have a lasting influence of enough magnitude on mesolimbic neurons to induce a significant rise in NAcc dopamine levels. At best, the rise would be short and difficult to measure.

Contrary to their VTA results, perfusion of ethanol into the NAcc had two profoundly different effects based on dose: 300 mM caused a 30% increase in NAcc DA levels, while 1000 mM caused a 50% decrease in NAcc DA levels. These findings seem oddly familiar, as they seem to mimic the findings of Xiao et al. (2009) when measuring glutamate release from presynaptic terminals in the VTA. In the case of Xiao, it was determined that presynaptic D_2R activation only occurs at high levels of ethanol and inhibits firing of the terminal. While in this case we are dealing with presynaptic

dopamine terminals, it still seems likely that this mechanism or a similar negative feedback mechanism is taking place.

In the second part of their study, Ericson and colleagues tested the effect of intraperitoneal pre-administration of a vesicular acetylcholine storage inhibitor – vesamicol – 40 minutes prior to administering 300 mM ethanol directly into the NAcc. They found that vesamicol completely prevented the ethanol-induced enhancement of NAcc dopamine levels, suggesting that cholinergic transmission – mediated by either nAChRs or mAChRs – mediates this effect of ethanol in the NAcc. While it is beyond the scope of this paper to attempt to explain this finding, cholinergic interneurons are present in the midbrain and could be responsible for this result. Interestingly, in the third part of the experiment perfusion of the nonselective nAChR antagonist mecamylamine (MEC) into the VTA 40 minutes prior to NAcc ethanol administration antagonized the NAcc dopamine-elevating effect of ethanol, yet administration of MEC directly into the NAcc did not. While this result is yet again beyond the scope of this paper, it should be noted that the latter result suggests that nAChRs in the NAcc do not play a role in ethanol's effect on NAcc dopamine levels, but nAChRs in the VTA do. In light of this fact, it seems both necessary and proper to continue to focus on the VTA to investigate these two drugs' interactions.

In the fourth part of the experiment, the selective β_2-containing nAChR antagonist DHBE (1 mM) was perfused into the VTA 40 minutes prior to NAcc ethanol perfusion. DHBE did not block the ethanol induced increase in NAcc dopamine levels. This would suggest that the nAChRs which seemed to be playing a role in ethanol's effects on NAcc DA levels are non- β_2-containing nAChRs. In the fifth part of the study,

the NMDA antagonist MK-801 was perfused into the VTA 40 minutes prior to NAcc ethanol perfusion. It was found that MK-801 alone increased NAcc DA levels, and that ethanol perfusion further increased this elevation in NAcc dopamine. While I personally believe that this effect was due to the predominance of AMPA receptor firing in the absence of NMDA signaling, such a discussion is beyond the scope of this paper and should be reserved for neuroelectrophysiologists. This paper serves as an example of the complexities encountered when trying to determine the neuroanatomical effects of ethanol – a drug with no specific receptor. The diffuse, widespread effects of ethanol on many different neurotransmitter systems make it a frustratingly difficult drug to study. Nonetheless, we will continue to take a look at its effects on glutamate release and glutamate receptor function in an attempt to understand how it can affect the glutamate-dopamine synapses we have explored so in depth already.

A good place to start our review of ethanol's effects on glutamate transmission is a microdialysis experiment performed on rats which sought to determine if there is a correlation between ethanol withdrawal and glutamate release in the striatum. In the experiment by Rosetti & Carboni (1995), one group of rats was given an intragastric dose of ethanol every 6 hours for six days with dosage varying based on level of intoxication. This was done in order to maintain a steady level of intoxication in the ethanol group of rats, while the control group received sucrose. Dialysate samples taken two hours after the last ethanol dose showed no significant difference in striatal glutamate levels between the two groups, however at 12 hours post-ethanol, glutamate output in the ethanol group reached a peak of 255% of control glutamate levels and remained significantly elevated

until 24 hours post-ethanol. By 36 hours post-ethanol, striatal glutamate levels had gradually returned to control value.

This finding is significant to the author's study as it shows a correlation between ethanol withdrawal and glutamate release, however it is significant to our exploration for another reason: The implication of an adaptive mechanism in response to ethanol. It appears that ethanol in some way suppresses glutamate release, and that the chronic ethanol rats' striatal circuits had developed a compensatory mechanism to keep glutamate at homeostatic levels – an assertion supported by the fact that both groups had approximately the same glutamate levels 2 hours post-ethanol. In fact, a closer look at the data shows that ethanol-intoxicated, non-withdrawn rats had glutamate levels of 3.87 pmol/min while sucrose rats had glutamate levels of 4.7 pmol/min, which would suggest that although the numbers are not statistically different, there is a clear trend toward decreased glutamate levels in chronic ethanol rats. While this initial chronic ethanol data only has limited application to clear alcoholics, let's look at the rest of the author's findings.

Rosetti & Carboni (1995) found that at 12 hours post-ethanol, an ethanol challenge reduced glutamate output to control values within 3 hours of administration and remained at control values for 8 hours post-challenge. Withdrawal symptoms were quelled within 1 hour post-challenge – even more quickly than glutamate output. Administration of the NMDA antagonist MK-801 to 12-hour post-ethanol rats reduced glutamate output to 36% above control within 2 hours and remained at this level for 1 hour before gradually returning to the pre-MK-801 value. Most importantly, at 2 hours post-injection, MK-801 significantly reduced withdrawal scores to approximately 20% of

control. Administration of the anti-anxiety benzodiazepine diazepam did not affect glutamate levels but reduced withdrawal scores to 46% of control in 2 hours and maintained this reduced score for another 2 hours.

The data from MK-801 and diazepam administration is compelling, leading the authors to conclude that striatal glutamate is a neurochemical correlate of ethanol withdrawal. The reduced efficacy of diazepam and its failure to affect glutamate levels supports this conclusion, as well as the fact that MK-801 both quelled withdrawal symptoms to a greater extent and reduced glutamate levels. In regards to our research, this data is encouraging: NMDA receptors are the primary mediators of nicotine-induced DA release in the mesolimbic system (Kenny et al., 2009). This common receptor thus seems to be a crucial point of interaction for both ethanol and nicotine's effects on the mesolimbic dopamine system. NMDA receptor hyperexcitability may be partially to blame for this heightened level of glutamate, as this could be a compensatory mechanism for chronic suppression of function by ethanol. MK-801's efficacy at quelling heightened glutamate levels lends a great deal of support to this assertion. Furthermore, the fact that chronic ethanol suppresses glutamate levels opens up the possibly that heavy alcohol consumption – such as the amounts consumed during binge drinking – could acutely suppress glutamate transmission in the VTA, driving those who have consumed nicotine before to seek it out in order to restore this transmission and subsequent dopaminergic transmission in the mesolimbic system. Now that we have determined NMDA receptors to play a critical function in mediating ethanol's effects, let's take a closer look at how ethanol can affect these receptors.

In an experiment on halothane-anaesthetized rats, Stobbs, Ohran, Lassen, Allison, Brown, & Steffensen (2004) explored the effect of ethanol and MK-801 on GABA neuron discharge in the VTA. While this experiment may seem unrelated to our endeavor, it is important to note that glutamatergic projections from the PFC have been found to synapse on both dopamine and GABA neurons in the VTA (Carr & Sesack, 2000). While we would prefer to have this same experiment performed on DA neurons in the VTA, the results of Stobbs et al. (2004) can still be considered relevant. Stobbs and colleagues found that intraperitoneal administration of ethanol significantly decreased VTA GABA neuron discharge rates in a dose-dependent manner. Furthermore, MK-801 administration also decreased VTA GABA neuron discharge rates in the same dose-dependent manner. At a dose of MK-801 which reduced GABA neuron discharge by 40%, doubling the dose doubled the degree of inhibition. Most importantly, at this 40% inhibition dose of MK-801, addition of an equipotent dose of ethanol *also* nearly doubled inhibition of GABA neuron discharge.

While we will see in our next section that ethanol exerts inhibitory effects on GABA transmission by MOR activation (Xiao, Zhang, Krnjevic, & Ye, 2007; Xiao & Ye, 2008), this data provides strong evidence for the assertion that ethanol antagonizes and suppresses NMDA receptor function in the VTA. It follows that chronic, continued suppression of NMDA receptor function could elicit compensatory mechanisms in which the brain either increases the sensitivity of NMDA receptors or the total number of NMDARs in order to compensate. In fact, this compensatory hyperexcitability in response to chronic ethanol has been documented in the microdialysis experiment by Rosetti & Carboni (1995). Furthermore, an experiment performed in 1995 appears to

present evidence of this hyperexcitability and upregulation in mammalian cortical neurons (Hu & Ticku, 1995).

In the experiment by Hu and Ticku (1995), cultured mammalian cortical neurons were treated with 50 mM ethanol for 5 consecutive days before being treated with NMDA. The authors then used changes in intracellular calcium to measure the function of these NMDA receptors compared to controls. The chronic ethanol exposure did not change the morphological characteristics, protein content, or resting intracellular calcium levels of the cortical neurons. However, when cells were treated with NMDA in a magnesium-free environment, the E_{max} value for for intracellular calcium was significantly enhanced in cells treated with ethanol for 5 days when compared to controls. The same treatment also elicited a 30% increase in MK-801 binding, and both effects were reversed after 48 hours of ethanol withdrawal. Additionally, incubation with noncompetitive NMDAR antagonist MK-801 or an alternative competitive NMDA receptor antagonist CPP in addition to ethanol prevented both the increase in the E_{max} of intracellular calcium and upregulation of NMDARs. It appears that the manner in which ethanol suppresses NMDAR function is different than that of these antagonists, and does so in a manner which elicits compensatory action from NMDA receptors.

All of this data strongly supports our assertion that chronic ethanol suppresses NMDAR function, causing compensatory NMDAR upregulation and hyperexcitability. While alcohol and MK-801 appear to have additive suppressing effects on NMDA receptor function in the VTA (Stobbs et al., 2004), it appears they have differing pharmacology as concomitant MK-801 and ethanol exposure prevents compensatory action in response to chronic ethanol (Hu & Ticku, 1995). In my opinion, this disparity

likely arises from complex interactions between ethanol's stimulation of mu-opioid receptors on local GABA interneurons and suppression of GLUTergic terminals in the VTA.

MK-801 quells ethanol withdrawal-induced glutamate overflow in the striatum and related symptoms with greater efficacy than benzodiazepenes and nearly as efficaciously as re-administration of ethanol (Rosetti & Carboni, 1995). If this ethanol-induced suppression of NMDA receptor function can occur acutely in response to heavy ethanol consumption – such as that of binge drinking – a user who has previously consumed nicotine with alcohol could crave nicotine in order to "reignite" glutamate transmission at glutamate-dopamine synapses in the VTA, causing mesolimbic neuron stimulation and firing. Nicotine's activation of presynaptic α_7 nAChRs on glutamate terminals as well as postsynaptic β_2-containing nAChRs on mesolimbic DA neuron cell bodies would heavily stimulate the mesolimbic system and induce firing despite ethanol interference with glutamate levels and NMDAR responsiveness. While the contribution of nAChR-NMDAR cooperativity would not be as stark or effective, nAChR stimulation would still induce firing, especially in those who do not smoke regularly and have fully sensitive nAChRs. Furthermore, smokers with desensitized nAChRs would be driven to smoke in excess in order to reignite firing of mesolimbic neurons to the greatest extent possible. Given all of this, it seems abundantly clear how ethanol can interact with glutamatergic PFC afferents and synaptic function in the VTA to elicit pharmacologically-induced nicotine craving.

GABAergic Transmission is Primarily Affected by Ethanol in the Mesolimbic Dopamine Pathway

1). Ethanol's effects on GABAergic Transmission in the Mesolimbic Pathway

Before we begin an in-depth discussion of ethanol's effects on GABAergic transmission in the mesolimbic system, let's recapitulate what we know about GABA neurons in the mesolimbic system: GABA interneurons are present in the VTA, synapse on local VTA DA neurons, and a subpopulation has been found which projects to the NAcc (Johnson & North, 1992b; Johnson & North, 1992a; Kitai et al., 1999; Van Bockstaele & Pickel, 1995). These GABA neurons are innervated by reciprocal, inhibitory GABA neurons which project back from the NAcc (Walaas & Fonnum, 1980; Von Bockstaele & Pickel, 1995), and additionally contain mu-opioid receptors (MORs) which – upon activation – greatly hyperpolarize these local VTA GABA interneurons – inhibiting their activity (Johnson & North, 1992a). In the VTA, these MORs are only found on GABA neurons, and are distributed primarily on the somata and dendrites of these neurons (Dilts & Kalivas, 1989; Garzon & Pickel, 2001).

Now that we've had a quick refresher on GABA circuitry in the mesolimbic system, we can dive in to the effects ethanol has on its transmission. Since our primary focus is on how the effects of ethanol on these GABA neurons affect firing of mesolimbic dopamine neurons, I will be careful to continually relate the ensuing complex interactions back to dopaminergic transmission on a regular basis. First we'll start with a few simple facts, then progress into the meat and bones of this neural network.

Let's start with this fact: Ethanol has been shown to potentiate GABAergic transmission in the spinal cord by potentiating $GABA_A$ receptor chloride channels

(Mehta & Ticku, 1988). This seems easy enough to swallow, but the adept reader will immediately spot a conflict: How does *increasing* inhibitory transmission increase VTA DA neuron firing? Well that's simple: It doesn't. In fact, it prevents firing of this reward system. Remember how we discussed the difficulties of studying the diffuse, widespread effects of ethanol? Well, nothing will showcase this frustrating study more than this section. As a reader, you will find that it will be most easy to reconcile what will at first seem like conflicting pieces of evidence by adhering to one simple principle: Evaluating the *magnitude* of each of ethanol's specific effects. We will see that some effects of ethanol *do* in fact potentiate GABA transmission, but it is the *net* effect which is most important. With this in mind, let us begin.

A very recent study by Theile, Morikawa, Gonzales, & Morrisett (2008) sought to examine the effect of acute ethanol on $GABA_A$-receptor mediated inhibitory postsynaptic currents (IPSCs) in ethanol-naïve VTA dopamine neurons. The authors used midbrain slices prepared from Sprague-Dawley rats, conventional whole-cell patch clamp techniques to measure IPSCs, and Kynurenic acid (1mM) to prevent interference by AMPA & NMDA currents. In 5 of the 6 neurons tested, 50 mM of ethanol resulted in an increase in spontaneous IPSCs (sIPSCs) and a significant enhancement of paired-pulse depression (PPD) – indicating an increase in first-pulse GABA release. Exploring further, the authors decided to investigate whether or not presynaptic inhibitory $GABA_B$ receptors mediated this enhancement of GABAergic transmission. The authors found that administration of baclofen alone – a $GABA_B$ agonist – decreased sIPSC frequency. However, when 50 mM ethanol was administered to a baclofen-depressed slice, it still raised sIPSC frequency by 30%. Conversely, when a $GABA_B$ antagonist – SCH50911 –

was administered, sIPSC frequency was enhanced. When ethanol was administered to this SCH50911-enhanced slice, it *still* increased sIPSC frequency by 23%, suggesting that ethanol potentiates sIPSC frequency regardless of presynaptic $GABA_B$ receptor activation or inhibition.

The authors knew that these tests were not enough to determine whether ethanol was enhancing GABA release via action at the terminals or by increasing the overall excitability of the GABA neuron. In order to delineate between these two possibilities, the authors recorded miniature IPSCs (mIPSCs) in the presence of tetrodotoxin (TTX) in order to block all action potential-mediated events. Even in the presence of TTX, ethanol still produced a significant enhancement in mIPSC frequency, indicating that ethanol is acting at the presynaptic GABA terminal to enhance GABA release. So, despite evidence from previous studies indicating that activation of these $GABA_B$ autoreceptors may limit ethanol-induced potentiation of GABA release at terminals (Ariwodola & Weiner, 2004), at these clinically relevant concentrations of ethanol it seems that acute ethanol still potentiates GABA release at GABAergic terminals in the VTA.

So, there we have it. Ethanol seems to be *enhancing* GABA transmission at the terminal. In fact, it seems to enhance GABA transmission at these GABA neuron→DA neuron synapses *despite* activation of GABA terminal autoreceptors. This enhanced inhibitory transmission would in fact *prevent* VTA DA neuron firing if it were the only effect of ethanol. However, we will see later in this section it is not. For now though, let's continue to explore this seemingly conflicting effect of ethanol by reviewing another experiment performed on these synapses in 2002. This time, rather than looking at the immediate, acute effects of a dose of ethanol on these GABA terminals, we will be

looking at the effects of a single dose of ethanol on these terminals 24 hours after *in vivo* administration.

Seeking to determine what short-term effects a single dose of ethanol may have on GABA synapses in the VTA, Melis, Camarini, Ungless, & Bonci (2002) chose to administer a single, introperitoneal (I.P.) dose of either ethanol (2 g/kg) or saline to C57BL/6J mice. 24 hours later, slice preparations were made and whole-cell voltage clamp recordings were used to measure $GABA_A$-mediated IPSCs in VTA dopamine neurons. The bicarbonate-buffered solution used in these slice preparations contained AP5, CNQX, strychnine, and eticlopride to antagonize and prevent interference from NMDA, AMPA, glycine, and D_2 dopamine receptors, respectively.

As you may have noticed, this recording method is the same as that used by Theile et al. (2008). The inhibitory currents are being measured on the VTA DA neuron's membrane, providing an accurate measure of the inhibitory input received by these VTA DA neurons from local VTA GABA interneurons. However the primary differences in this experiment are twofold: 1) ethanol was administered to the mice while they were still alive, and 2) slices were made and tested 24 hours after this *in vivo* administration. The *in vivo* application and subsequent 24 hour delay before decapitation and slice preparation allows the mice's own pharmacokinetic elimination of the drug. Meanwhile, testing of these GABAergic inputs 24 hours later allows us to examine whether or not the acute, potentiating effects of ethanol observed on these GABA terminals by Theile et al. (2008) pervades for longer than simply the duration of ethanol intoxication.

The results of Melis et al.'s (2002) experiments are complex, but enlightening. They found that saline control mice displayed paired-pulse facilitation (PPF), while

ethanol mice displayed paired-pulse depression (PPD) 24 hours after a single *in vivo* exposure to ethanol. As we've discussed already, paired-pulse ratio (PPR) is equal to $ISPC_2/ISPC_1$. In essence, if the second current is stronger than the first, we can deduce that repeated stimulation either causes *greater* neurotransmitter release or that the postsynaptic receptors being measured – in this case $GABA_A$ receptors – are functionally sensitized by the first release of neurotransmitter – either of which would result in PPF. Conversely, a weakened second current – PPD – can indicate that either 1) the first stimulation caused release of so much neurotransmitter that there is not as much left immediately available for release following a second pulse, 2) there is lower overall probability of neurotransmitter release, 3) there is a decrease in the functional sensitivity of the postsynaptic receptors being measured, or 4) another mechanism or any combination of the above mechanisms.

In order to delineate between the above mechanisms, Melis and colleagues manually bath-applied GABA in the presence of the $GABA_B$ antagonist phosphinic acid. By doing this, they took the presynaptic GABA terminal completely out of the equation and manually tested the postsynaptic $GABA_A$ receptors. Furthermore, by antagonizing $GABA_B$ receptors, they could be sure that all IPSCs recorded under these parameters were from postsynaptic $GABA_A$ receptors and not postsynaptic $GABA_B$ receptors. Sure enough, they found no significant difference between the amplitude of inward IPSCs recorded in both saline and ethanol mice VTA DA neurons, essentially confirming that ethanol-induced modification of these VTA GABA neuron→DA neuron synapses occurs by modification of GABA release *at the terminal* and not modification of postsynaptic $GABA_A$ receptors.

In further exploration of how ethanol modifies GABA terminal function, these researchers examined spontaneous $GABA_A$ miniature IPSCs (mIPSCs) in these VTA slices. While the amplitudes of these mIPSCs were the same in both groups, the frequency was found to be significantly higher in slices from ethanol-treated mice. Such a result appears to indicate that ethanol administration *increases* the probability of GABA release from these GABAergic terminals in the VTA, and that this effect is still seen 24 hours after exposure to ethanol. It seems that despite the fact that initial PPD evidence pointed to a lower probability of GABA release, the result of ethanol exposure is in fact *increased* spontaneous GABA release from these terminals.

