CHARACTERIZATION OF THE EFFECTS OF NOVEL 5-HT2C RECEPTOR AGONISTS ON NEUROTRANSMISSION AND VOLUNTARY ALCOHOL CONSUMPTION IN RATS

By

JAMES MICHAEL KASPER

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To my wife Reyna and my parents: Dan and Peggy

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LIST OF ABBREVIATIONS

5-HT	Serotonin
ADE	Alcohol Deprivation Effect
FR	Fixed Ratio
GABA	Gamma-Aminobutyric Acid
IP	Intraperitoneal
K+	Potassium
NAc	Nucleus Accumbens
PR	Progressive Ratio
SC	Subcutaneous
VTA	Ventral Tegmental Area

Abstract of Dissertation Presented to the Graduate School
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James Michael Kasper

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This dissertation project studies the ability of 5-HT_{2C} receptor modulators to alter voluntary ethanol intake and to investigate the changes in neurotransmission that accompany 5-HT_{2C} agonists in a neuronal pathway associated with reward, the mesolimbic pathway. The 5-HT_{2C} receptors are expressed throughout this pathway including in the nucleus accumbens, NAc. Due to high transmembrane sequence homology of the 5-HT_2 subfamily of receptors it is difficult to find selective agonists for the 5-HT_{2C} receptor that do not activate 5-$HT_{2A/B}$ receptors as well. This is problematic because while 5-HT_{2C} agonists are predicted to be a pharmacotherapy for alcoholism, agonists for 5-HT_{2A} cause psychotomimetic effects and agonists for 5-HT_{2B} cause cardio-valvulopathy. Recently, a promising series of phenylaminotetralin-based structures, PAT, have been discovered and certain PATs have been shown to have functional selectivity at the 5-HT_2 subfamily of receptors. Of special interest are compounds that can act as agonists at 5-HT_{2C} while acting as antagonists/inverse agonists at 5-$HT_{2A/B}$ receptors. The goal of this dissertation is to determine if these functionally selective PATs can alter voluntary ethanol consumption, both under basal

conditions and in ethanol deprived animals, and examine changes in neurotransmission that might mediate this effect.

CHAPTER 1
LITERATURE REVIEW

Substance Addiction

Substance addiction in humans is characterized by continued drug-seeking and drug-taking despite negative or dangerous consequences. These drugs often initially induce euphoria or remove distress. Over time and repeated drug use, homeostatic changes in the body occur which result in tolerance, sensitization, physical dependence, and/or craving (Cami and Farre, 2003). Many of these changes are mediated by altered receptor populations, synaptic turnover, and cellular protein expression in the central nervous system (Wolf, 2002). For this reason, key areas of the brain have been identified and studied to discover the process of addiction development and identify therapeutic targets.

Alcoholism

An estimated half of Americans over the age of 12 are reported to be ethanol drinkers in a 2006 study by the Department of Health and Human Services. This study also found that a fifth of Americans over 12 engaged in binge drinking (five or more drinks on one occasion).

Ethanol's mechanism of action for reward is not fully understood but there is evidence demonstrating ethanol's mechanism of action for sedative and amnestic effects. At low acute doses ethanol is perceived as a stimulant due to suppressing inhibitory drive in the CNS. As doses increase, sedation and motor discoordination can be observed (Holdstock and de Wit, 1998). Pharmacologically relevant doses of ethanol have been shown to act on a wide variety of proteins. $GABA_A$ receptors can be stimulated by ethanol while glutamate NMDA receptors are inhibited (Melis et al., 2009).

Neuronal calcium channels (Wang et al., 1992) and potassium channels (Chu et al., 1992) are also altered by ethanol. But ethanol does not only affect extracellular proteins, it can also act directly on second messenger pathways like adenylyl cyclase (Yoshimura and Tabakoff, 1999) and protein kinase C (Solem et al., 1997). Ethanol's many mechanisms of action make alcoholism difficult to study because there are numerous ethanol induced effects to treat or block by potential pharmacotherapy.

Mesolimbic Pathway

The pluripotent nature of ethanol's mechanisms of action can alter numerous parts of the brain but the mesolimbic pathway has been shown to mediate the rewarding effects of ethanol (Söderpalm and Ericson, 2009) and the acquisition of alcohol consumption (Vengeliene et al., 2007). The mesolimbic pathway consists of dopaminergic neurons that project from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) and is known for its role in reward. The neuronal cell bodies in the NAc are primarily GABAergic and project either back to the VTA or to other brain regions including the striatum and raphe nucleus. The raphe nucleus has serotonergic cell bodies that can project to the VTA or NAc. These projections explain why dopamine, GABA and serotonin receptors in the mesolimbic pathway are expressed in those regions (Westerink et. al., 1996, Clemett et al., 1999) and are able to alter neurotransmitter release (Alex and Phek, 2007).

The importance of the mesolimbic pathway in voluntary ethanol intake is demonstrated by studies that show that rats will self-administer ethanol directly to the VTA (Rodd et al., 2004). This indicates that the VTA is a site of action for ethanol and that ethanol in the VTA has reinforcing value. Ethanol self administration directly to the VTA is blocked by coinfusion of a D2/3 agonist, indicating the importance of dopamine

in ethanol's ability to be a reinforcing agent (Rodd et al., 2005). It is clear that the mesolimbic pathway plays a critical role in ethanol reinforcement and therefore the development of alcoholism. However, there are probably more neurotransmitters involved in this circuitry besides just dopamine.