Fortunately, the authors were not OK with simply accepting this conflict. They posited that the increased probability of GABA release which resulted from ethanol exposure could raise overall GABA levels in the VTA, resulting in activation of presynaptic $GABA_B$ receptors and causing inhibition of second-pulse GABA release - effectively decreasing $ISPC_2$ magnitude, resulting in PPD. It has already been demonstrated that these $GABA_B$ receptors exist on presynaptic GABAergic terminals (Ariwodola & Weiner, 2004). Furthermore, Theile et al. (2008) confirmed that application of a $GABA_B$ agonist decreases sIPSC frequency, and that ethanol still increases the sIPSC frequency despite this depression, albeit to a lesser extent. When Melis and her colleagues administered a $GABA_B$ antagonist, it completely shifted the PPR of ethanol-treated mouse slices from PPD to PPF, despite having no effect on either $IPSC_1$ or $IPSC_2$ in saline-treated mouse slices! Such results seem to confirm that the first pulse elicited by electrical stimulation of these neurons causes release of enough GABA to activate these presynaptic $GABA_B$ receptors, resulting in decreased amplitude of the

second evoked current – $IPSC_2$ – and the observation of PPD in ethanol-exposed mice! Melis and her colleagues did note that PPD and PPF in this experiment did *not* depend on the the size or intensity of the stimulus, indicating that these PPR results were not due to differences in first-pulse GABA release. In light of this, it seems that the ethanol-induced increase in GABA release probability is *just* enough to elevate GABA in the synapse to a level at which stimulation of the neuron results in release of enough GABA to activate these presynaptic $GABA_B$ receptors, resulting in inhibition at the terminal and the observation of PPD.

Despite this cohesive evidence the authors had one last question regarding PPR: What if the sensitivity of presynaptic $GABA_B$ receptors is enhanced in ethanol-treated animals? To test this, the authors compared the inhibition caused by administration of baclofen – a $GABA_B$ receptor agonist – in both ethanol- and saline-treated mice. They found that the concentration-response curves for baclofen administration were similar in both groups, indicating that this shift from PPD to PPF was *not* due to increased presynaptic $GABA_B$ receptor sensitivity to endogenous GABA, but rather likely due to increased GABA levels in the synapse.

So, after all of this testing it seems that Melis et al. (2002) finally confirmed that ethanol increases the probability of GABA release at GABA→DA synapses in the VTA, and this increase appears to result in observation of PPD during paired-pulse experiments due to activation of presynaptic $GABA_B$ receptors by increased GABA in the synapse. While this would be enough for most researchers, Melis and her colleagues wished to do one last set of experiments to dive further into ethanol's effects on these GABA neurons. The authors chose to explore the adenylyl cyclase (AC) → cyclic AMP (cAMP) →

Protein Kinase A (PKA) pathway in order to determine whether it mediated any of ethanol's effect on GABAergic terminals. They found that application of forskolin – an adenylyl cyclase activator – increased $IPSC_1$ amplitude in saline mouse slices but had no effect on ethanol mouse slices. This increase in $IPSC_1$ amplitude shifted the PPR of these control slices towards PPD, mimicking the effects of a single ethanol exposure and supporting the idea that AC activation increases the probability of GABA release. The frequency of spontaneous mIPSCs also increased in these saline control slices, further mimicking the effects of ethanol and suggesting that ethanol may exert its potentiating effects on GABA release at GABAergic terminals by saturating AC and maximizing its activity. In further investigation, the authors used the PKA antagonist H89 to see if antagonization of the pathway could reverse ethanol's effects. In fact, H89 reduced the amplitude of $IPSC_1$ in ethanol mouse slices – shifting the PPR towards PPF – but had no effect in slices from saline mice. Furthermore, application of forskolin in the presence of H89 was unable to alter $IPSC_1$ in either group, suggesting that the effects of AC activation are indeed mediated by PKA activity – i.e. AC activation increases cAMP, resulting in increased PKA levels and activity which evokes changes in GABA release probability at GABA terminals. Lastly, H89 reduced the spontaneous mIPSC frequency in slices from ethanol mice but not in slices from saline mice, further corroborating the claim that ethanol exerts its potentiating effect on GABA release at GABAergic terminals in the VTA by enhancing the activity of the adenylyl cyclase pathway.

It is safe to say that we have now clearly established ethanol's potentiating effect on GABA release at GABAergic terminals in the VTA. Ethanol exerts this effect at the presynaptic GABAergic terminal in the VTA and increases the overall probability of

GABA release at these synapses, potentially raising the level of GABA in the synapse (Theile et al., 2008; Melis et al., 2002). Now, we will begin to look at a series of *in vivo* experiments which all seem to support a seemingly conflicting conclusion: Ethanol depresses GABA neuron function in the mesolimbic system. Remember how I said to keep in mind the *magnitude* of each discussed effect of ethanol? Well, now is the time to do so. While ethanol enhances GABA release at the terminal itself, it seems that this effect pales in comparison to the net effect of ethanol: Inhibition of GABA neuron firing in the mesolimbic system.

An earlier, two-part experiment on both freely-behaving and halothane-anaesthetized rats performed by Gallegos, Lee, Criado, Henriksen, & Steffensen (1999) is the best place to start our exploration of ethanol's inhibiting effects on GABA neurons in the mesolimbic system. In the first portion of the experiment, 10 male Sprague-Dawley rats had microwire electrodes implanted into their VTA, were allowed 1 week to recover, and then divided into two "freely behaving" groups: The first consisted of 4 rats treated first with two acute introperitoneal (I.P.) doses of ethanol (0.4 and 0.8 g/kg), and then 2 weeks of twice-daily I.P. ethanol injections. The second group consisted of 6 rats treated with the same initial I.P. doses of acute ethanol, followed by 2 weeks of overnight ethanol vapor exposure.

When 3 of these freely behaving rats were given their first doses of ethanol sequentially at 20, 30, and 70 minutes apart, it was found that the acute ethanol administered consistently resulted in a significant, marked decrease in VTA GABA neuron firing which peaked between 5 and 10 minutes post-injection and was followed by a moderate increase in firing. Out of the 18 total VTA GABA neurons recorded in

freely moving rats, 16 responded to these initial, acute ethanol doses with a rapid decrease in firing rate. Overall, all I.P ethanol doses of greater than 0.2 g/kg significantly inhibited the firing rate of these VTA GABA neurons. Interestingly, increasing the ethanol concentration did *not* increase the magnitude of GABA neuron inhibition, but rather increased the relative *duration* of the inhibition. We'll discuss possible reasons for this later in this section.

After these initial ethanol tests, one of the groups of rats was subjected to twice daily ethanol injections for a 2 week period. These injections of 2 g/kg ethanol occurred in the morning, and then 8 hours later. GABA neuron activity was recorded before and after the second administration. The authors found that ethanol significantly decreased the firing rate in these rats and still decreased this rate after 7 days of this dosing regimen. However, by the 11^{th} day of this regimen, ethanol lacked any effect on the VTA GABA neurons being measured and no alteration in firing rate was recorded upon ethanol administration from that point through the 14^{th} day of treatment when compared with baseline firing rates of saline controls. The authors noted that pre-injection baseline firing rates were significantly increased after this 14-day ethanol regimen, suggesting that these rats were experiencing mild symptoms of withdrawal between doses of ethanol. These data suggest that acute ethanol inhibits the firing rate of VTA GABA neurons, and some form of compensatory GABA mechanism is established after two weeks of twice-daily ethanol exposure.

The other group of rats in the freely-behaving experiment was subjected to a more complex regimen of ethanol dosing. The ethanol-naïve rats were given the same initial ethanol injections as those in the twice-daily injection group, but then were exposed to

continuous ethanol vapor each night for 15 hours. For the first 7 days, the ethanol vapors were the rats' only exposure to ethanol. On days 8-13, the rats would receive the ethanol vapors for these 15 hours at the beginning of the night, then go without ethanol for approximately 8 hours during the day before receiving an I.P. dose of either saline or one of 4 doses of ethanol on a random schedule. On days in which the rats received a larger dose of ethanol, they were withheld from the ethanol vapor chamber for the time necessary to lower their blood alcohol and avoid overexposure. Then, some of the rats were returned to the overnight vapor for an additional five days. The authors found that in rats exposed to 7-12 days of ethanol vapor, doses of ethanol between 0.4 and 2.0 g/kg significantly inhibited the firing rate of VTA GABA neurons when compared to saline controls. However, in rats exposed to 14-19 days of ethanol vapor, the same doses of ethanol did not alter the neurons' firing rates. This data again suggests that acute ethanol inhibits the firing rate of VTA GABA neurons, and 2 weeks of chronic ethanol exposure is sufficient to elicit compensatory changes in these GABA neurons.

In the second portion of their experiment, 20 additional male Sprague-Dawley rats were anesthetized with 3-4% halothane and placed in a stereotaxic apparatus, after which ethanol was delivered either via microelectrophoresis (0.3 M ethanol) or I.P. injection (1.2 g/kg). The authors found that I.P. administration of ethanol first slightly increased the VTA GABA neuron's firing rate, then moderately reduced it. This moderate decrease in firing rate occurred within 5-10 minutes of the injection, however 30-60 minutes after the injection a mild increase or "rebound" in firing rate occurred. This spurred the authors to ask a critical question: Is the tolerance of the VTA GABA neuron firing rate to ethanol inhibition due to the effects of ethanol directly on the VTA, or due to its effect on

structures which project to it? Seeking an answer to this question, the authors recorded the VTA GABA neurons while simultaneously using in situ application of ethanol to apply it directly into the VTA of these anaesthetized rats. The authors found that regardless of application time, no rebound excitation was observed. Additionally, the firing rate of all VTA GABA neurons in these locally treated, ethanol-naïve, anesthetized rats was reduced by 60% when compared to local saline. Furthermore, when a few of the 14-day chronic ethanol vapor rats were given this same local application under anesthesia – the same rats which showed no inhibition of VTA GABA neuron firing in response to I.P. ethanol – they displayed a significant, 34% reduction in the firing rates of these VTA GABA neurons.

These last results are crucial. If only *systemic* alcohol produced rebound excitation in VTA GABA neurons 30-60 minutes after an initial depression in function, then some sort of afferent input to these neurons must be getting modified by ethanol. The authors propose, both based on evidence and experience, that this is the afferent GABA input from the NAcc we have already discussed (Van Bockstaele & Pickel, 1995). They point out that other experiments by members of their research team have shown that "systemic ethanol inhibition of NAcc firing rates persists for 1 hour (Criado, Lee, Berg, & Henrikson, 1995), whereas the inhibition of VTA GABA neurons lasts less than 15

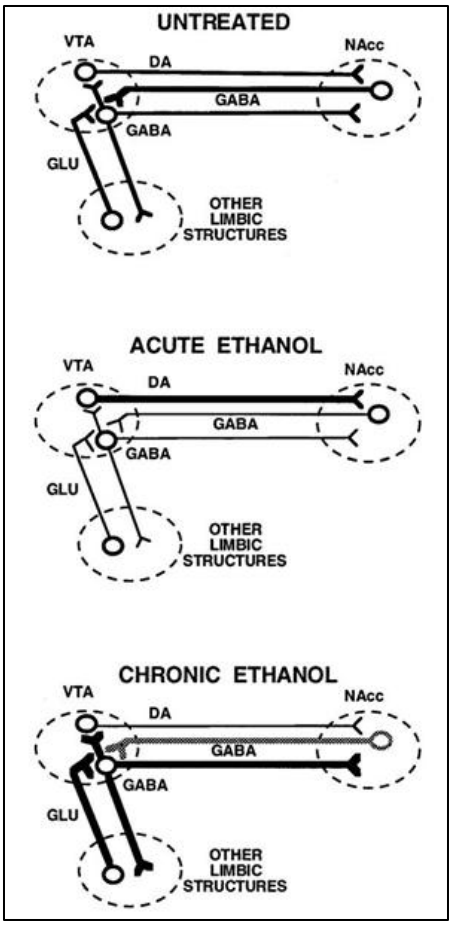

Figure 7. Adaptations of GABA Circuitry to Ethanol.

minutes" (Gallegos et al., 1999). Such persistent inhibition of afferent inhibitory inputs beyond the time course of local GABA interneuron inhibition would in fact explain this rebound in the local VTA GABA neuron's firing rate (see Fig. 7, previous, from Gallegos et al., 1999). However, the authors are quick to point out that a delayed increase in excitatory synaptic input to NMDA receptors – i.e. glutamatergic input on these VTA GABA neurons – could also provide an explanation for this finding. Such an explanation is valid: Glutamatergic afferents from the PFC have been shown to synapse on GABA neurons in the VTA (Carr & Sesack, 2000).

The authors, citing experience, claim that they believe the long-term adaptations observed – such as the complete tolerance of VTA GABA neurons to acute ethanol observed after 11 days of chronic treatment – are likely due to a long-term diminishing of this inhibitory NAcc GABA input. However, they admit that when it comes to making a final decision, the jury is still out. Regardless of which effect is most responsible in this circumstance, this study still clearly demonstrates that ethanol inhibits GABAergic neurons in the VTA! It appears that the potentiating effects of ethanol at the level of the GABA terminal has a minimal impact on overall function when compared to the drastic inhibition it exerts over the GABA neuron as a whole. We may have even caught a glimpse of this potentiation in Gallegos et al.'s (1999) experiment: The authors noted a slight initial increase in GABA firing before it was moderately reduced by acute systemic administration of ethanol. However, this could also be due to inhibition of the accumbotegmental GABA input to this local VTA GABA neuron occurring faster than inhibition of the local neuron itself. Regardless, we now can see that the overall effect of

ethanol on GABA neurons is inhibition, so now we're left with a question: How does ethanol induce this inhibition? You guessed it: Mu-opioid receptors.

First off, let us recall that mu-opioid receptors (MORs) are located only on non-dopaminergic neurons in the VTA – i.e. GABA neurons (Dilts & Kalivas, 1989). These MORs are primarily distributed on the somata and dendrites of these GABA neurons, with less than 10% being found on synaptic terminals (Garzon & Pickel, 2001). Furthermore, activation of these MORs results in hyperpolarization of the GABA neurons on which they reside (Johnson & North, 1992a). These MORs are naturally stimulated by the release of endogenous beta-endorphins, however artificial stimulation using MOR agonists can be used as an effective means to indirectly stimulate mesolimbic DA neuron firing via disinhibition (Johnson & North, 1992a; Mansour et al.,1995). While this is all great information, our question still remains: How does ethanol induce the inhibition of GABA neurons? A series of experiments by Xiao, Zhang, Krnjevic, & Ye (2007) suggest that ethanol induces this inhibition by either enhancing or mimicking opioid-mediated inhibition of these GABA neurons.

In the experiment by Xiao et al. (2007), midbrain slice preparations were made from Sprague-Dawley rats of age PN14 – PN28. The authors used classic patch-clamp techniques to measure the firing rates of both VTA GABA neurons and VTA DA neurons in different portions of the experiment. The authors found that 40 mM of ethanol drastically but reversibly depressed the firing rate of a VTA GABA neuron by 63%. Further administration of 20 mM and 80 mM demonstrated that this ethanol-induced inhibition occurs in a dose-dependent manner. Upon recording a different VTA DA

neuron, the authors found that ethanol enhanced spontaneous firing activity of this neuron in a dose-dependent manner as well.

Considering this evidence, the authors went on to propose the following hypothesis: "If ethanol excites dopamine neurons indirectly by reducing GABA-mediated inhibition, this excitation should be [mimicked] by blockade of $GABA_A$ receptors" (Xiao et al., 2007). Upon testing this hypothesis, the authors found that 40 mM of ethanol accelerated the spontaneous firing rate of dopamine neurons by 22%. When two different selective $GABA_A$ antagonists – bicuculline and GABAzine – were applied in separate experiments, it was found 10 µM bicuculline increased VTA DA neuron firing by 45%, while 3 µM and 10 µM GABAzine increased firing by 32% and 57%, respectively. These results appear to confirm the authors' hypothesis, and suggest that VTA DA neurons are under strong, tonic GABA inhibition mediated by $GABA_A$ receptors. Upon adding 40 mM ethanol to a slice still containing a 10 µM bicuculline treatment, the authors found that ethanol still enhanced firing, but only by 12% - significantly less than ethanol alone. The authors repeated this dual bicuculline + ethanol test 5 times and found an average 13% enhancement by ethanol in the continued presence of bicuculline. Such data indicates that ethanol excites VTA DA neurons indirectly by inhibiting some portion of tonic GABA inhibition.

Returning to exploration of their original hypothesis, the authors decided to apply the MOR agonist DAMGO to slices and compare its effects to ethanol. Applied separately, 40 mM ethanol resulted in a 34% increase in VTA DA neuron firing rates, while 3 µM DAMGO increased VTA DA neuron firing by 42%. Once this response to DAMGO stabilized, the authors added 40 mM ethanol and found it to only increased

firing by 15%. The fact that a MOR agonist attenuated the indirect excitatory effects of ethanol on these DA neurons suggests that ethanol may act via a similar mechanism. While the exact mechanism of ethanol's action could not be determined from these experiments, the authors concluded that "agents which inactivate GABAergic inputs to DA cells significantly reduce the ethanol-induced excitation of VTA DA neurons." In an attempt to further narrow down the possible mechanisms ethanol may utilize to induce this GABA neuron inhibition, the authors administered an MOR antagonist – naloxone – and found that it's administration reduced the firing of 6 out of 8 VTA DA neurons and increased the firing of 3 out of 4 VTA GABA neurons. Such data demonstrates the sheer *magnitude* of MORs' influence on GABA neurons. Given the predominance of MORs on GABA neurons in the VTA (Garzon & Pickel, 2001), it seems reasonable to suggest that the magnitude of ethanol's agonist-like effect on these receptors could easily outweigh its potentiating effect at GABA terminals in the VTA. However, before we discuss this let's review the data from an even more detailed set of experiments conducted by these same researchers in further investigation of this ethanol – MOR paradigm.

One year later, an in-depth set of experiments using a whole-cell patch clamp technique to record electrophysiological activity was published by Xiao & Ye (2008). Similar to the experimental techniques used by Theile et al (2008) and Melis et al. (2002), the authors recorded $GABA_A$ receptor-mediated IPSCs on the membrane of VTA DA neurons in the presence of APV & DNQX to prevent interference by NMDA and AMPA receptor-mediated currents. Furthermore, suppression of IPSCs by bicuculline (BIC) confirmed that the IPSCs being measured were those mediated by $GABA_A$ receptors. The authors found that 40 mM ethanol significantly and reversibly decreased

the peak amplitude of evoked IPSCs (eIPSCs) in these VTA DA neurons by 23%. Interestingly, in paired-pulse ratio (PPR) experiments, ethanol shifted the PPR towards paired-pulse facilitation (PPF) by reducing the amplitude of $IPSC_1$, indicating that ethanol seemed to be inhibiting GABA release at the presynaptic terminal. Additionally, spontaneous IPSCs (sIPSCs) that were eliminated by BIC were lowered in frequency but not amplitude by ethanol application, indicating that these VTA GABA terminals may be sensitive to ethanol depression as well.

Citing the possibility of GABA inhibition by stimulation of presynaptic $GABA_B$ receptors (Ariwodola & Weiner, 2004), the authors administered 1 µM of the $GABA_B$ antagonist CGP52432 before applying 40 mM ethanol. They found that in the presence of CGP52432 ethanol lowered sIPSC frequency by 33%, while without CGP52432 ethanol lowered sIPSC frequency by 38%. Such data indicates that at this synapse $GABA_B$ activation does not play a major role in ethanol-induced inhibition of sIPSC frequency. The adept reader has probably noticed that these findings conflict with those of Theile et al. (2008) and Melis et al. (2002). We will address this conflict shortly. However, let's first finish this review of Xiao & Ye (2008).

Continuing on, Xiao and Ye used 1 µM tetrodotoxin (TTX) to block action potential (AP) induced sIPSCs. They found that TTX suppressed sIPSC frequency by 45% without altering amplitude, suggesting that nearly half of the spontaneous inhibitory currents mediated by $GABA_A$ receptors are due to depolarization of the innervating GABA neuron cell bodies, and not simply random terminal release of GABA. 40 mM of ethanol failed to elicit any change in the frequency of the remaining miniature IPSCs

(mIPSCs), suggesting that ethanol's actions are somehow linked to TTX-sensitive sodium channels on local GABA interneurons.

In order to delineate how ethanol may affect MORs on these local VTA GABA neurons, the authors recorded VTA GABA neuron firing rates in the presence of 3 μM DAMGO. They found that DAMGO hyperpolarized the membrane of this GABA neuron and blocked its continuous discharge. Additionally, this dose of DAMGO depressed evoked IPSCs from VTA DA neurons by 59% and reversibly decreased sIPSC frequency in these VTA DA neurons by 49% without altering amplitude. Most importantly, DAMGO did not in any way alter mIPSCs, indicating that DAMGO likely inhibits $GABA_A$-mediated IPSCs by silencing VTA GABA neurons.

In their most important set of experiments, Xiao and Ye looked at the effect of ethanol on these *remaining* eIPSCs and sIPSCs in the presence of 3 μM DAMGO. In the presence of DAMGO, 40 mM ethanol increased the amplitude of eIPSCs by 43% and decreased the PPR by 42% due to this large increase in the magnitude of $IPSC_1$. Such a result indicates that ethanol has a potentiating effect on the presynaptic GABA terminal. Additionally, this application of ethanol in the presence of DAMGO increased sIPSC frequency by 57%, but only increased the amplitude by 8% - an amount the authors purport to likely be physiologically insignificant. From this data, the authors conclude that when VTA GABA neurons are being hyperpolarized by MOR activation, ethanol enhances GABA transmission by potentiating GABA release at these presynaptic terminals.

With all of this in mind, let's address a conflict in the literature: Xiao and Ye (2008) found that administration of 40 mM ethanol in the presence or absence of a

$GABA_B$ antagonist lowered sIPSC frequency by 33% and 38%, respectively. Meanwhile, Theile et al. (2008) found that administration of 50 mM ethanol in the presence of either a $GABA_B$ agonist or antagonist still increased sIPSC frequency by 30% and 23%, respectively. Both groups of researchers used VTA DA neurons from ethanol-naïve rat midbrains, and both groups used AMPA and NMDA receptor antagonists to block interference from these receptors. The truth is that the reason for the difference between these two groups' results is difficult to pin down. MORs have been shown to be present on inhibitory terminals in the VTA, but only approximately 8% of the total MORs reside on these terminals while the large majority reside on the somata and dendrites of these VTA GABA neurons (Garzon & Pickel, 2001). It is possible that Xiao and Ye happened to come across one of these MOR-containing terminals. In fact, in later experiments they found that ethanol potentiated sIPSC frequency at the presynaptic terminal when GABA neurons were being inhibited by DAMGO application (Xiao & Ye, 2008). Additionally, when sodium channels were blocked with TTX ethanol had *no* effect on the remaining mIPSCs, suggesting it has an effect on TTX-sensitive sodium channels.

The truth is that there simply isn't enough information here to reach a solid conclusion. Ethanol could be affecting these VTA neurons via an entirely different receptor or mechanism that hasn't been elucidated yet. Regardless, we can make some solid general conclusions about the effects of ethanol on GABA neurons in the mesolimbic system based on the available data. I would propose these few basic tenets, ranked in order of importance according to their magnitude:

1) Ethanol severely hyperpolarizes GABA neurons by potentiating the stimulation of mu-opioid receptors (Xiao et al., 2007; Xiao & Ye, 2008). This

effect is the largest in magnitude due to ethanol's ability to stimulate beta-endorphin release, which then activates these MORs that are present only on GABA neurons (Herz, 1997; Marinelli & Gianoulakis, 2004; Dilts & Kalivas, 1989).

2) Ethanol potentiates action potential-dependent sIPSCs via an effect on TTX-sensitive Na^+ channels (Xiao & Ye, 2008). While this effect has only been documented in the experiment by Xiao and Ye, the potential magnitude of this effect is not to be ignored. If ethanol potentiates sodium channels at the nerve terminal of GABA neurons, the relative balance of terminal MORs to terminal Na^+ channels could explain the conflicting results between Xiao and Ye (2008) and Theile et al. (2008).
3) Ethanol enhances GABA release at the presynaptic terminal, and this change persists for as long as 1 week after a single exposure (Theile et al., 2008; Melis et al., 2002). The subsequent increase in GABA is regulated in part by presynaptic $GABA_B$ receptor activation (Melis et al., 2002). However, the magnitude of this terminal potentiation pales when compared to the hyperpolarization induced by MOR activation; it has been demonstrated that MOR activation indirectly facilitates firing of VTA DA neurons (Johnson & North, 1992a; Mansour et al., 1995; Kitai et al., 1999).

We can see now that *magnitude* of effect is clearly the most important principle to keep in mind when observing the effects of ethanol on GABA neurons in the VTA. Fortunately, nicotine's effects on GABA neurons are much more direct: They are mediated by its effects on nicotinic receptors present on presynaptic terminals of VTA

GABA neurons. In the next section we will discuss how nicotine differentially affects GABA and glutamate input to dopaminergic neurons, and more importantly we will begin to see how this difference may drive regular drinkers who have consumed nicotine previously to seek out nicotine under the influence of alcohol.

2). Nicotine's Differential Effects on GABA and Glutamate Transmission in the Mesolimbic Pathway.

While nicotine's effects on GABA transmission may at first seem trivial, they in fact play a crucial, indirect role in aiding the potentiation of mesolimbic dopamine neurons. Low doses of nicotine – such as the background levels present in the blood of smokers – can in fact have differential desensitizing effects on different types of nAChRs: For example, 20 nM nicotine can strongly desensitize some types of β_2-containing nAChRs, while having no significant desensitizing effect on α_7 homomeric nAChRs (Wooltorton, Pidoplichko, Broide, & Dani, 2003). With this in mind, it seems pivotal to examine whether this differential desensitization occurs in the mesolimbic dopamine system. In fact, a study of this phenomenon has already been carried out by Mansvelder, Keath, and McGehee (2002).

In the study by Mansvelder et al. (2002), these researchers sought to shed light on the differential effects nicotine receptor stimulation can have on inhibitory GABAergic neurons in the VTA when compared to their excitatory counterpart – glutamatergic neurons. In their study, Mansvelder and his colleagues prepared slices from the VTA of Sprague-Dawley rats 10-14 days old. Using whole-cell recording, they documented the transmission activity of both VTA glutamate and VTA GABA neurons in response to electrical or pharmacological stimulation.

The authors found that when nicotine was administered, 7 of the 11 GABA neurons tested showed a quick spike in sIPSC frequency, followed by a rapid decrease. The authors hypothesized that this drop-off in sIPSC frequency was due to rapid desensitization of presynaptic nAChRs on the GABA terminals. To further test this hypothesis, they administered an "antagonist cocktail" which demonstrated that this action of nicotine on GABA occurred independent of GLUTergic, muscarinic acetylcholinergic, DAergic, and serotonergic inputs, confirming that this action was mediated by nAChRs. It should also be noted that during this "antagonist cocktail" experiment, rapid desensitization of GABA input was still clearly observed. Further experimentation showed that this effect was TTX-sensitive, demonstrating that the nAChRs mediating this effect are located away from the terminal and do not influence GABA directly. Administration of methyllycaconitine (MLA) – a selective α_7 nAChR antagonist – did not alter this effect of nicotine, indicating that this receptor is not an α_7 nAChR. However, mecamylamine (MEC) and the selective β_2-containing nAChR antagonist DHBE completely abolished this effect, indicating that this receptor is in fact a β_2-containing nAChR.

In further experimentation, the authors bath-applied 250 nM nicotine – a dose equivalent to the average blood levels of nicotine achieved from smoking a single cigarette – for 10 minutes, after which GABAergic transmission in response to 1 µM nicotine was tested and found to be nearly completely abolished – indicating substantial desensitization. Citing the fact that smokers typically have a blood nicotine concentration of ~40 nM just before a cigarette, the authors tried administering 40 nM of nicotine for 10 minutes prior to administration of a 1 µM dose of nicotine. In fact, they found that this

pretreatment did not alter the sensitivity of GABA transmission to this acute dose of nicotine, suggesting "GABA neurons can recover from desensitization with resting levels of nicotine" (Mansvelder et al., 2002). Additionally, antagonization of these β_2-containing nAChRs with MEC in the presence of NMDA and AMPA receptor antagonists resulted in increased dopaminergic transmission, suggesting that blocking or desensitization of these β_2-containing nAChRs on GABA neurons can result in disinhibition of mesolimbic DA neurons! Most interestingly, the authors found that recovery of these β_2-containing nAChRs from 1 μM nicotine-induced desensitization took *over an hour*, highlighting the magnitude and extent to which this desensitization can occur.

In the most important portion of this experiment, the authors administered the same 250 nM dose of nicotine for 10 minutes in the presence of bicuculline – a GABA antagonist – in order to observe the effect of this nicotine administration of spontaneous excitatory postsynaptic currents (sEPSCs). The authors found that this administration of nicotine produced a gradual increase in sEPSC frequency without any decline for the entire 10 minute application period. Additionally, application of 1 μM nicotine at the end of this session increased sEPSC frequency further to 223% of control values! These experiments conclusively demonstrate that in the VTA, glutamatergic transmission can continue to increase amidst the same levels of nicotine that desensitize and eliminate nicotine-induced GABAergic enhancement!!

Such a finding is absolutely invaluable. Mansvelder et al. (2002) have decisively shown that nicotine does not simply increase excitatory stimulation of mesolimbic dopamine neurons, but also fails to induce inhibitory stimulation of these mesolimbic DA

neurons. It appears that smoking one cigarette not only stimulatse glutamatergic EPSCs, but desensitizes and nullifies β_2-containing nAChR stimulation of inhibitory GABA transmission!

Can you see how this could interact with ethanol? Ethanol administration hyperpolarizes GABA neurons in the VTA by stimulating the release of beta-endorphins which then stimulate mu-opioid receptors present on GABA neurons' somata and dendrites (Johnson & North, 1992a; Herz, 1997; Marinelli & Gianoulakis, 2004; Xiao et al., 2007; Xiao & Ye, 2008). This hyperpolarization decreases tonic inhibition of mesolimbic dopamine neurons in the VTA, increasing their firing rate and subsequently stimulating mesolimbic dopaminergic transmission (Johnson & North, 1992a; Mansour et al., 1995; Kitai et al., 1999). If nicotine's natural effect is to at first stimulate, and then drastically cease stimulation of these VTA GABA neurons (Mansvelder et al., 2002), the inhibition of their function by ethanol could serve as *yet another* trigger point for association of ethanol with nicotine. In essence, the regular drinker who has previously smoked cigarettes while drinking could be neuroanatomically cued or "reminded by" the normal effects of nicotine on this system by the alcohol-induced inhibition of VTA GABA neurons and subsequent indirect stimulation of mesolimbic DA transmission!

With all of this in mind, we will now take a look at yet another important point of interaction between ethanol and nicotine: The nicotinic receptor itself. As we will see, alcohol can exert some of its stimulatory effects on mesolimbic DA pathway transmission by potentiating some configurations of this receptor while mildly inhibiting others.

Nicotinic Acetylcholine Receptors Mediate Ethanol's Effects on Mesolimbic Dopamine Pathway Transmission

While ethanol's suppressing effects on glutamate efflux, NMDA receptor activity, and GABA function are compelling and crucial to understanding its interactions with nicotine, these interactions do not stop here. We previously mentioned that perfusion of the nonselective nAChR antagonist mecamylamine (MEC) into the VTA prior to NAcc ethanol administration antagonized the NAcc dopamine-elevating effect of ethanol, while administration of MEC directly into the NAcc did not (Ericson et al., 2003). This seems to indicate that nicotinic receptors (nAChRs) play a direct role in mediating the effects of ethanol. Heteromeric $\alpha_4\beta_{2\text{-}4}$ nAChRs and homomeric α_7 nAChRs make up 90% of nAChRs in the brain (Lindstrom, 2003). Additionally, we have seen just how abundant these nAChRs are throughout the mesolimbic dopamine system, and demonstrations of their cooperativity with other receptors such as NMDA receptors (Cheramy et al., 1996). So if ethanol does interact directly with these receptors, it would have a profound effect on mesolimbic dopamine neuron function.

In fact – as mentioned in the introduction – behavioral studies have shown physiological evidence of cross tolerance between nicotine and ethanol in mice that isn't explained by alterations in affinity or number of nAChRs in mice (Burch et al., 1988). Genetic polymorphism studies on mice have shown $\alpha_4\beta_2$ nicotinic receptors to be heavily involved in mediating the effects of ethanol withdrawal (Butt, King, Stitzel, & Collins, 2004). Even more recent behavioral studies have shown that α_7 homomeric nAChRs buffer some of the behavioral responses to ethanol (Bowers, McClure-Begley, Keller, Paylor, Collins, & Wehner, 2005). In this experiment, Bowers and colleagues used wild-type and α_7 knockout mice to determine whether or not the deletion of α_7 nAChRs would

have an effect on behavioral responses to ethanol. While no differences were seen in acoustic startle, pre-pulse inhibition, and Y-maze tests, significant differences were found between the groups in the open field test, righting response test, and measures of thermoregulation. The α_7 knockout mice showed greater activity in response to ethanol, a greater decrease in body temperature, and a greater loss of righting response – indicating increased sensitivity to the sedative hypnotic effects of ethanol. From this experiment we can conclude that at least some of the effects of alcohol are buffered by α_7 nAChR activity, a conclusion which highlights the urgent need for further investigation of ethanol's effects on nAChRs. In fact, we will see in this section that ethanol has profound and greatly differing effects on many of the various subunit compositions of nAChRs.

An experiment performed by Blomqvist, Ericson, Engel, & Soderpalm (1997) serves as a great place to start our exploration of ethanol's interactions with nAChRs. In this experiment, microdialysis samples of dopamine from the NAcc were taken from awake, freely moving male Wistar rats in order to measure mesolimbic activity in response to intraperitoneal (I.P.) doses of 2.5 g/kg ethanol. In some trials, the nAChR antagonists mecamylamine (MEC) and hexamethonium (HEX) were administered either intraperitoneally or directly perfused into the VTA or NAcc 40 minutes prior to ethanol administration. I.P ethanol administration resulted in a large efflux of dopamine in the NAcc. Mecamylamine – a nonselective, central nAChR antagonist – injected intraperitoneally completely prevented this accumbal dopamine efflux. However, I.P. administration of hexamethonium did not prevent this NAcc DA efflux. This latter result is expected, as hexamethonium is a nonselective peripheral nAChR antagonist that is incapable of crossing the blood brain barrier. When either MEC or HEX was perfused

directly into the VTA, accumbal DA efflux was completely prevented, indicating that VTA nAChR stimulation is a *critical* step in mediating the mesolimbic pathway-activating effects of ethanol. Interestingly, perfusion of MEC directly into the NAcc did *not* prevent ethanol-induced accumbal dopamine efflux, further indicating that ethanol's actions on nAChRs in the mesolimbic pathway are primarily mediated by nAChRs in the VTA. Altogether, the results evidenced by this experiment suggest that central nAChRs in the VTA play a critical role in mediating ethanol-induced activation of the mesolimbic dopamine system.

A similar experiment by Larsson, Svennson, Soderpalm, & Engel (2002) found that 2.0 g/kg I.P ethanol increased accumbal dopamine efflux by approximately 40%. In line with the findings of Blomqvist et al. (1997), Larsson and colleagues found that pretreatment with I.P. mecamylamine completely prevented this accumbal dopamine efflux. However, pretreatment with I.P. methyllycaconitate (MLA) – a selective α_7-nAChR homomer antagonist – did not prevent accumbal DA efflux. Pretreatment with subcutaneous DHBE – a selective β_2-containing nAChR antagonist – did not prevent this efflux either, despite the fact that the same form of administration of these antagonists blocked nicotine-induced NAcc DA efflux. The results from this experiment suggest that high doses of ethanol do not utilize α_7 nAChRs or β_2-containing nAChRs to induce dopamine efflux in the NAcc. While this at first may seem to confound our hypothesis, we will see that these results are perfectly in line with our findings regarding how ethanol and nicotine receptors interact in the mesolimbic system.

Now that we've seen some general experiments on ethanol and nAChR interactions we'll begin to look at more specific studies on specific subtypes of nAChRs

and how they respond to low and high doses of ethanol administered with acetylcholine. An experiment by Covernton & Connolly (1997) used voltage clamp electrophysiological recordings of different nAChR subtypes expressed in Xenopus *oocytes* to determine how these different nAChRs respond to low (1-30 mM) and high (100-300 mM) doses of ethanol. They found that $\alpha_3\beta_4$ nAChRs were either potentiated (237%) or inhibited (25%) by low concentrations of ethanol when compared to baseline, but highly potentiated at high doses of ethanol (135-305%). Meanwhile, $\alpha_3\beta_2$, $\alpha_{4\text{-}1}\beta_2$, and $\alpha_{4\text{-}1}\beta_4$ nAChRs were predominately insensitive to low ethanol concentrations, but were highly potentiated at high concentrations, showing firing rates 178%, 226%, and 154% of baseline, respectively. The α_7 homomeric nAChR also displayed relative insensitivity to low doses of ethanol, but showed mild inhibition or no alteration of control at high doses of ethanol. These numbers are crucial, and we will surely revisit them when discussing neuroanatomical pairing mechanisms for nicotine and ethanol at the end of this paper. We have already seen evidence of how critical the α_7 nAChR is to glutamatergic function in the VTA: It is the only presynaptic nAChR located on glutamatergic terminals in the striatum. Given this high level of importance, let's look at two more experiments regarding the behavior of the α_7 nAChR in response to ethanol.

A whole-cell patch clamp experiment in 1999 sought to determine how α_7 homomeric nAChRs respond to various doses of ethanol. In the experiment by Aistrup, Marszalec, & Narahashi (1999), α-bungarotoxin (aBTX) sensitive currents were measured to determine the behavior of the α_7 nAChRs. These currents are considered to represent α_7 nAChRs because aBTX is a nAChR antagonist specific to α_7 homomeric nAChRs. They found that these currents were inhibited by 5-29% by 10-300 mM of

ethanol in a dose-dependent fashion when administered with acetylcholine. Furthermore, they found that aBTX-insensitive currents – or those from other nAChRs – were potentiated by ethanol. Such a finding is concurrent with those of Covernton & Connolly (1997) and helps corroborate their evidence. Additionally, Aistrup and his colleagues found NMDA receptor currents to be inhibited by ethanol by as much as 35%, a finding in line with those of Stobbs et al. (2004). From the results of this experiment and those of Covernton & Connolly (1997), it appears that α_7 homomeric nAChRs are indeed inhibited by ethanol.

One question follows: If α_7 nAChRs are inhibited by ethanol in a dose-dependent fashion, what part of the α_7 nAChR is ethanol acting on? A chimaera experiment, which combined the N-terminal domain of the α_7 nAChR with the C-terminal domain of the 5-HT_3 serotonin receptor, has demonstrated that the amino-terminal (N-terminus) or extracellular domain of the α_7 nAChR is involved in the ethanol-induced inhibition of the α_7 nAChR (Yu, Zhang, Eisele, Bertrand, Changeux, & Weight, 1996). The authors used expression of both the wild-type α_7 nAChR and chimaeric α_7-V201-5HT_3 in Xenopus *laevis* and a two-electrode voltage-clamp technique to record and determine the participation of the extracellular N-terminal α_7 nAChR domain in ethanol-induced inhibition of α_7 nAChRs. This result, along with the voltage- and patch-clamp results of Covernton & Connolly (1997) and Aistrup et al., (1999) provide overwhelming evidence that α_7 homomeric nAChRs are inhibited by ethanol.

Before we wrap up this section, let's look at one more experiment which will give us a preview of the complexity of potential ethanol and nicotine interactions. This experiment in 2002 compared the effects of varying doses of I.P. ethanol and direct VTA-

perfused nicotine on dopamine release in the NAcc (Tizabi, Copeland, Louis, & Taylor, 2002). Both drugs administered alone showed a dose-dependent effect on increases in NAcc dopamine, however when administered together the results were more interesting: When the lowest doses of each drug – 0.5 g/kg ethanol, 0.25 µg nicotine – were administered together, ethanol and nicotine together increased the dopamine efflux in the NAcc with a nearly-perfect additive effect. However, when higher doses of each drug – 1.0 g/kg ethanol, 1.0 µg nicotine – were administered together, the effect was *much less* than additive.

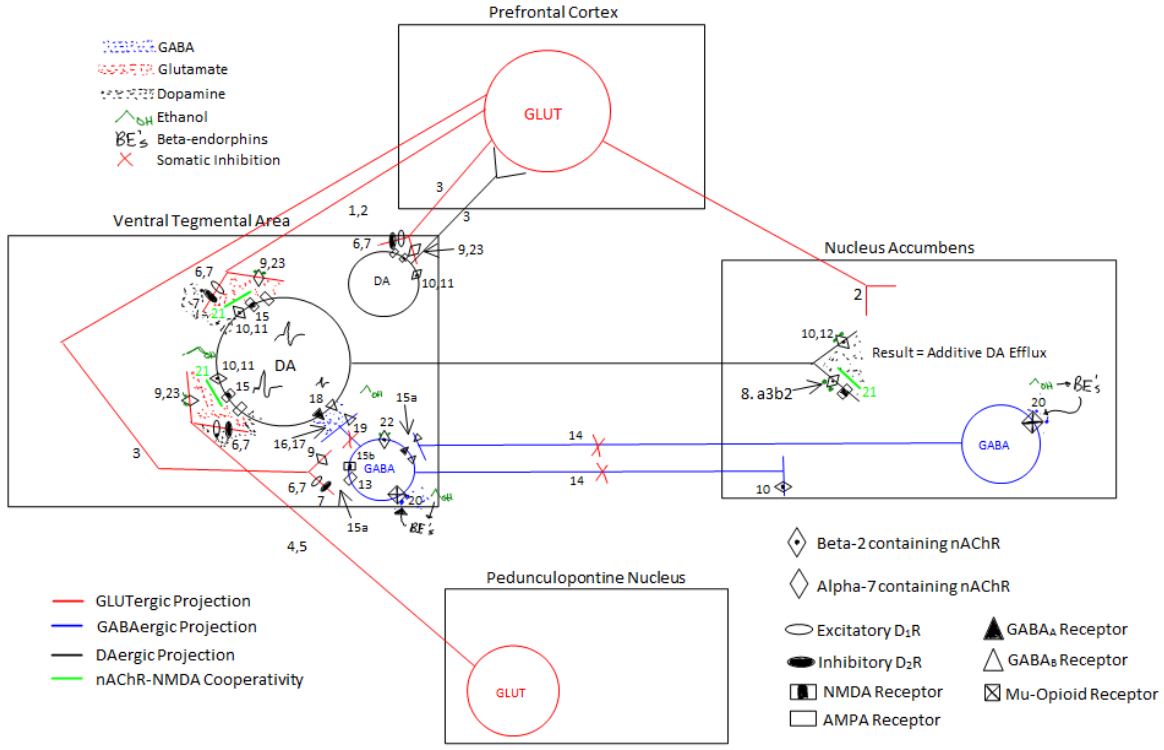

Figure 8. Interactions of Nicotine and Ethanol in the Mesolimbic System at Low Concentrations of Ethanol. (See Appendix for larger version).

While the authors propose this to represent a "ceiling effect," I would propose the interaction to be much more complex: At low doses of ethanol, the positive feedback loop evidenced by Deng et al. (2009) and Xiao et al. (2009) propagates glutamate release at glutamate-dopamine synapses, activating postsynaptic glutamate receptors (Wang &

French, 1993) and causing depolarization and firing of mesolimbic dopamine neurons, resulting in dopamine efflux in the NAcc. These low doses of ethanol have only a mild, near-irrelevant effect on most nAChRs (Covernton & Connolly, 1997), and therefore at low doses of ethanol these receptors respond normally to stimulation by nicotine. GABA neurons are inhibited via MOR stimulation to a level which correlates with the dose of ethanol, as this effect has been found to be dose-dependent (Xiao et al., 2007). Since nicotinic receptors are located presynaptically on glutamate terminals in the VTA (Marchi et al., 2002), on the cell bodies of mesolimbic dopamine neurons in the VTA (Maskos et al., 2005), and on dopaminergic terminals in the NAcc (Kaiser et al., 1998), all of these receptors stimulate dopamine efflux to the same level as they would without the presence of ethanol, resulting in an additive effect of ethanol and nicotine on dopamine efflux in the NAcc (see Figure 8, previous).

However, under high levels of ethanol conditions change: presynaptic α_7 nAChRs become partially inhibited by ethanol (Covernton & Connolly, 1997; Aistrup et al., 1999), while other $\alpha_*\beta_*$ nAChRs become potentiated. The glutamatergic positive feedback loop which was still active at low levels of ethanol is both desensitized from prolonged stimulation (Deng et al., 2009) and inhibited by presynaptic D_2R activation (Xiao et al., 2009), and the nicotinic stimulation of presynaptic α_7 homomeric nAChRs is not as effective anymore due to inhibition by ethanol (Covernton & Connolly, 1997; Aistrup et al., 1999). This culminates to result in an increase in excitation from β_2-containing nAChRs on mesolimbic neuron cell bodies and terminals in the NAcc, but loss of excitation from glutamate terminals synapsing on mesolimbic dopamine neurons in the VTA. The loss of glutamatergic input to mesolimbic cell bodies in the VTA results in a

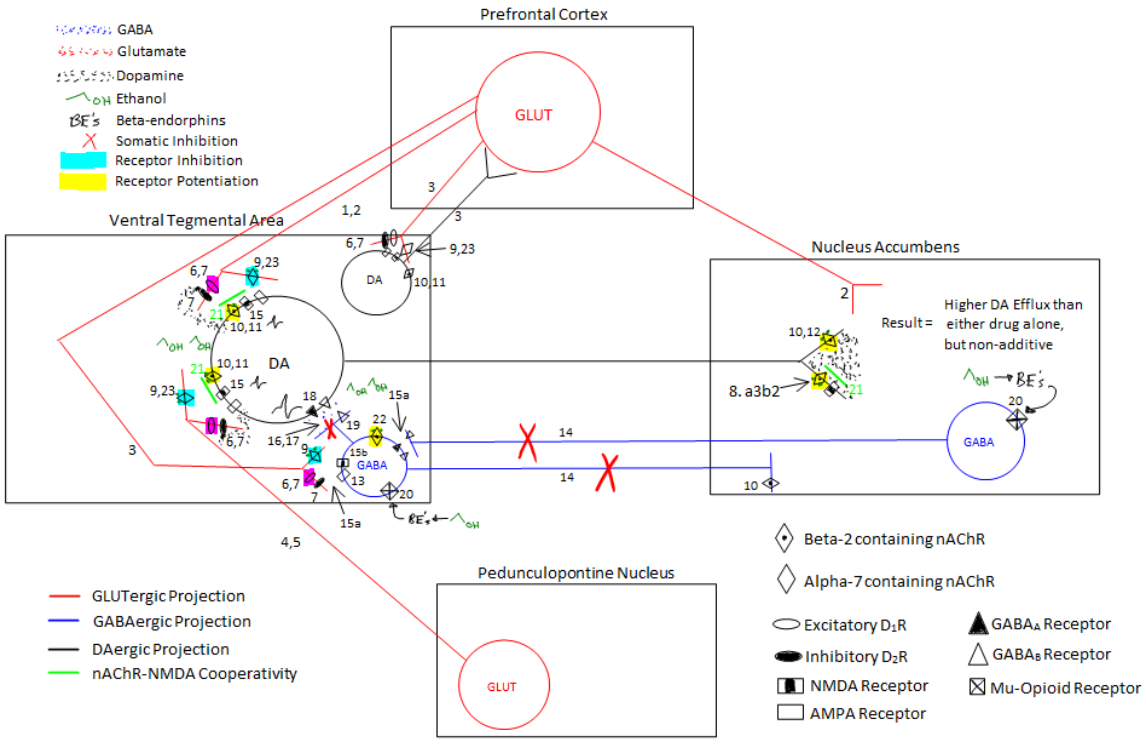

Figure 9. Interactions of Nicotine and Ethanol in the Mesolimbic System at High Concentrations of Ethanol. (See Appendix for larger version).

heavy decrease of stimulation, as postsynaptic NMDA receptors (Wang & French, 1993) – unblocked by nearby β_2-containing nAChRs (Maskos et al., 2005; Cheramy et al., 1996) – are no longer activated by glutamate. However, this decrease in stimulation is partially compensated by the potentiation of $\alpha_3\beta_2$ nAChRs on dopamine terminals in the NAcc, the potentiation of other β_2-containing nAChRs both on these dopaminergic terminals and on the cell bodies of mesolimbic DA neurons in the VTA (Kaiser et al., 1998; Maskos et al., 2005), as well as the dose-dependent disinhibition of VTA DA neurons by ethanol (Xiao et al., 2007). NMDA receptors present on dopaminergic terminals in the NAcc would still be active and unblocked by nearby, now-potentiated nAChRs, and thus would also help compensate for this loss of transmission. At this higher dose of ethanol, the end result would be dopamine efflux higher than either drug

alone, but not purely additive in nature (see Fig 9, previous). This is precisely what was discovered by Tizabi et al. (2002).

We can now see just how dynamic the interactions of nicotine and ethanol are in the mesolimbic dopamine pathway! Before we conclude our paper with a few exciting articles regarding more dynamic interactions in the VTA, we'll first take a look at documented changes that occur in the mesolimbic pathway as a result of exposure to nicotine, ethanol, or both drugs. Once we have done these things, we will conclude our paper by attempting to determine what repeated neuroanatomical interactions could result in these changes.

CHAPTER III

Changes that Occur as a Result of Nicotine or Ethanol Exposure

The Mesolimbic Dopamine Reward Pathway Undergoes Changes as a Result of Exposure to Nicotine

To begin our discussion on nicotine-induced changes in the mesolimbic system, let's look at an experiment which directly tested the sensitivity of nAChRs over prolonged stimulation. A study by Pidoplichko, DeBiasi, Williams, & Dani (1997) used a current-clamp method to record the depolarizations of isolated midbrain dopamine neurons from the VTA in response to nicotine. These researchers found that after 5 minutes of prolonged exposure to 0.5 μM of nicotine, the nAChRs on these midbrain dopamine neurons were so desensitized that currents evoked by acetylcholine injection fell almost to pre-nicotine baseline measurements. A smoker's level of blood nicotine reaches approximately 0.5 μM immediately after smoking a cigarette, with anywhere between 0.1-0.5 μM nicotine being the average level reached (Henningfield et al., 1993). Therefore, it is entirely possible for a smoker to have a large number of desensitized nicotinic receptors immediately after smoking a cigarette. In fact, the low concentration of nicotine found in a typical smoker's blood can be enough to maintain this desensitization.

In further investigation, Pidoplichko and colleagues applied the selective α_7 homomeric nAChR antagonist methyllycaconitine (MLA) to determine what effect it would have on the currents of these midbrain DA neurons. They found that MLA blocked the strong, fast depolarization elicited by acetylcholine injection, but not the slow current. Follow up tests found that this fast current desensitizes quickly even under normal

physiological conditions when acetylcholine injections are given 5 seconds apart, suggesting that these α_7 nAChRs desensitize quickly regardless of the presence of nicotine. This experiment's findings present yet another dynamic way in which the environment of the mesolimbic system can change in response to nicotine. In the next experiment, we will see how even a single exposure to nicotine can elicit changes that last for 24 hours.

Ferrari, Le Novere, Picciotto, Changeux, and Zoli (2001) conducted a set of experiments in which they gave rats an acute dose of nicotine via one of three routes of administration: intraperitoneal, intra-accumbal perfusion, or intra-VTA via a microinjection cannula. The dosages of nicotine were adjusted to be equivalent based on route of administration, and saline controls were used for each route of administration, as well as a cohort of rats which received intraperitoneal amphetamine. 24 hours later, all NAcc dopamine levels were found to be approximately the same as control groups and the amphetamine group with the exception of one: The intra-VTA nicotine microinjection group showed NAcc DA levels still elevated to 200% of the previous day's basal values. In addition, this group of rats also showed tyrosine hydroxylase (TH) mRNA levels that were 40% higher than their respective saline controls and GluR1 mRNA levels in the dopaminergic portion of the VTA that were 55% higher than controls!

We have already seen that stimulation of mesolimbic neurons in the VTA with a nicotine perfusion elicits a much longer-lasting elevation in NAcc DA levels than direct perfusion of nicotine into the NAcc (Nisell et al., 1994). I have already hypothesized that activation of presynaptic α_7 nAChRs on glutamatergic terminals in the VTA could result in a much greater stimulation of mesolimbic DA neurons and thus greater NAcc DA

efflux. In fact, this experiment has given us strong evidence that these glutamate-dopamine synapses are active during nicotine stimulation: GluR1 subunit expression increased 55%! The authors conclude that this could be a sign of long-term potentiation taking place at these synapses, and given the presence of nicotine receptors both pre- and postsynaptically at these synapses (Marchi et al., 2002; Maskos et al., 2005) this seems to be the prime logical conclusion. Altogether, the data from this experiment demonstrate how even single doses of nicotine can elicit changes in the mesolimbic system that pervade long past excretion of nicotine.

So if one acute dose of nicotine can elicit changes in both mesolimbic function and AMPA receptor subunit mRNA levels, can chronic nicotine exposure induce changes in other glutamate receptors' function also? Data from an experiment testing the functionality of NMDA receptors in the striatum after 10 days of chronic nicotine appears to answer the question with a "yes!" In this experiment by Risso, Parodi, Grilli, Molfino, Raiteri, and Marchi (2004), adult male Sprague-Dawley rats were given a continuous infusion of nicotine or saline into the striatum for 1 or 10 days via an osmotic minipump. After the 1 or 10 day infusion period, the rats were quickly decapitated and synaptosomes were made which isolated dopaminergic terminals from the striatum.

When tested in a magnesium-free medium, Risso et al. (2004) found that administration of 100 μM NMDA evoked radioactive dopamine (rDA) release 40% greater than baseline in 1 day saline, 10 day saline, and 1 day nicotine rats. However, administration of this same dose of NMDA to dopamine terminals isolated from 10 day nicotine rats resulted in an increase in rDA efflux nearly 60% over baseline! Interestingly, administration of AMPA did not evoke a release of rDA greater than

controls in any group, indicating that AMPA receptors on dopaminergic terminals in the striatum are not affected by chronic nicotine administration. These data corroborate other evidence of NMDA receptor sensitization on terminals in the NAcc in response to 14 days of chronic nicotine (Grilli et al., 2009). NMDA receptors have been shown to be the primary mediators of nicotine-induced mesolimbic activation (Kenny et al., 2009), as well as cooperative with nearby nicotinic receptors on dopamine terminals in the NAcc (Cheramy et al., 1996). NMDA receptor function has also been found to be suppressed by chronic ethanol administration (Stobbs et al., 2004). The fact that chronic nicotine and ethanol have opposite effects on NMDA receptor function adds yet another dimension to our theory of compensatory action: A person who begins to use both drugs could potentially create a precarious, self-medicated balancing act in which ingestion of either drug elicits compulsive seeking of the other drug in order to appropriately balance NMDA receptor function. In light of the crucial role NMDA receptors play in long-term potention and associative mechanisms in our cortex and limbic system (Perkinton et al., 1999), this possibility is especially scary as playing with NMDA receptor function could severely impair the recall of previously learned associations and general cognitive ability. Such a "balancing act" would therefore be inherently addicting if the function of NMDA receptors became predominately influenced by the relative concentrations of these two drugs in the users' central nervous system. The changes elicited by chronic nicotine use do not end at NMDA receptors however, as evidenced in our next piece of experimental evidence.

In 2000, a study was carried out which sought to determine whether nicotine exposure during the first, rapid period of growth in the brain would affect the ratio of

high-affinity (HA) to low-affinity (LA) nicotinic receptors found in the cortex or cause a difference in nicotine-induced behavior (Eriksson, Ankarberg, & Fredriksson, 2000). The authors of the study found that a group of mice injected with nicotine twice daily for 5 days between PD 10 – PD 14 of the mouse neonatal period showed a shift in the ratio of low-affinity vs. high-affinity receptors from an approximately 3:1 HA:LA ratio to only having detectable HA receptors when tested 4 months later. Additionally, this group of neonatal mice was the only group to show a hypoactive response to nicotine administration 4 months later, with all other groups showing no significant responses. Spontaneous behavior was also tested 4 months after the 5 day nicotine administration period, however no differences were observed between the nicotine group and saline controls.

While this experiment deals with adolescent exposure to nicotine during a specific developmental period, it serves as a prime example of the degree of change in nicotinic receptor function that can occur as a result of exposure during a vulnerable period. Additionally, the shift towards high-affinity receptors demonstrates that even the adolescent brain reacts to nicotine by sensitizing its reward circuits after prolonged exposure. It appears that the tenets of IST regarding addiction are even more applicable in the developing, adolescent brain.

An intra-cranial self-stimulation (ICSS) threshold experiment performed in 2006 sought to find out just how long the sensitizing effects of chronic nicotine on the mesolimbic DA system last. In the experiment by Kenny & Markou (2006), adult male Wistar rats were taught to self-administer 0.03 mg/kg nicotine on a FR5TO20 schedule for either 1 or 12 hours per day for 20 days. The self-administration of nicotine was

found to significantly lower ICSS reward thresholds in both 1h and 12h rats when compared to controls – indicating sensitization to the rewarding effects of nicotine. Interestingly, the 1h nicotine rats showed a reward threshold that was even lower than that of 12h nicotine rats, suggesting that limiting exposure time to self-administration of nicotine may increase the sensitizing response of the mesolimbic system.

In further investigation of which nAChRs may be mediating this change, Kenny and Markou administered DHBE – a selective β_2-containing nAChR antagonist – on day 15 of the 20 day self-administration period. They found that DHBE administration completely reversed the lowering of the ICSS threshold in these rats, indicating that β_2-containing nAChRs are critical in mediating this ICSS threshold-lowering - or reward-sensitizing - effect of chronic nicotine self-administration. The most exciting discovery of this experiment, however, was that the sensitizing effect of nicotine on reward systems persisted for the entire 36 day post-nicotine period during which thresholds were continually measured in the absence of nicotine! Such data implies that the self-administration of nicotine can activate and elicit changes in the mesolimbic system that can last for longer than the period of usage.

These pervasive, lasting changes to reward sensitivity are extremely important to our exploration of alcohol-cued nicotine craving: A person who drinks 1-3 times per week socially and only smokes during these outings may experience nicotine-induced mesolimbic sensitization that is temporally associated with alcohol consumption. If repeated enough times, such sensitization could last long enough to cue nicotine craving upon alcohol consumption. In fact, even a single dose of nicotine has been shown to increase NAcc dopamine efflux for as long as 24 hours following administration (Ferrari

et al., 2001). Given these data, it seems that repeated self-administration of nicotine via cigarettes while intoxicated could quickly and strongly associate this reward sensitization with alcohol consumption. Fortunately, post-mortem examinations of human brains have shown that even long-term smokers can reverse most of this sensitization, as we will see in this next experiment by Breese, Marks, Logel, Adams, Sullivan, Collins, & Leonard (1997).

In this postmortem experiment, Breese et al. (1997) used radioactive nicotine (rNIC) binding to assess the number of nAChRs present in the hippocampus and thalamus of nonsmokers, lifelong smokers, and long-term smokers who had quit for anywhere between 2 months and 30 years before their death. Breese and his colleagues found that subjects with lifelong histories of smoking had significantly higher levels of rNIC binding in the hippocampus and thalamus than nonsmokers, manifested as a net increase in the number of nAChRs present. Furthermore, there was a positive correlation between the degree of cigarette smoking (average number of packs per day) and the number of nicotine binding sites in these areas. Results from the radioactive binding study on nonsmokers, however, suggest that this nAChR upregulation is reversible: nAChR levels in smokers who had quit were not significantly different from those of non-smokers. Interestingly, this mean number of nAChRs in smokers who had quit was noticeably *lower* than the mean number of nAChRs in nonsmokers, however as previously stated this difference did not reach significance.

These results are – at the same time – both damning and hopeful. From the data in Breese et al. (1997) it appears that even long-term smokers can experience a return to normal nAChR levels and reactivity. The only exception appears to be in those that

smoke during vulnerable periods in adolescence – rat studies suggest that nicotine exposure during this time can cause long-term, irreversible shifts towards high-affinity nAChRs (Eriksson et al., 2000). In light of all of the experiments covered here, it is readily apparent that changes can occur 1) immediately – such as the rapid desensitization of α_7 nAChRs in response to 5 minutes of nicotine exposure (Pidoplichko et al., 1997), 2) for short periods – such as the 24 hour increase in NAcc DA levels observed in response to a single intra-VTA perfusion of nicotine (Ferrari et al., 2001), and 3) for long periods – such as the 36-day perseverance of reward sensitization seen in rats which were allowed to self administer nicotine for 1 or 12 hours per day for 20 days (Kenny & Markou, 2006). We now can see that nicotine administration can cause both rapid and long-lasting changes in the mesolimbic reward pathway, as well as nAChR upregulation and changes in sensitivity.

The Mesolimbic Dopamine Reward Pathway Undergoes Changes as a Result of Exposure to Ethanol

While the literature pertaining to nicotine-induced changes in the mesolimbic system is vast, research regarding ethanol-induced changes in this system is more limited. Here, I have chosen three studies which highlight the conflicting findings regarding whether dopaminergic neurons in the mesolimbic system are sensitized or desensitized by chronic ethanol administration. From these studies, it appears that both *frequency* of ethanol administration and time of experimentation play the most influential role in how mesolimbic dopamine neurons respond to stimulation after chronic treatment. To start, we will look at a mice study by Mark S. Brodie (2002) which tested the excitability of

these mesolimbic DA neurons in slice preparations made 12 hours after cessation of a 21 day ethanol treatment regimen.

In the study by Brodie (2002), mice were given an intraperitoneal injection of either saline or 35 g/kg ethanol twice daily for at least 21 days. The last ethanol injection was given at least 12 hours prior to decapitation and slice preparation, and no overt signs of withdrawal hyperactivity were observed before this decapitation. Once mounted, Brodie found no significant difference between baseline spontaneous firing rates of neurons from either saline or ethanol treated mice. However, upon construction of a concentration-response curve for ethanol perfusion, Brodie found that the VTA dopamine neurons from ethanol-treated mice were excited to a significantly greater extent by ethanol than VTA DA neurons from saline-treated mice. For example, at the highest dose used – 120 mM ethanol – the firing rates of VTA DA neurons from saline mice increased to approximately 13% above baseline, but those from ethanol mice increased nearly 43% above baseline!

In further investigation of this difference, Brodie administered various doses of GABA to determine the response of these neurons to GABA-mediated inhibition. Ethanol has long been posited to exert its CNS-depressing effects by enhancing GABA receptor function (Mehta & Ticku, 1988). Brodie found that GABA inhibition of VTA DA neuron firing was significantly less effective on VTA DA neurons from ethanol-treated mice than those of saline controls. For example, 200 μM GABA produced a 38% decrease in the firing of neurons from saline-treated mice, but only a 6% decrease in the firing of neurons from ethanol-treated mice. This lessened response of VTA DA neurons to GABA inhibition suggests that these VTA DA neurons have become desensitized to

ethanol-potentiated GABA inhibition. It would appear that either GABA receptors on the cell bodies of VTA DA neurons have become desensitized or the functional response of GABA terminals in the VTA to stimulation has become desensitized. In fact, research into GABA receptor subunit has shown a downregulation of $GABA_A$ subunits after 12 weeks of ethanol treatment (Charlton, Sweetnam, Fitzgerald, Terwilliger, Nestler, & Duman, 1997).

In his final experiment, Brodie administered NMDA to both sets of neurons and found that VTA DA neurons from saline- and ethanol-treated mice were equally potentiated by NMDA stimulation. This is at first alarming: We have already summarized many articles which give ample evidence for the assertion that ethanol suppresses NMDA receptor function. Administration of the NMDA receptor antagonist MK-801 12 hours after the last dose of a chronic ethanol regimen has been shown to significantly reduce ethanol withdrawal-induced heightened glutamate output and withdrawal symptoms to 36% and 20% of control values, respectively (Rosetti & Carboni, 1995). Furthermore, MK-801 administration reduces GABA neuron discharge in a dose-dependent manner to the same degree as ethanol administration and has an additive effect when administered with ethanol, suggesting that ethanol either suppresses or antagonizes NMDA receptor function (Stobbs et al., 2004).

So why did Brodie's findings conflict with these experiments? The answer is simple: He tested these neurons in a bath solution which contained $MgSO_4$, and furthermore did not include glycine with his NMDA administration. Dissociated Mg^{2+} ions naturally block the ion channel of NMDA receptors and glycine is a critical co-transmitter required for activation and opening of NMDA receptor channels. Therefore,

the presence of Mg^{2+} and absence of glycine makes these findings irrelevant as this is an environment not normally found in the brain.

So it appears that our conclusion that ethanol suppresses NMDA receptor function is still valid. Despite this null NMDA experiment, Brodie's findings regarding the sensitization of VTA DA neurons' response to ethanol are still very pertinent. The data elicited suggests that chronic ethanol sensitizes mesolimbic DA neurons to ethanol-induced stimulation in a manner that is at least partially mediated by desensitization of these VTA DA neurons to GABA-mediated inhibition. With this mechanism of ethanol-induced sensitization in mind, let's look at another experiment which also supports the assertion that chronic ethanol sensitizes mesolimbic DA neurons.

In one of the most well-designed experiments summarized in this thesis, Nestby et al. (1999) used a novel approach to the free-choice ethanol self-administration paradigm to investigate adaptations in dopamine and acetylcholine efflux in the NAcc. Male Wistar rats were first given free choice between water and ethanol every-other day during a 17 day acquisition period. The rats were given only water during their off days. On each day of free-choice ethanol consumption, the concentration of ethanol was increased by 1%, resulting in a gradual, every-other day increase from 2% ethanol to 10% ethanol. Upon completion of the 17 day acquisition phase, the rats were given unrestricted free choice access to 10% ethanol for 2 weeks, followed by a 3 week ethanol withdrawal period. The highest drinkers (HD) and lowest drinkers (LD) of the ethanol rat group were selected for further experimentation, with each group representing approximately 25% of the total initial ethanol rat cohort. Part of the LD and HD rat groups were given free-choice 10%

ethanol again after this 3 week period to observe how much ethanol would be consumed, while the rest were decapitated for slice preparation and further experimentation.

The LD group was found to average approximately 1.1 g/kg ethanol during the 2 week unrestricted period, comprising 11% of their total drinking volume. Meanwhile, the HD group was found to average approximately 3.1 g/kg ethanol during this 2 week period, comprising 31% of their total drinking volume. After the 3 week withdrawal period, the rats which were again allowed unrestricted access to 10% ethanol drank a significantly greater amount than they averaged during the initial unrestricted 2 week period: The LD group drank approximately 1.9 g/kg ethanol – 27% of their total drinking volume – while the HD group drank approximately 4.5 g/kg ethanol, or 65% of their total drinking volume. Clearly, these rats were affected by chronic ethanol and withdrawal.

The rats that were decapitated for slice preparation after the three week withdrawal period showed significant signs of long-term adaptations in the NAcc. Using radioactive dopamine (rDA) and acetylcholine (rACh) release as measures of sensitivity to 10 minutes of electrical stimulation, Nestby and colleagues found significant differences between the LD and HD groups and controls. LD rats showed rDA release 114% greater than that of control rats, but no significant difference in rACh release when compared with controls. HD rats, however, showed rDA release 150% greater than control rats and rACh release 120% greater than controls.

Such data indicates that even rats which only self-administered low doses of ethanol developed an increased sensitivity to ethanol - manifested as increased sensitivity of mesolimbic DA neurons in the NAcc. While I would posit that this is specifically an increase in the sensitivity of mesolimbic DA neuron *terminals* in the NAcc, this

experiment would need to be repeated using synaptosomes in addition to these slice preparations to confirm this assertion. This data also shows that the amount of alcohol self-administered seems to have a dose-dependent effect on the degree of sensitization which occurs at these dopamine terminals in the NAcc. Finally, the fact that consumption of high levels of ethanol for a 2 week period sensitized the release of acetylcholine in the NAcc has profound implications for this paper: This is yet another possible link between ethanol and nicotinic acetylcholinergic receptors.

While there is not enough evidence from this single experiment to overemphasize the importance of this connection here, this experiment serves as a fantastic model of differential self-administration of ethanol. It uses the rats' natural self-administration tendencies to determine which group each rat belongs to, and then proceeds to study the neuroanatomical changes elicited by this free choice self-administration in each group. This is perhaps the closest any experiment summarized in this paper has come to reproducing the natural, free-choice nature of alcohol consumption in human beings, and should be used as an example for future studies on ethanol consumption.

Our third experiment is one which presents evidence of desensitization of VTA dopamine neurons. As we will see, this set of experiments by Bailey, Manley, Watson, Wonnacott, Molleman, and Little (1998) were intentionally carried out 24 hours after the last dose of ethanol when withdrawal symptoms had completely subsided. In essence, the following data was recorded in an attempt to record the behavior of these mesolimbic neurons during their post-withdrawal "slump," when symptoms such as the withdrawal hyperexcitability observed 12 hours post-ethanol by Rosetti & Carboni (1995) had subsided. In their experiments, Bailey et al. (1998) used a liquid diet to slowly increase

the ethanol consumption of male TO mice from an initial 3.5% of total caloric intake to 8% over the first 12 days, then maintained this 8% ethanol administration for another 8 days. The mice then had ethanol withdrawn from their diet and withdrawal responses measured every 6 hours. By 24 hours post-ethanol, withdrawal symptoms were completely absent and slice preparations were made.

Interestingly, active VTA DA cells were only found in 1 of 7 initial ethanol-group slices, despite the fact that all 5 of 5 control slices contained active VTA DA neurons. The authors added 5 µM NMDA to elicit baseline firing rates in all slices. Even in the presence of 5 µM NMDA, ethanol slices displayed significantly lower baseline firing rates than controls. The authors went on to administer both dopamine and amphetamine, and each drug elicited lower firing rates in control and ethanol slices. Additionally, the ethanol slices still showed a significantly lower firing rate than controls in both the dopamine and amphetamine trials. Bailey and his colleagues go on to cite research which has shown dopamine autoreceptor stimulation to be responsible for this decrease in firing in response to dopamine and amphetamine, however this is irrelevant in regards to this research.

What *is* relevant here is that VTA DA neurons showed extreme depression of firing rates after cessation of ethanol withdrawal symptoms. What *is* relevant here is that it took administration of NMDA to elicit any level of recordable mesolimbic neuron firing in these slice preparations. These results seem to indicate that after the initial hyperexcitability of glutamate systems 12 hours post ethanol (Rosetti & Carboni, 1995) and hypersensitivity of VTA DA neurons 12 hours post ethanol (Brodie et al., 2002) has subsided, these mesolimbic DA neurons are desensitized to stimulation. However, *we*

can't be sure because Bailey and his colleagues never re-administered ethanol. Without this re-administration data, the implications of this article are restricted to the baseline data collected 24 hours later. This was a crucial mistake that surely needs to be fixed in future studies and I will address this error later in the conclusion.

So then, what can we conclude? Surprisingly, quite a bit. Brodie et al. (2002) found that neurons from chronic ethanol mice were significantly less sensitive to GABA inhibition 12 hours after their last ethanol injection. It is entirely possible that by the 24 hour post-ethanol mark, these mesolimbic DA neurons had regained their sensitivity to GABA inhibition and were subsequently suppressed by lingering GABA transmission in the slice preparations. On the other hand, perhaps after the initial, drastic rise in glutamate levels 12 hours post ethanol (Rosetti & Carboni, 1995) a period of hypoexcitability set in as a rebound to this withdrawal-induced over-stimulation. Unfortunately, this experiment provides no evidence to validate either of these suppositions. Even if *both* of these mechanisms were taking place, ethanol still would likely reignite sensitivity, perhaps even to a greater extent than that controls – a finding which would corroborate those of Brodie et al (2002) and Nestby et al. (1999). In light of the relative levels of thorough investigation used in these three experiments, I would suggest that even 24 hours after cessation of chronic ethanol these mesolimbic DA neurons would *still* be hypersensitive to ethanol stimulation. Even if they were to not be immediately hypersensitive, once the GABA-insensitivity discovered by Brodie et al. (2002) was re-established, it is likely that this hyperexcitability would be recovered.

Overall, it appears that the mesolimbic system does become hypersensitive in response to chronic ethanol administration, although through a much-more complex set

of neurological interactions centered around the establishment of GABA-insensitivity. It is also quite possible that ethanol has long-term effects on nAChR function; however this is a much more obscure subject that has - to this day - received little attention in the academic world. In our final section discussing changes that occur as a result of these drugs, we will look at the three studies which have attempted to determine what changes take place in the brain in response to both ethanol and nicotine exposure. As we will see, these studies posit that everything from cholinergic systems, to CNS cytochrome enzyme expression, to protein kinase and CRE-mediated gene expression could be the points of interaction at which these drugs induce major changes in mesolimbic function. Hang on, for soon we will begin to synthesize the major findings discussed in this paper into working models of mesolimbic stimulation and adaptive change.

The Mesolimbic Dopamine Reward Pathway Undergoes Pronounced Changes as Result of Exposure to both Nicotine and Ethanol

Before we move on to discussing current, major models of nicotine receptors in the limbic system, LTP induction, and imbalanced desensitization in the mesolimbic system, let's look at three studies which used trial administration of both alcohol and nicotine to determine what common places these drugs interact to induce changes in mesolimbic function. In our first study, we will look at a recent experiment which sought to determine what effects chronic alcohol and/or nicotine can have on the $\alpha_4\beta_2$ nAChR.

In the study by Ribeiro-Carvalho, Lina, Filgueiras, Manhaes, & Abreu-Villaca (2008), C57BL/6 mice were exposed to nicotine (50 µg/mL in drinking water), ethanol (2 g/kg introperitoneal), or both on an every-other day schedule between postnatal (PN) days 30-45. This period is considered a critical adolescent period for mice during which the brain undergoes much of the same growth observed in human adolescents.

Additionally, the authors used an every-other day schedule of drug administration in order to mimic cyclical patterns of alcohol consumption and intermittent nicotine consumption. Saline controls underwent the same injection and water administration as these mice in order to control for any stress induced by the treatment. On PN45, the mice were decapitated during their 12 hour dark cycle and slice preparations made for analysis of [^{3}H]cytisine binding and choline acetyltransferase (ChAT) activity.

The authors found that concomitant exposure to ethanol and nicotine for 15 days during adolescence produces marked upregulation of $\alpha_4\beta_2$ nicotinic acetylcholine receptors (nAChRs) in the cortex and midbrain. In the cortex, it appeared that nicotine and ethanol had an additive effect on nAChRs: ethanol induced a 9% increase in nAChRs binding when compared to controls (non-significant), nicotine induced a 13% increase ($p< .05$), and ethanol + nicotine induced a 22% increase in nAChR binding ($p< .001$). While statistically ethanol alone only showed a trend towards upregulation, it would be imprudent to disregard the clearly additive effect between ethanol and nicotine in the cortex. Additionally, in the midbrain ethanol and nicotine demonstrated a marked synergistic effect on nAChR upregulation: ethanol induced a 1% increase, nicotine induced an 8% increase (both non-significant), while ethanol + nicotine induced a significant 26% increase ($p< .001$) in nAChR binding. ChAT activity was less predictable, and differed between the cortex and midbrain: In the cortex, no significant difference in ChAT activity was seen between any experimental group and controls, however midbrain ChAT activity was significantly increased in the ethanol-only group, slightly increased in the nicotine-only group, and no different in the nicotine+ethanol group. Despite the inconclusive ChAT results, the marked synergistic effect of alcohol

and nicotine on $\alpha_4\beta_2$ nAChR upregulation in the midbrain suggests that ethanol and nicotine both act on the central cholinergic system, and that these pronounced upregulatory effects may partially underlie motivation for concomitant consumption of these drugs.

Given that high levels of ethanol have been shown to drastically potentiate $\alpha_4\beta_2$ nAChR-mediated currents (Covernton & Connolly, 1997), and that β_2-containing nAChRs are present both on the cell bodies of mesolimbic DA neurons in the VTA and on DA terminals in the NAcc (Maskos et al., 2005; Wonnacott et al., 2000), it isn't surprising to see that ethanol, combined with nicotine, potentiated the upregulation of nAChRs in the midbrain. I would posit that the ethanol-induced potentiation of nicotine receptors in the mesolimbic system, combined with heavy nicotinic stimulation of these receptors led to overstimulation and subsequent desensitization of these nAChRs. Such desensitization then triggered a compensatory upregulating response by the brain in order to restore homeostatic nAChR sensitivity. In fact, we will soon look at a meta-analysis which has found that the brain slows down nAChR turnover and maintains nAChR production in order to compensate for nAChR desensitization and retain more nAChRs at the neuronal membrane (Dani & Heinemann, 1996). Overall, the data from Ribeiro-Carvalho et al. (2008) seems to corroborate the evidence we have already seen for nAChRs playing the primary role in nicotine-ethanol interactions in the mesolimbic DA system.

While these receptor interactions are surely the core of our discussion, they are not the only place researchers have looked in order to understand the synergistic effects of ethanol and nicotine. Other researchers such as Howard, Miksys, Hoffman, Mash, &

Tyndale (2003) have looked into each drugs' effect on cytochrome P450 enzymes – a critical enzyme group present in the liver and CNS responsible for metabolizing nearly 75% of drugs used by humans (Guengerich, 2008) – in order to determine whether or not the use of either drug could increase the metabolic capacity - and subsequently tolerance - for both drugs.

In this study by Howard et al. (2003), the levels of CNS cytochrome p450 2E1 (CYP2E1) induction were measured in response to ethanol administration by gavage or subcutaneous (SC) nicotine administration in order to determine whether or not nicotine administration could also induce CYP2E1 expression in the CNS. CYP2E1 induction has been proposed to contribute to the increased metabolic capacity for ethanol observed in alcoholics (Lieber, 1999). It was found that nicotine and ethanol administered separately both increased CYP2E1 expression in the olfactory bulbs, frontal cortex, and cerebellum. Additionally, ethanol increased CYP2E1 expression in the hippocampus, while nicotine increased CYP2E1 expression in the olfactory tubercle and brainstem. The authors also conducted postmortem immunocytochemical staining studies on human brains and found that CYP2E1 was significantly increased in alcoholic smokers when compared to controls in both glial cells and layers II-VI of the prefrontal cortex, as well as purkinje cells of the cerebellum. The authors conclude that CYP2E1 induction in the brain by ethanol or nicotine may influence the development of nervous system pathologies typified by alcoholics and smokers.

While this study may not directly pertain to our study of the mesolimbic system's response to ethanol and nicotine, it does give us a fresh perspective on another process which can affect ethanol and nicotine's ability to stimulate our reward pathways:

Metabolism. The results from Howard et al. (2003) suggest that use of either drug can potentiate our ability to metabolize the other, lowering the total amount of time for which either drug can remain active in our central nervous system. This reduced efficacy time of each drug results in a user of either drug needing to consume more of the substance desired in order to achieve the same level of mesolimbic pathway stimulation previously attained - i.e. classic tolerance.

With this in mind, we can begin to see why chronic administration of a drug – such as ethanol – may not always produce the same level of NMDA receptor sensitization in all trials: Differences in metabolic capacity – resulting from time and frequency of exposure – may render these changes unnecessary as cytochrome p450 enzyme induction increases and the half-life of the drug is reduced. In fact, this could be a key factor behind different results obtained between ethanol experiments which use constant ethanol perfusion vs. dosing regimens. While we have already discussed the major error made by Brodie et al. (2002) by including Mg^{2+} but not glycine when testing NMDA functionality of neurons from mice which had received two daily injections of ethanol for 21 days, it is still possible that *had* he used a proper medium there may still have been a difference between the NMDA receptor functionality of his mice and that of the cultured mammalian cortical neurons exposed to constant ethanol for five days in the experiments of Hu & Ticku (1995). While this is hypothetical, it highlights the dynamic, multivariate way in which our body can maintain homeostasis when exposed to an exogenous substance such as ethanol or nicotine.

Now that we've discussed an alternative manner in which the brain may regulate its response magnitude to nicotine or ethanol, let's look at an excellent study which used

a host of experimental techniques in order to devise a specific pathway for interactions between nicotine and ethanol at the terminal synapses of mesolimbic VTA DA neurons in the NAcc. In this *in vitro* experiment carried out by Inoue et al. (2007), the author and colleagues grew VTA neurons, NAcc neurons, or both neuron groups in separate laboratory cultures. The cells were harvested from 17-day old embryos of pregnant Sprague-Dawley rats in primary cultures and CRE-mediated genetic expression levels were measured using fluorescent luciferase activity in response to 10 minute exposure to either a low or high dose of nicotine, a low dose of ethanol, or combined low doses of both drugs. In this *in vitro* setting, the fluorescent luciferase activity is a reliable measure of CRE-mediated genetic expression triggered by protein kinase A (PKA) activity. The cultures of separate VTA and NAcc neurons showed no significant differences in CRE-mediated genetic expression when compared to controls, however the combined culture displayed significantly enhanced luciferase activity in response to the largest dose of nicotine - 10 μM and to concomitant low doses of ethanol (25 mM) and nicotine (3 μM).

In a further investigation of this enhanced luciferase activity, Inoue et al. (2007) tested a numerous antagonists and found that the nicotine-only enhancement of luciferase activity was inhibited by nAChR, D_1R, or D_2R antagonists as well as the PKA inhibitor H-89. This led the authors to conclude that activation of somatic and presynaptic nicotinic acetylcholine receptors caused dopamine release at the VTA →NAcc synapse which in turn simultaneously activated D_1Rs and D_2Rs. Since administration of *either* D_1 or D_2 receptor antagonists blocked this enhanced luciferase activity the authors suggested that this simultaneous activation was a critical step to the process, and that the subsequent

increase in CRE-mediated gene expression in the NAcc neuron was mediated by PKA activity.

The low-level ethanol+nicotine enhancement of luciferase activity was also intensely investigated, and it was found that this CRE-mediated gene expression was blocked by nAChR, D_2R, or adenosine A_{2A} receptor antagonists, as well as by a G-protein receptor G_i βγ subunit inhibitor, PKA inhibitor, or adenosine deaminase. This led the authors to conclude that dopamine released from VTA DA terminals – due to stimulation of somatic and presynaptic nAChRs – activated D_2Rs on the postsynaptic NAcc neuron, while elevated levels of adenosine – due to the presence of ethanol – activated A_{2A} receptors. The A_{2A} activation, coupled with the release of G_i βγ from $G_{i/o}$-coupled D_2 receptors then synergistically stimulated the adenylyl cyclase pathway (AC→cAMP→PKA→CREB) which induced CRE-mediated gene expression and subsequent increased levels of luciferase activity as observed by Inoue and his colleagues (see Figure 10, above, from Inoue et al., 2007).

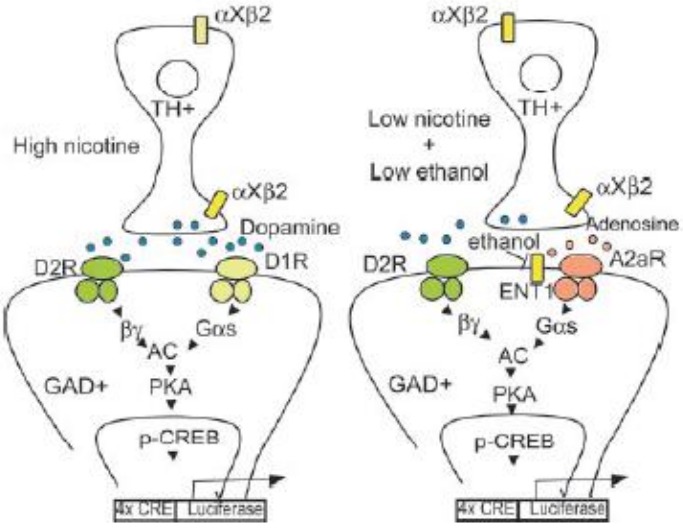

Figure 10. Interactions of Ethanol and Nicotine in the Adenylyl Cyclase Pathway.

The major breakthrough made by this study is that it proposes a specific mechanism for the synergistic interaction of ethanol and nicotine in the mesolimbic dopamine reward pathway. This mechanism could in fact be a potential target for future

pharmaceutical action, for it suggests that the creation of drugs which specifically inhibit G_i βγ function or synergy between A_{2A} and D_2 receptors may better attenuate or help block some of the synergistic reward effects that lead to concomitant addiction with potentially less side effects. This study also provides a well-supported pathway from receptor activation down to genetic transcription that could account for some of the changes in receptor morphology and number that result from exposure to both drugs. Despite having small, insignificant effects alone, low doses of ethanol and nicotine together have been found to cause drastic upregulation of nAChRs in the midbrain (Ribeiro-Carvalho et al., 2008). Given this evidence presented by Inoue et al. (2007), it seems that a similar, if not identical pathway could mediate this change in nAChR availability. More so, with this evidence in hand even if this *isn't* the pathway at work it seems that devising an experiment to explore the pathways which lead to nAChR upregulation in response to these drugs' exposure would not be so difficult!

And so here we – at least temporarily - end our discussion of changes elicited in the mesolimbic system as a result of exposure to nicotine and ethanol. Now that we have seen how incredibly detailed the exploration of these interactions can be, we can move forward with confidence as we review and discuss the major model of nAChRs' roles in the mesolimbic system, as well as a crucial mechanism in addiction formation - LTP induction - that may also be partially responsible for the association of nicotine with alcohol consumption.

CHAPTER IV

Receptor Mechanisms that could Lead to Pairing of Nicotine with Ethanol Consumption

The Current Model of the Role of Nicotinic Receptors in the Mesolimbic System

Throughout this paper, it has become evident that nAChRs themselves are one of the primary points of interaction for ethanol and nicotine. Nicotine always has a stimulatory effect on these receptors, unless they are over-stimulated or desensitized by long-term low levels of nicotine in the body. Meanwhile, ethanol has a host of different effects: Ranging from potentiation to inhibition depending on the dose of ethanol and the nAChR subtype in question (Covernton & Connolly, 1997). I have already reviewed a great deal of evidence to support these assertions and created working models of this balance in the mesolimbic system. Now it is time to broaden our horizon. Here, we will briefly summarize and discuss a review article written by John A. Dani and Steve Heinemann (1996) which solely focuses on the effect of sustained smoking on nAChR levels in the CNS as a whole. We will find that the dynamic principle of desensitization – one which has been critical to my proposed models thus far – lies behind many of the changes which occur in response to sustained nicotine abuse. In this case, desensitization is prolonged, predictable, and has been found to reliably result in nAChR upregulation in all portions of the CNS (Dani & Heinemann, 1996).

Dani & Heinemann (1996) begin by citing two articles which have shown evidence that "long-term nicotine exposure causes an increase in the actual number of nAChRs in humans, mice, and rats" (Dani & Heinemann, 1996). We have indeed already detailed the data of a postmortem brain tissue study conducted in the labs of A.C. Collins

which found both an increased number of nAChRs in the brain tissue of lifelong smokers, as well as a positive correlation between degree of smoking and the number of nAChRs present in the hippocampus and thalamus (Breese et al., 1997). After this discussion, the authors go on to cite evidence that, "…low levels of nicotine cause significant receptor desensitization, and over the long term, nAChRs enter long-lasting inactive states" (Dani & Heinemann, 1996). In fact, we have already seen that 5 minutes of nicotine exposure can result in such severely desensitized nAChRs that currents elicited by acetylcholine injection fall nearly to baseline measurements (Pidoplichko et al., 1997). Furthermore, the same experiment found that fast currents elicited by α_7 homomeric nAChR stimulation desensitize even when only stimulated 5 seconds apart by acetylcholine, suggesting that these nAChRs desensitize even under normal physiological conditions. As we can see, in light of the evidence we have already discussed this is not at all surprising. However, it is Dani & Heinemann's explanation of the compensatory mechanism – nAChR upregulation – which is utterly enlightening:

> "…[the] number of nAChRs seems to be regulated by a post-transcriptional mechanism that decreases nAChR turnover…exposure to low levels of nicotine inactivates some nAChRs, which turn over more slowly…the number of nAChRs increases, distributed among various states [of sensitization]" (Dani & Heinemann, 1996, p. 906).

While the nicotine-induced, CRE-mediated genetic expression observed by Inoue et al. (2007) is likely a process involved in mediating some of the changes to the nAChR population in the brain, it appears that decreasing nAChR turnover is another mechanism by which the CNS upregulates nAChR levels. By decreasing nAChR turnover on the back end and continuing to produce nAChRs at the same rate, these receptors "pile up" in the membrane, resulting in increased membrane sensitivity to nicotine and acetylcholine

(ACh) and the reestablishment of homeostatic levels of ACh sensitivity. Increasing nAChR transcription at the genetic level could further increase the rate of this upregulation (Inoue et al., 2007), resulting in a relatively quick response to the widespread desensitization of nAChRs by prolonged, low levels of nicotine. After establishing these facts, Dani and Heinemann (1996) go on to explain how these changes result in nicotine dependency in regular smokers: A regular smoker has an excess number of nAChRs due to activation of compensatory upregulation mechanisms discussed above, however many of these are constantly inactivated by low levels of background nicotine in the bloodstream (See Fig. 11, above, from Dani & Heinemann, 1996). During sleep, the level of background nicotine in the bloodstream falls, resulting in many of the excess nAChRs becoming responsive to acetylcholine. The problem, however, is that these excess nAChRs are present not just in reward pathways, but in *all* pathways, including non-rewarding pathways such as those involved with anxiety and agitation. The abnormal potentiation of these anxiogenic pathways by the excess, re-sensitized nAChRs drives the smoker to the next cigarette, which not only stimulates his hypersensitive mesolimbic pathway, but also desensitizes the excess number of responsive nAChRs in non-reward pathways back to their normal desensitized homeostatic state. In this state only the appropriate number of functional nAChRs are sensitive, resulting in normal function of these non-reward pathways.

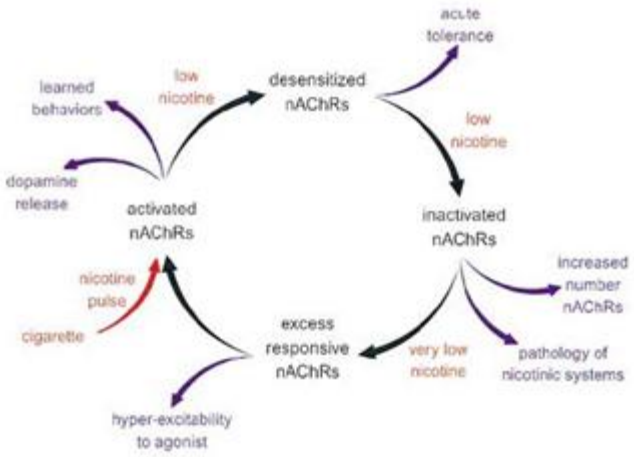

Figure 11. Cycle of nAChR Desensitization in Smokers.

It therefore appears that long-term smokers are driven to keep smoking by the anxiogenic effects of having excess nAChRs in the CNS. Before we conclude this discussion on ethanol and nicotine interactions, we need to take a look at a vital occurrence in the mesolimbic system: Selective long-term potentiation, or LTP. As we will see, the selective potentiation of certain excitatory synapses in the VTA provides a mechanism by which many of the long-term effects of nicotine exposure – and some of its associations with ethanol – can be explained.

Long-Term Potentiation of Glutamatergic and Dopaminergic Synapses in the VTA

One of the critical characteristics of synaptic transmission is its plasticity, or ability to change strength based on patterns of pre- and postsynaptic transmission. One form of this synaptic plasticity is long-term potentiation (LTP), defined as enhancement in synaptic transmission between two neurons following synchronous pre- and postsynaptic stimulation (Cooke & Bliss, 2006). As we will see in this section, the selective strengthening of certain synapses in the mesolimbic system can contribute to short- and long-term changes in transmission that sensitize the user's mesolimbic reward system to stimulation by nicotine or ethanol. In this section, we will look at two studies of the VTA which have found evidence of selective synapse enhancement in response to nicotine, as well as differences in the innate ability of certain excitatory synapses to express NMDA receptor-dependent LTP.

In an effort to determine the effects of nicotinic stimulation on excitatory synaptic plasticity, Mansvelder and McGehee (2000) used whole-cell voltage clamp techniques to record the activity of glutamatergic synapses on VTA DA neurons in midbrain slices from 10-20 day old Sprague-Dawley rats. The authors inhibited GABAergic transmission

by perfusing 20 μM of bicuculline (BIC) – a GABA antagonist – during all recordings. The authors found that application of 1 μM nicotine for 2 minutes did in fact evoke glutamatergic EPSCs which increased in amplitude to 151% of control values. In light of the fact that the amplitude of these EPSCs was unaltered during the nicotine bath, the authors concluded that nicotine was likely increasing the probability of glutamate release via a presynaptic mechanism. In fact, nicotine still elicited this increase in GLUTergic EPSCs in the presence of tetrodotoxin, further supporting the assertion that this effect of nicotine was being mediated by the presynaptic glutamate terminal and not the presynaptic fibers or soma of the neuron.

In further exploration of this presynaptic EPSC enhancement, the authors sought to determine the type of nAChR mediating this potentiation. Mansvelder and McGehee found that even 0.5 μM nicotine nearly doubled spontaneous EPSC frequency. This enhancement was completely prevented by application of the selective α_7 nAChR antagonist MLA, while the nonselective nAChR antagonist MEC prevented the postsynaptic effects of nicotine application but not this effect on sEPSC frequency. All of these data indicate that α_7 nAChRs are mediating a presynaptic increase in glutamate release – resulting in higher sEPSC frequency – while non- α_7 nAChRs are mediating the postsynaptic effects on the VTA DA neuron soma.

Now that the authors had established the presence of nAChRs on the presynaptic GLUT terminal, as well as the postsynaptic DA membrane, they decided to test the synaptic plasticity of this particular synapse. After establishing control EPSC recordings with maximum strength stimulation, they paired presynaptic stimulation with transient postsynaptic depolarization 200 times at a rate of 1 Hz. Mansvelder and McGehee found

that after the pairing session, EPSC amplitude increased to 122% of control values and persisted for at least 40 minutes, indicating the induction of LTP. In an effort to determine whether or not nicotine's enhancement of GLUTergic transmission could also contribute to LTP, the authors paired the same postsynaptic stimulation with a 200 second application of 1 μM nicotine. They found that this nicotine pairing increased evoked EPSCs to 116% of control for at least 40 minutes! Additionally, the amount of LTP induced by this nicotine pairing correlated with the recorded increase in EPSC frequency.

This discovery is crucial, for it makes credible the assertion that any substance which stimulates these VTA neurons heavily enough can be paired with presynaptic enhancement of glutamatergic transmission by nicotine – including ethanol. However before we discuss this let's finish reviewing the results of their experiment. Mansvelder and McGehee (2000) wanted to know whether stimulating somatic postsynaptic nAChR stimulation with presynaptic fiber stimulation – a form of stimulation which mimics action potential-mediated firing – could also evoke LTP. While postsynaptic nAChR stimulation did potentiate the AP frequency of the postsynaptic DA cell by 242%, no LTP was observed, indicating that postsynaptic nAChR stimulation alone does not contribute to LTP induction at this synapse. Final tests showed that the LTP induction observed earlier in the experiment could be blocked by the NMDA antagonist APV, indicating that both the initial pairing-induced LTP and nicotine-induced LTP are dependent on NMDA receptors. MLA was found to block the nicotine-induced LTP but not pairing-induced LTP, indicating that presynaptic α_7 nAChRs are crucial for this nicotine-induced LTP.

In light of this evidence, it seems entirely possible for LTP to be induced at these excitatory GLUT neuron → DA neuron synapses and evoked by many combinations of stimulation. For example, the firing evoked by ethanol-induced disinhibition of these VTA DA neurons could be paired with this nicotine-induced presynaptic stimulation of GLUTergic transmission. In other words, concomitant consumption of both drugs could induce LTP at these synapses, sensitizing the mesolimbic dopamine system and thus the user to cravings (Robinson & Berridge, 2001). Additionally, this mechanism provides a way in which we could make deep, limbic system-driven associations between the pleasure evoked from alcohol with the craving and subsequent satisfaction of smoking a cigarette.

The potential associations don't stop at just this synapse. We have already seen evidence that afferent glutamatergic input from the prefrontal cortex synapses on local GABA neurons in the VTA (Carr & Sesack, 2000). These local interneurons have been found to both tonically inhibit local VTA DA neurons, as well as project to the cortex and NAcc (Johnson & North, 1992a; Von Bockstaele & Pickel, 1995; Carr & Sesack, 2000). Previous research conducted on the mesolimbic system has sought to determine the synaptic plasticity of these GLUT→DA and GLUT→GABA synapses in the VTA (Bonci & Malenka, 1999). They found that after 200 pairing stimulations at a rate of 1 Hz, a 20% increase in synaptic strength was elicited for 30-35 minutes at the GLUT→DA synapse. However, the same protocol did *not* elicit LTP at the GLUT→GABA synapse. Interestingly, administration of the $GABA_B$ agonist baclofen (1 μM) had no effect on EPSCs from DA neurons, but significantly depressed EPSCs elicited from the GABA neurons recorded. Such data indicates that $GABA_B$ receptors are located on the

presynaptic glutamate terminals of these GLUT→GABA synapses, providing a negative feedback mechanism that could prevent overstimulation of these GABA neurons by innervating glutamate neurons.

The fact that LTP induction can occur at excitatory synapses on mesolimbic dopamine neurons but not excitatory synapses on local GABA interneurons is yet another crucial find. This discovery indicates that while nicotine application can elicit LTP at these GLUT→DA synapses, it cannot strengthen the transmission at GLUT→GABA synapses (Mansvelder & McGehee, 2000; Bonci & Malenka, 1999). This neural wiring essentially creates a one way street for potentiation in which excitatory stimulation of these "craving and reward" mesolimbic DA neurons can be strengthened, but stimulation of the inhibitory, counterbalancing force – local GABA neuron input to the mesolimbic DA neurons – *cannot* be strengthened. In light of this fact, the ability of nicotine to elicit stronger craving than alcohol, cocaine, or opiates (Carter & Tiffany, 1999) makes complete sense – it strengthens excitatory stimulation of mesolimbic DA neurons without strengthening the opposing inhibitory input!

Now, we can readily see both why nicotine can elicit such strong cravings, as well as how such craving can be readily paired with ethanol consumption. In the following, final section I will recap the major associative mechanisms presented throughout this paper. Additionally, I will attempt to make clear the most important steps which must be taken to further explore these mechanisms. Surely, this area of research is still ripe with potential and deserves continued attention in the coming decades.

Conclusion

To begin our conclusion, let's first refresh ourselves on the locations of receptors and synapses in the mesolimbic system: $\alpha_3\beta_2$ nAChRs as well as other β_2-containing nAChRs are located presynaptically on dopaminergic terminals in the striatum and cortex (Kaiser et al., 1998). Additionally, β_2-containing nAChRs are present on mesolimbic DA projections and DA terminals in the NAcc (Maskos et al., 2005). β_2-containing nAChRs have also been found on the cell bodies of dopamine neurons in the striatum and ventral tegmental area (VTA), and have been proven to be essential to nicotine-induced firing of mesolimbic DA neurons (Maskos et al., 2005; Picciotto et al. 1998). Furthermore, the homomeric α_7 nAChR has been found to be exist on glutamatergic terminals in both the striatum and cortex (Marchi et al., 2002). Since glutamatergic input to the VTA and and NAcc has been shown to come from the PFC and PPT (Sesack & Pickel, 1992; Tong et al., 1996; Carr & Sesack, 2000; Charara et al., 1996; Lokwan et al., 1999), the presence of α_7 nAChR on these terminals provides another means by which nicotine can indirectly increase the firing of mesolimbic dopamine neurons.

Afferent glutamatergic inputs from the PFC not only synapse on dopaminergic neurons, but also on local GABA interneurons in the VTA which both innervate local DA neurons and project to the NAcc (Carr & Sesack, 2000; Johnson & North, 1992b). These GABA neurons are the only neurons in the VTA which contain mu-opioid receptors (MORS) located primarily on their somata and dendrites; however as much as 8% have been found on GABAergic terminals (Dilts & Kalivas, 1989; Garzon & Pickel, 2001). These GABA neurons are greatly hyperpolarized by ethanol due to activation of MORs by ethanol-induced beta-endorphin release (Johnson & North, 1992a; Xiao et al., 2007;

Xiao & Ye, 2008; Herz, 1997; Marinelli & Gianoulakis, 2004). Hyperpolarization of these VTA GABA neurons by ethanol results in indirect facilitation of VTA DA neuron firing via disinhibition (Johnson & North, 1992a; Mansour et al., 1995). This effect of ethanol on GABA neurons – net inhibition – occurs despite ethanol's apparent ability to facilitate GABA release at presynaptic GABAergic terminals in the VTA (Melis et al., 2002; Theile et al., 2008). I have posited that this is due to ethanol's effect on beta-endorphin release, and subsequent MOR stimulation, is much greater in magnitude than this potentiating effect at the terminal.

Dopamine receptors play a unique role in the mesolimbic system as autoreceptors in the VTA. Both excitatory D_1Rs and inhibitory D_2Rs are present on presynaptic glutamatergic terminals which synapse on dopamine neurons in the VTA (Deng et al., 2009; Xiao et al., 2009). These same experiments have shown that ethanol causes release of dopamine from the somata and dendrites of VTA DA neurons, which acts in a retrograde fashion on excitatory dopamine D_1 receptors (D_1Rs) located on glutamate terminals, facilitating glutamate transmission and subsequent firing of the VTA DA neurons in a positive feedback loop. This positive feedback loop desensitizes rapidly however – within 30-60 seconds – due to desensitization of the presynaptic D_1Rs (Deng et al., 2009). This loop can also be shut off by high levels of ethanol due to D_2R activation (Xiao et al., 2009).

With regards to stimulating nicotine cravings, I have hypothesized that because nicotine potentiates the firing of these excitatory synapses GLUT→DA synapses – and consequentially the firing of the mesolimbic dopamine pathway – consumption of nicotine while intoxicated could be seen as a compensatory response to the slumping of

this synapse's firing frequencies at high levels of ethanol. For someone who had previously experienced the high firing rates or "reward" of using both drugs at the same time, the experience of a slump in mesolimbic firing after the initial burst – due to desensitization of D_1Rs or activation of D_2Rs – could trigger a response to seek out a cigarette to prevent this decrease in reward circuitry firing. Nicotinic receptors are indeed present presynaptically on glutamatergic and dopaminergic terminals (Marchi, Risso, Viola, Cavazzani, & Raiteri, 2002; Kaiser, Soliakov, Harvey, Leutje, & Wonnacott, 1998), as well as postsynaptically on the dendrites and cell bodies of mesolimbic dopamine neurons (Maskos et al., 2005), making this a plausible contributor to alcohol-induced nicotine cravings.

The potential for interaction does not stop at this feedback loop. A significant portion of nicotine's activation of the mesolimbic dopamine pathway is due to indirect activation of the excitatory ionotropic glutamate NMDA receptor. While direct AMPA receptor activation does elicit DA release in the NAcc, it does not appear that this receptor plays a part in nicotine's activation of the mesolimbic DA system. Nicotine appears to primarily utilize NMDA receptor activation in the mesolimbic DA pathway. Nicotinic receptors have already been found to be able to depolarize the membrane of a neuron enough to remove the Mg^{2+} block from NMDA (Cheramy et al., 1996). Additionally, nicotinic receptors are present both pre- and post-synaptically at these glutamate-dopamine synapses, and NMDA receptors are present postsynaptically on the cell bodies of these mesolimbic DA neurons (Marchi et al., 2002; Maskos et al., 2005; Picciotto et al. 1998; Wang & French, 1993). Therefore, in the VTA, administration of nicotine can activate presynaptic α_7 nAChRs, causing release of glutamate from

glutamatergic terminals which then activates postsynaptic NMDA receptors that have had their Mg^{2+} block removed by the membrane depolarization from nearby β_2-containing nAChRs.

Ethanol, however, appears to have an mild inhibitory effect on these presynaptic α_7 nAChRs, as well as a suppressing effect on NMDA receptors (Covernton & Connolly, 1997; Stobbs et al., 2004). If this ethanol-induced suppression of NMDA and α_7 nAChR receptor function can occur acutely in response to heavy ethanol consumption – such as that of binge drinking – a user who has previously consumed nicotine with alcohol could crave nicotine in order to "reignite" glutamate transmission at glutamate-dopamine synapses in the VTA, causing mesolimbic neuron stimulation and firing. Nicotine's activation of presynaptic α_7 nAChRs on glutamate terminals as well as postsynaptic β_2-containing nAChRs on mesolimbic DA neuron cell bodies would heavily stimulate the mesolimbic system and induce firing despite ethanol interference with glutamate levels and NMDAR responsiveness. While the contribution of nAChR-NMDAR cooperativity would not be as stark or effective, nAChR stimulation would still induce firing, especially in those who do not smoke regularly and have fully sensitive nAChRs. Furthermore, smokers with desensitized nAChRs would be driven to smoke in excess in order to reignite firing of mesolimbic neurons to the greatest extent possible.

While these "reignition" theories have their vulnerabilities, they are attractive as they explain much of what has been observed in studies which administered both drugs, Such as that of Tizabi et al. (2002). This *in vivo* microdialysis experiment compared the effects of varying doses of I.P. ethanol and direct VTA-perfused nicotine on dopamine overflow in the NAcc (Tizabi, Copeland, Louis, & Taylor, 2002). Both drugs administered alone

showed a dose-dependent effect on increases in NAcc dopamine, however when administered together the results were more interesting: When the lowest doses of each drug – 0.5 g/kg ethanol, 0.25 µg nicotine – were administered together, ethanol and nicotine together increased the dopamine efflux in the NAcc with a nearly-perfect additive effect. However, when higher doses of each drug – 1.0 g/kg ethanol, 1.0 µg nicotine – were administered together, the effect was *much less* than additive.

While the authors propose this to represent a "ceiling effect," I have proposed the interaction to be much more complex: At low doses of ethanol, the positive feedback loop evidenced by Deng et al. (2009) and Xiao et al. (2009) propagates glutamate release at glutamate-dopamine synapses, activating postsynaptic glutamate receptors (Wang & French, 1993) and causing depolarization and firing of mesolimbic dopamine neurons, resulting in dopamine efflux in the NAcc. These low doses of ethanol have only a mild, near-irrelevant effect on most nAChRs (Covernton & Connolly, 1997), and therefore at low doses of ethanol these receptors respond normally to stimulation by nicotine. Since nicotinic receptors are located presynaptically on glutamate terminals in the VTA (Marchi et al., 2002), on the cell bodies of mesolimbic dopamine neurons in the VTA (Maskos et al., 2005), and on dopaminergic terminals in the NAcc (Kaiser et al., 1998), all of these receptors stimulate dopamine efflux to the same level as they would without the presence of ethanol, resulting in an additive effect of ethanol and nicotine on dopamine efflux in the NAcc.

However, under high levels of ethanol conditions change: presynaptic α_7 nAChRs become partially inhibited by ethanol (Covernton & Connolly, 1997; Aistrup et al., 1999), while other $\alpha_*\beta_*$ nAChRs become potentiated. The glutamatergic positive

feedback loop which was still active at low levels of ethanol is both desensitized from prolonged stimulation (Deng et al., 2009) and inhibited by presynaptic D_2R activation (Xiao et al., 2009), and the nicotinic stimulation of presynaptic α_7 homomeric nAChRs is not as effective anymore due to inhibition by ethanol (Covernton & Connolly, 1997; Aistrup et al., 1999). This all combines to result in an increase in excitation from non-α_7 nAChRs on mesolimbic neuron cell bodies and terminals in the NAcc, but loss of excitation from glutamate terminals synapsing on mesolimbic dopamine neurons in the VTA. The loss of glutamatergic input to mesolimbic cell bodies in the VTA results in a heavy decrease of stimulation, as postsynaptic NMDA receptors (Wang & French, 1993) – unblocked by nearby β_2-containing nAChRs (Maskos et al., 2005; Cheramy et al., 1996) – are no longer activated by glutamate. However, this decrease in stimulation is partially compensated by the potentiation of $\alpha_3\beta_2$ nAChRs on dopamine terminals in the NAcc, as well as the potentiation of other β_2-containing nAChRs both on these dopaminergic terminals and on the cell bodies of mesolimbic DA neurons in the VTA (Kaiser et al., 1998; Maskos et al., 2005). NMDA receptors present on dopaminergic terminals in the NAcc would still be active and unblocked by nearby, now-potentiated nAChRs, and thus would also help compensate for this loss of transmission. At this higher dose of ethanol, the end result would be dopamine efflux higher than either drug alone, but not purely additive in nature. This is precisely what was discovered by Tizabi et al. (2002).

Even if these "reignition" theories were to be proved false, there are still many ways in which nicotine craving could become associated with ethanol. Mansvelder et al.'s (2002) discovery that β_2-containing nicotine receptors on the somata of VTA GABA

interneurons desensitize at concentrations of nicotine which still greatly stimulate α_7 nAChRs on glutamatergic terminals synapsing on VTA DA neurons decisively showed that nicotine does not simply increase excitatory GLUTergic stimulation of mesolimbic dopamine neurons. Rather, it also *fails* to induce inhibitory stimulation of these mesolimbic DA neurons. It appears that smoking one cigarette does not only stimulate glutamatergic EPSCs, but desensitizes and nullifies β_2-containing nAChR stimulation of inhibitory GABA transmission!

Based on what we know about ethanol's effects on local GABA neurons in the VTA, it seems obvious how ethanol could become associated with nicotine via synergy with this mechanism. Ethanol administration hyperpolarizes GABA neurons in the VTA by stimulating beta-endorphin release which stimulates mu-opioid receptors present on their somata and dendrites (Johnson & North, 1992a; Herz, 1997; Marinelli & Gianoulakis, 2004; Xiao et al., 2007; Xiao & Ye, 2008). This hyperpolarization decreases tonic inhibition of mesolimbic dopamine neurons in the VTA, increasing their firing rate and subsequently stimulating mesolimbic dopaminergic transmission (Johnson & North, 1992a; Mansour et al., 1995; Kitai et al., 1999). If nicotine's natural effect is to at first mildy stimulate, and then drastically cease stimulation of these VTA GABA neurons (Mansvelder et al., 2002), I would suggest that the inhibition of these GABA neurons' function by ethanol could serve as *yet another* trigger point for association of ethanol with nicotine. In essence, the regular drinker who has previously smoked cigarettes while drinking could be neuroanatomically cued or "reminded by" the normal effects of nicotine on this system by the alcohol-induced inhibition of VTA GABA neurons and subsequent indirect stimulation of mesolimbic DA transmission.

The induction of long-term potentiation cannot be ignored either, as it appears to be key in facilitating addictive cravings to these drugs. Gao et al. (2010) have demonstrated that a single intraperitoneal dose of nicotine given to Wistar rats significantly shifts the AMPA/NMDA receptor current ratio towards AMPA receptors in the VTA as quickly as one hour later, and furthermore this alteration persists for up to 72 hours. This significant increase in the contribution of AMPA receptor currents occurred presumably due to mechanisms such as the activation of silent synapses and/or sensitization of pre-existing AMPA receptors in the VTA. In fact, 24 hours post-injection, new AMPA receptors were found to be contributing to the functional responses, confirming this assertion. The most important data gathered from this experiment, however, was the fact that only antagonization of both α_7 homomeric and β_2 – containing nAChRs prevented this shift towards AMPA receptor currents. This evidence suggests that LTP can occur at this synapse via activation of either presynaptic α_7 nAChRs or postsynaptic β_2–containing nAChRs, making this an extremely dynamic, high-plasticity synapse that could undergo long-term potentiation even if one of the nAChR subtypes were to become completely desensitized.

This corresponds with the observations of Mansvelder & McGehee (2000). They found that after a 200 stimulation pairing session of the presynaptic terminal and postsynaptic membrane of a GLUT→DA synapse in the VTA, EPSC amplitude increased to 122% of control values and persisted for at least 40 minutes, indicating LTP induction. When the authors paired the same postsynaptic stimulation with a 200 second application of 1 μM nicotine, they found that this nicotine pairing increased evoked EPSCs to 116%

of control for at least 40 minutes. Most importantly, the amount of LTP induced by this nicotine pairing correlated with the recorded increase in EPSC frequency.

As I have already stated, this discovery is crucial. It makes possible the assertion that any substance which stimulates VTA DA neurons heavily enough – including ethanol – can be paired with the presynaptic enhancement of glutamatergic transmission by nicotine. For example, the firing of VTA DA neurons evoked by ethanol-induced disinhibition could be paired with this nicotine-induced presynaptic stimulation of GLUTergic transmission. In other words, concomitant consumption of both drugs could induce LTP at these synapses, sensitizing the mesolimbic dopamine system and thus the user to cravings (Robinson & Berridge, 2001). I conclude that this mechanism provides a way in which we could make deep, limbic system-driven associations between the pleasure evoked from alcohol with the craving and subsequent satisfaction of smoking a cigarette. To top everything off, it has been shown that this form of LTP *cannot* be induced at GLUT→GABA synapses in the VTA (Wooltorton et al., 2003). In other words, these same glutamatergic afferents cannot strengthen their ability to stimulate firing of local inhibitory GABA neurons, creating an imbalance which may *only* be shifted towards hyperexcitation of mesolimbic DA neurons.

I would assert that the next major point of exploration should focus on antagonization of the α_7 nAChR homomer. This receptor is present and active in the majority of the mechanisms we have discussed, and could prove to be a turning point in breaking the interactions of nicotine and ethanol with the mesolimbic dopamine system. This receptor is one of the few nAChRs that doesn't show strong desensitization in response to low background levels of nicotine – such as those present in a smoker's blood

overnight (Mansvelder et al., 2002). It is possible that for this reason this receptor is not proliferated as greatly as other nAChRs in response to constant desensitization by nicotine (Dani & Heinemann, 1996). However, we cannot be sure. If this receptor still underwent proliferation, such proliferation would greatly enhance the presynaptic stimulation of glutamate transmission induced by nicotine consumption, making this mechanism of mesolimbic stimulation more resilient to both desensitization by nicotine as well as partial inhibition by high levels of ethanol (Covernton & Connolly, 1997).

At the same time, resilience of this nAChR subtype to proliferation could give it unique characteristics that may provide the opportunity for concentrated treatment of maladapted, sensitized mesolimbic systems. Perhaps antagonization of this receptor could help prevent LTP of these GLUT-DA synapses in the VTA. Methyllycaconitine (MLA) is the current standard for specific antagonization of this receptor, however its use as a therapeutic agent has been limited by its muscle paralysis-inducing effect in mammals (Panter et al., 2002). Pursuit of new antagonists which do not have diffuse effects on peripheral nervous system nAChRs could result in the development of new anti-craving treatments for nicotine addiction, as well as other drugs of addiction which interact with nAChRs – such as ethanol.

The effect such a drug would have on clinical treatment of nicotine addiction would be equivocal or greater than that of Chantix – otherwise known as varenicline. This partial $\alpha_4\beta_2$ agonist has been shown to aid smoking cessation in clinical trials (Jorenby et al., 2006). Interestingly, a comprehensive working model of the changes it induces in mesolimbic circuit transmission has yet to be produced. I believe that the production of such a model would greatly aid our understanding of what effective anti-

craving treatments should look like in the future, and more importantly *why* varenicline is so efficacious. Furthermore, it would provide an opportunity for criticism of the current treatments and realization of the areas of research necessary for further improvement in anti-craving drug pharmacology. Certainly, there is much room left for improvement in this field.

On this note, I now consider this thesis concluded. Yes, there are plenty of questions left to be answered: What other pharmacological interventions can we derive from this knowledge? Can we "de-couple" these limbic associations without causing severe side effects in patients? I personally believe that finding the answers to these questions will only be achieved with both improvements in our understanding of this addiction circuitry, as well as improvements in pharmacokinetic administration techniques. Can you imagine being able to give a patient a drug which will only carry out its pharmacological action on a certain region of the brain? It seems impossible to most, but to me it seems only a matter of time. It is the pursuit of answers to questions such as these, the discovery of techniques such as these for which I hope to spend a lifetime pursuing the answers.

APPENDIX

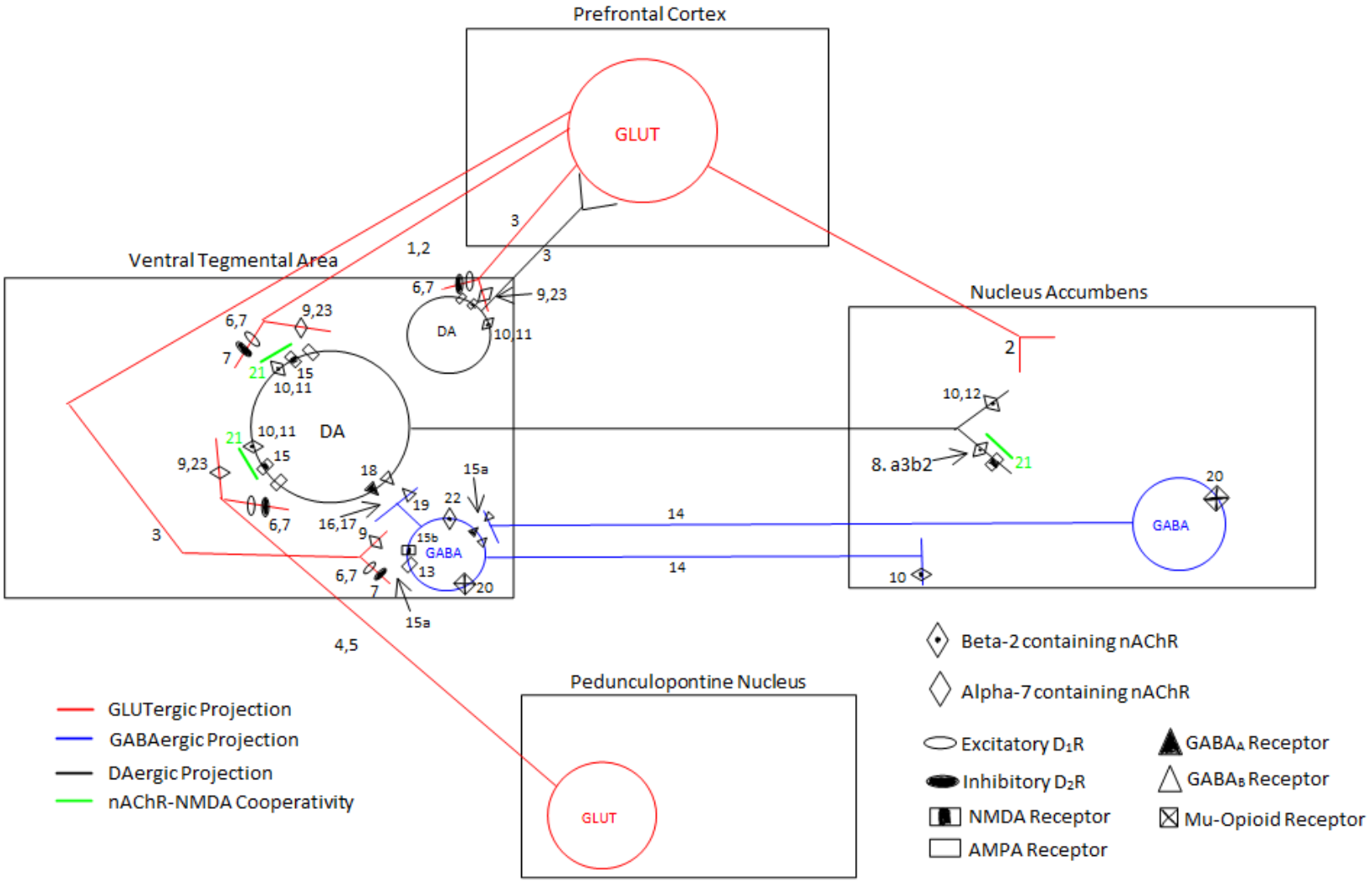

Figure 2. Diagram of Pertinent Synapses Involved in Nicotine-Ethanol Interactions in the Mesolimbic Dopamine System.

Figure 2 Citations

1. Sesack & Pickel, 1992.

2. Tong, Overton, & Clark, 1996.

3. Carr & Sesack, 2000.

4. Lokwan, Berry, Overton, & Clark, 1999.

5. Charara, Smith, & Parent, 1996.

6. Deng, Ke-Yong, Zhou, & Ye, 2009.

7. Xiao et al., 2009.

8. Kaiser, Soliakov, Harvey, Leutje, & Wonnacott, 1998.

9. Marchi, Risso, Viola, Cavazzani, & Raiteri, 2002.

10. Maskos et al., 2005.

11. Piccioto et al., 1998.

12. Wonnacott, Kaiser, Mogg, Soliakov, & Jones, 2000.

13. Johnson & North, 1992b.

14. Van Bockstaele & Pickel, 1995.

15a. Steffensen, Svingos, Pickel, & Henriksen, 1998.

15b. Steffensen, Svingos, Pickel, & Henriksen, 1998.

16. Johnson & North, 1992a.

17. Kitai, Shepard, Callaway, & Scroggs, 1999.

18. Theile, Morikawa, Gonzales, & Morrisett, 2008; Xiao & Ye, 2008.

19. Ariwodola & Weiner, 2004.

20. Dilts & Kalivas, 1989; Garzon & Pickel, 2001.

21. Cheramy, Godeheu, L'Hirondel, & Glowinski, 1996.

22. Mansvelder, Keith, & McGehee, 2002.

23. Mansvelder & McGehee, 2000.

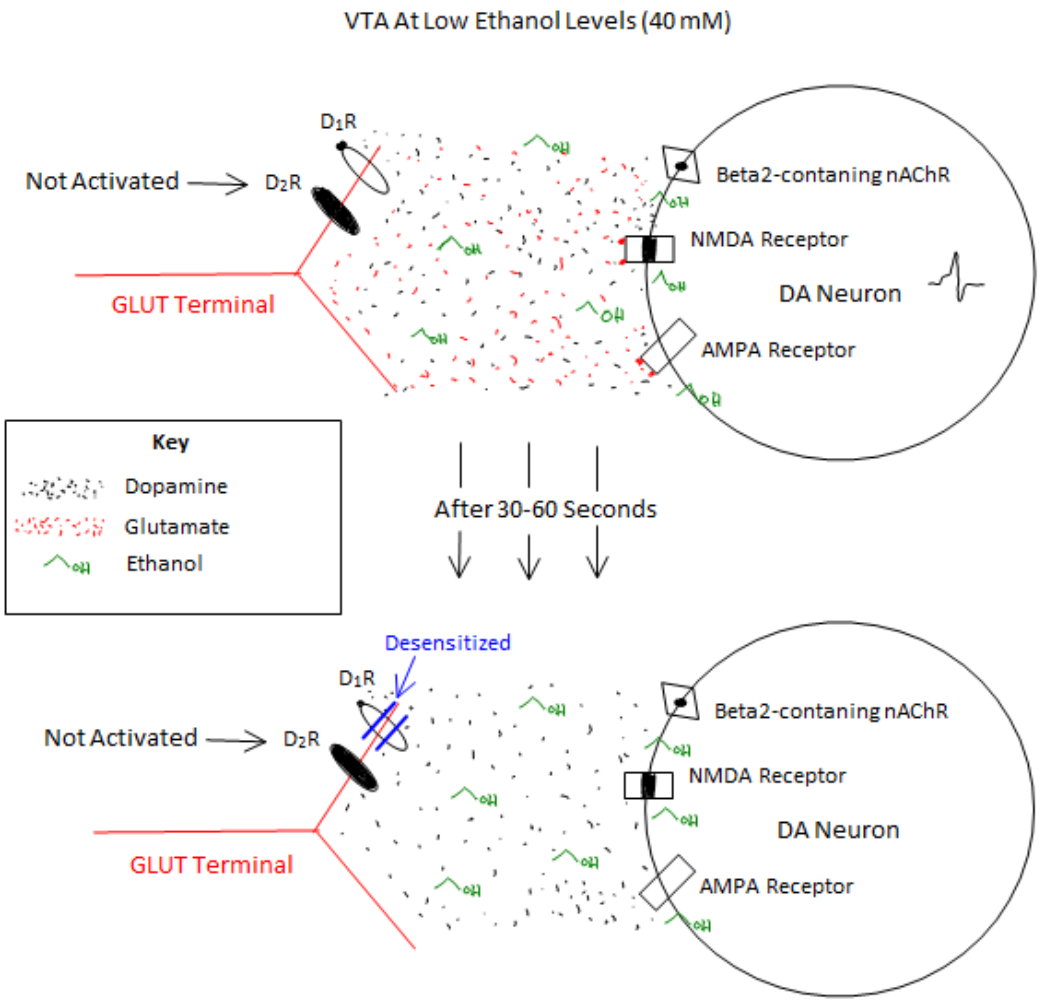

Figure 3. Short-term Positive Feedback Loop at Dopamine-Glutamate Synapse in VTA.

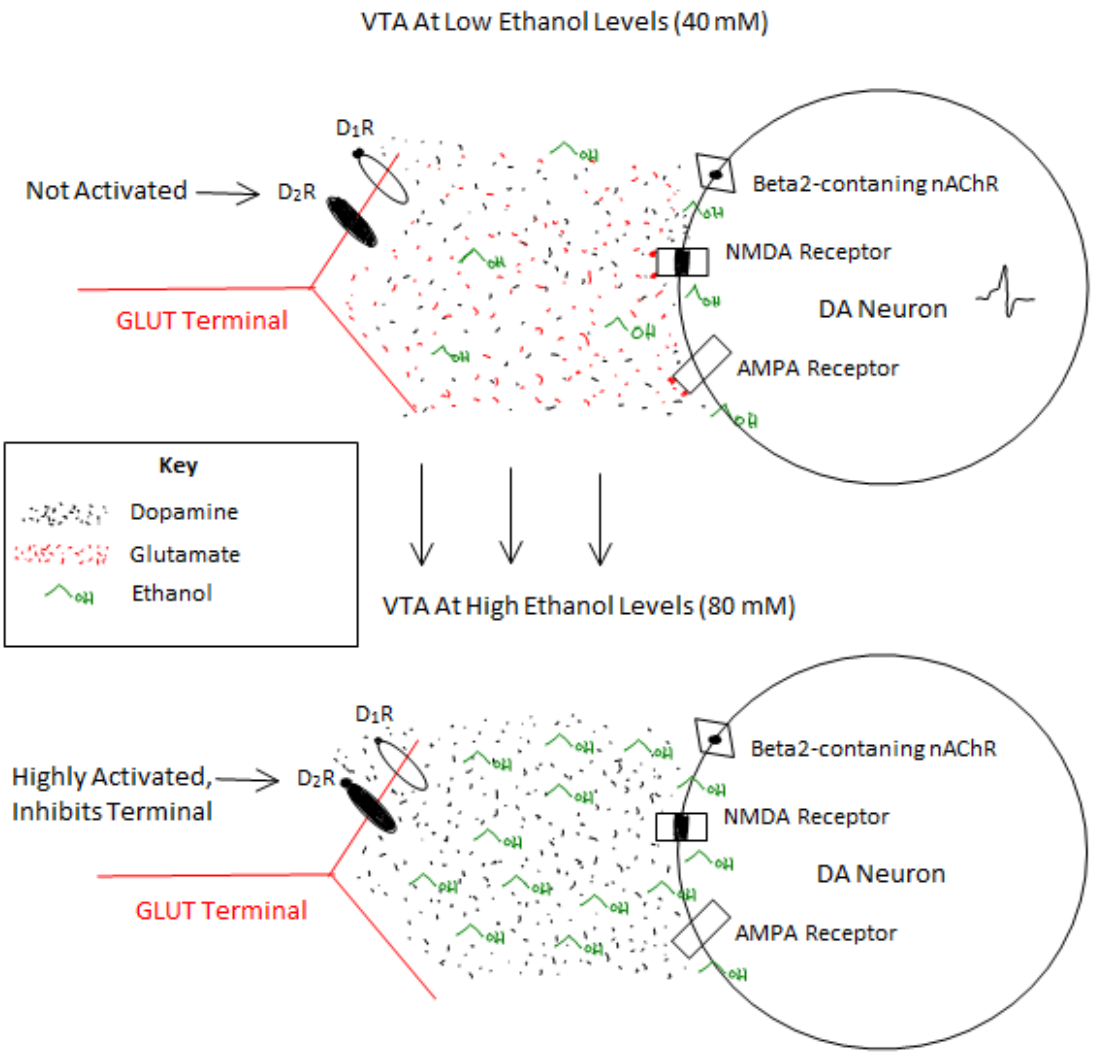

Figure 4. D_2R Activation Occurs at High Ethanol Levels, Inhibiting Glutamate Transmission.

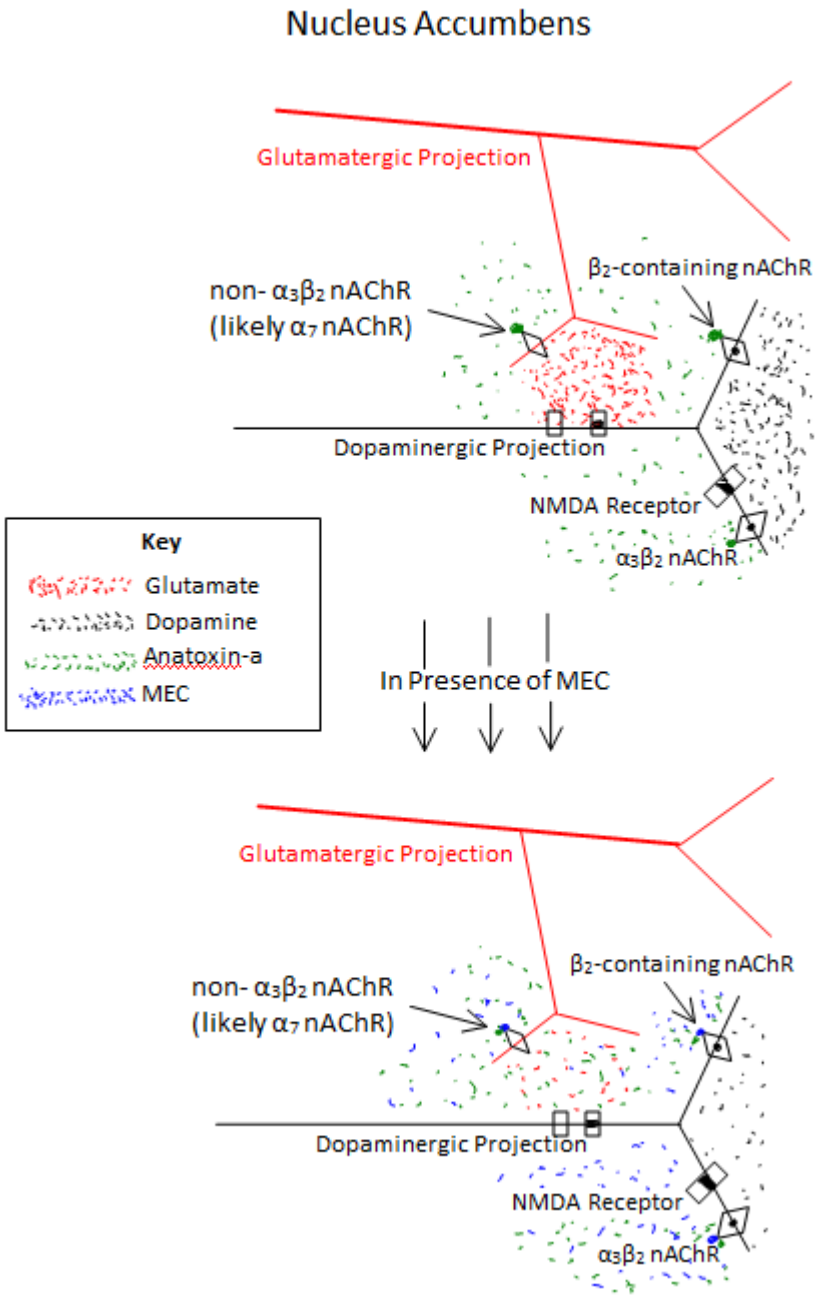

Figure 5. Mecamylamine Inhibits non-$\alpha_3\beta_2$ nAChRs on Axoaxonal Glutamate Terminals.

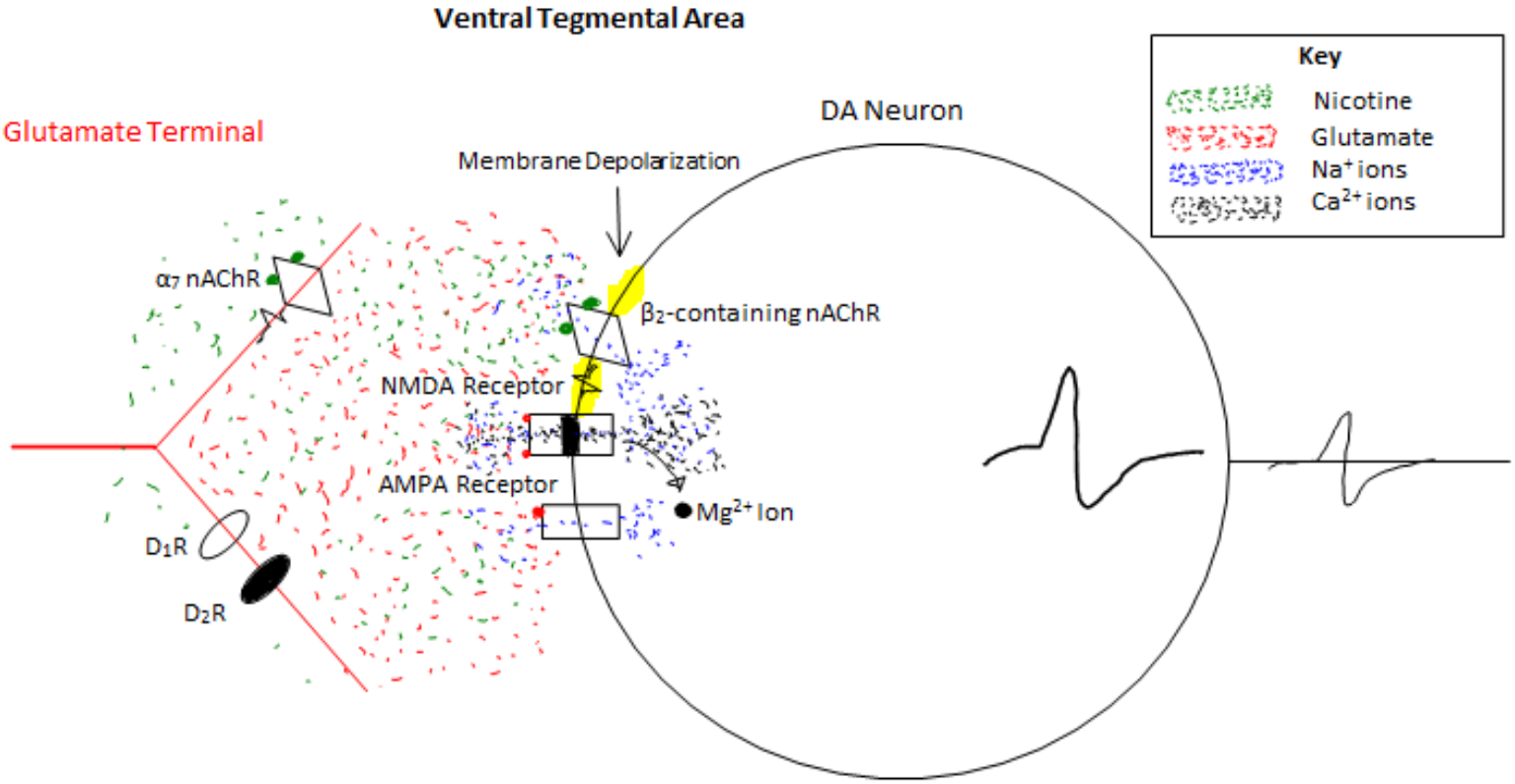

Figure 6. Cooperativity of nAChRs with NMDA Receptors in the Ventral Tegmental Area.

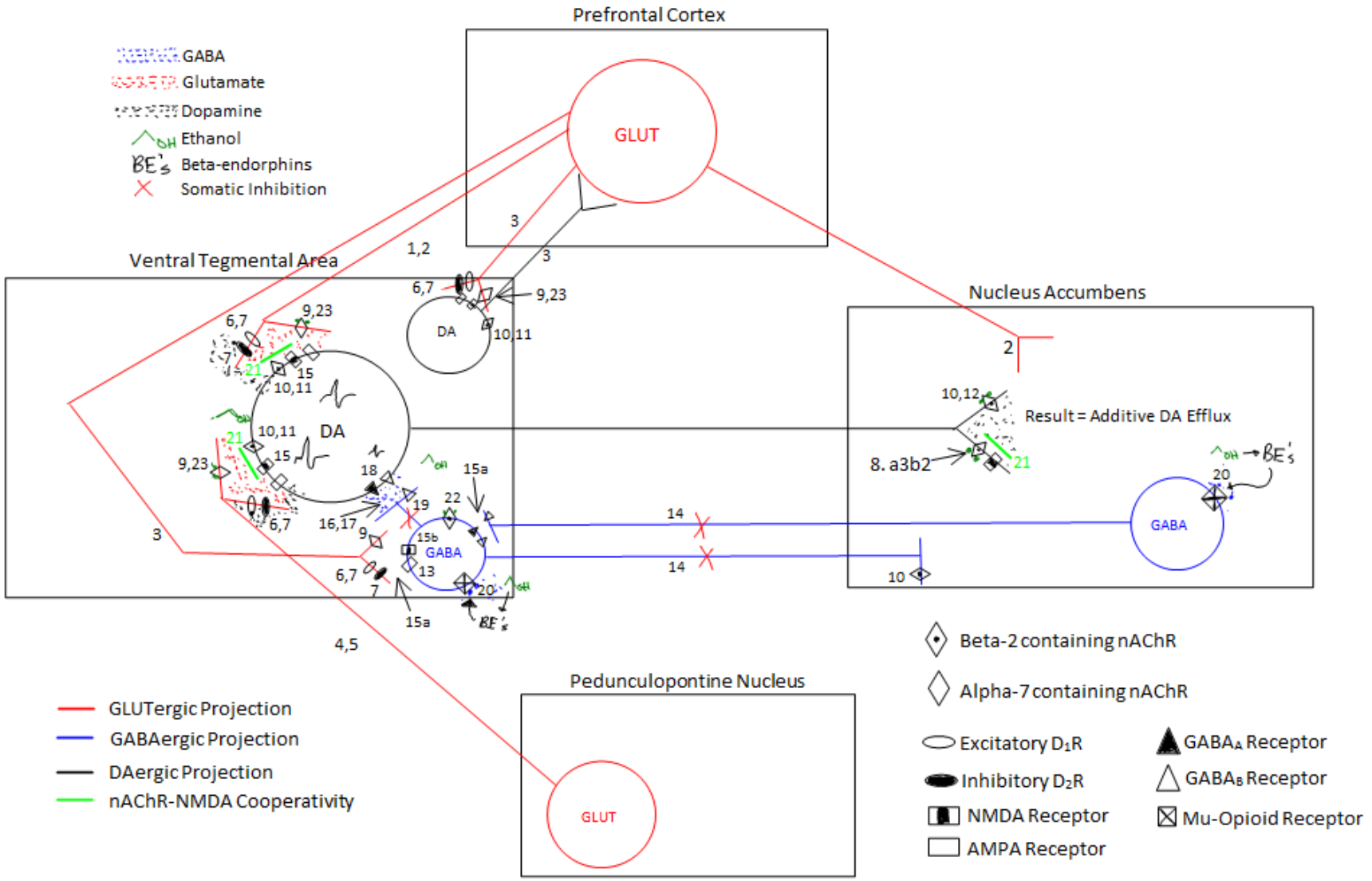

Figure 8. Interactions of Nicotine and Ethanol in the Mesolimbic System at Low Concentrations of Ethanol.

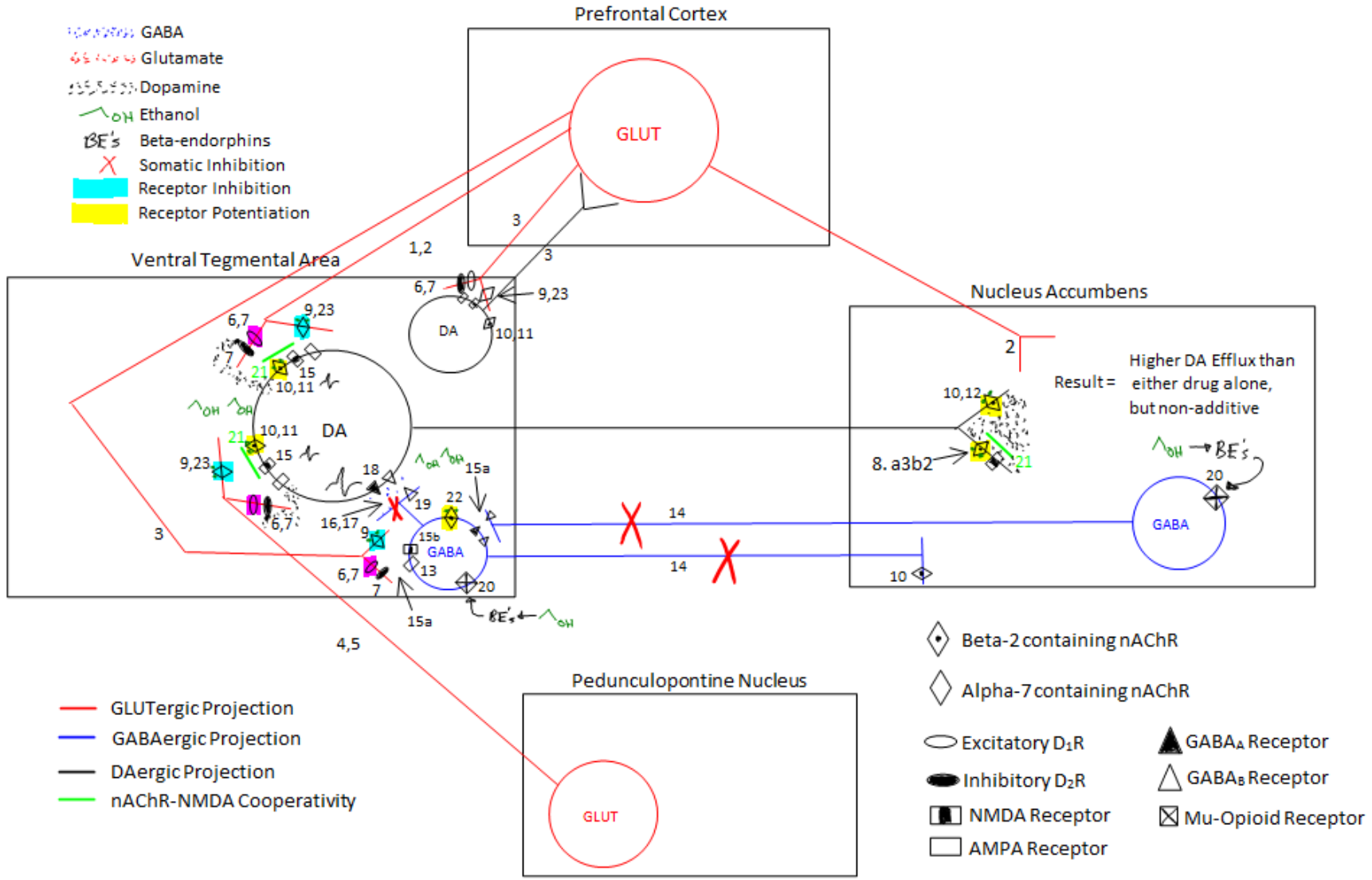

Figure 9. Interactions of Nicotine and Ethanol in the Mesolimbic System at High Concentrations of Ethanol.

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