Neurotransmitters Implicated In Alcoholism

Dopamine

Dopamine is a catecholamine neurotransmitter that plays an important role in addiction (Söderpalm and Ericson, 2011). This is because drugs of abuse often increase dopamine concentrations in the NAc (Sulzer, 2010). Dopamine receptors are sorted into 5 subtypes, D_1 through D_5. Those subtypes are often grouped into D_1-like and D_2-like receptor families (Hurely and Jenner, 2006). D_1-like receptors include D_1 and D_5 receptors which couple to Gαs. Activation of the D_1-like receptors will cause activation of adenylyl cyclase and an increase in the second messenger cAMP in most brain regions. This can lead to increase in neurotransmitter release of the neuron. The D_2-like family includes D_2, D_3, and D_4 receptors that mostly couple to Gαi. Activating this family will generally inhibit adenylyl cyclase and thus inhibit neurotransmission (Neves et al., 2002).

In the mesolimbic pathway, both D_1 and D_2-like receptor families have been shown to be important regulators of neurotransmission. The D_1 and D_5 receptors are expressed in the NAc and can mediate both the effects of ethanol and ethanol self-administration (Söderpalm and Ericson, 2011). D_1-like receptor antagonists in the NAc can decrease ethanol self-administration (Hodge et al., 1997). D_2 and D_3 receptors are highly expressed in the NAc but their role is more complicated. D_2-like receptor

agonists have biphasic effects; with low doses increasing and high doses decreasing ethanol self-administration (Hodge et al., 1997).

Ethanol causes dose dependent excitation of dopamine neurons in the VTA (Gessa et al., 1985). Ethanol directly administered to the VTA causes dopamine release in the NAc (Ding et al., 2009) and increased ethanol seeking behavior (Hauser et al., 2010), but the $D_{2/3}$ receptor agonist quinpirole reduced this seeking when microinjected alongside the ethanol. Ethanol's actions in the VTA are reinforcing and that reinforcement can be modulated by dopamine receptors. It is clear that ethanol induced changes in dopamine in the mesolimbic pathway are related to the rewarding value of ethanol.

GABA

GABA, γ-Aminobutyric acid, is one of the main inhibitory neurotransmitters. It is widely present throughout the CNS and its receptors have been implicated in addiction as therapeutic targets (Lingford-Hughes, 2010). GABA receptors include the $GABA_A$ and $GABA_B$ receptor families.

The $GABA_A$ receptor is a ligand gated ion channel that will selectively allow chloride ions to enter the neuron. In the majority of neurons, this hyperpolarizes the cell, preventing action potentials, and thus inhibiting neurotransmission. Ethanol acts as a positive allosteric modulator at this receptor and increases the effect of GABA when present (Weiner and Valenzuela, 2006). Antagonists for $GABA_A$ receptors administered directly to the NAc or VTA decrease ethanol self-administration in rats (Chester and Cunningham, 2002). Interestingly, Agonism of the $GABA_A$ receptors by muscimol in the NAc also causes a decrease in ethanol self-administration in rats (Hodge et al., 1995).

The $GABA_B$ receptor is coupled to a G protein and can inhibit adenylyl cyclase and open potassium channels. As with the $GABA_A$ receptor, the flow of ions causes hyperpolarization and inhibits the neuron from firing (Kelm et al., 2011). Positive modulation of $GABA_B$ receptors inhibits the reinforcing ability of numerous substances of abuse including ethanol (Vlachou and Markou, 2010). This decrease in reinforcing ability is thought to be mediated by $GABA_B$ receptors expressed in the VTA on dopaminergic cell bodies (Boehm et al., 2002). Activation of $GABA_B$ receptors on VTA dopamine neurons decreases the mesolimbic dopamine release induced by ethanol (Westerink et al., 1996).

Ex vivo studies have demonstrated that bath applied ethanol causes a dose dependent decrease in VTA GABA neuron firing along with an increase in dopamine neuron firing (Xiao et al., 2007). The increase in dopamine firing was able to be blocked by a $GABA_A$ receptor antagonist. This suggests that ethanol causes a disinhibition of dopamine neurons through presynaptic effects on GABA neurons.

In vivo acute ethanol administration (2g/kg; large enough to cause loss of righting reflex) given i.p. causes a decrease of VTA GABA neuron firing in the VTA (Gallegos et al., 1999). This decrease in GABA firing can be blocked by systemic administration of a D_2 receptor antagonist, eticlopride (Ludlow et al., 2009). However, when animals are given a lower ethanol dose (0.1 g/kg; small enough to induce euphoria and stimulate locomotor activity) i.v., there is an increase in VTA GABA neuron firing (Steffensen et al., 2009).

In addition to these electrophysiology studies, microdialysis studies showed there were no significant changes in VTA GABA concentrations (2 g/kg ethanol i.p.) observed

using both alcohol preferring and non-preferring rats (Kemppainen et al., 2010). Indeed, few microdialysis studies on ethanol induced changes in overall GABA concentrations show significant changes in any brain region (Klem et al., 2011). Two notable exceptions were in the NAc: GABA concentrations decrease after ethanol administration in ethanol-tolerant (Piepponen et al., 2002) and ethanol-dependent rats (Dahchour and De Witte, 2000). These seemingly conflicting results from microdialysis and electrophysiology are currently unresolved.

Even though the mesolimbic pathway is thought of as a dopaminergic pathway, the importance of GABAergic neurons is great. GABA cell bodies represent a large portion of the cell bodies in the NAc and form a major pathway back to the VTA (Conrad and Pfaff, 1976). Mesolimbic GABA provides important regulation of dopaminergic neurons in the mesolimbic pathway and represents an interesting pharmacotherapeutic target for addictive substances (Hodge et al., 1995).

Serotonin

Serotonin, 5-HT, is a monoamine neurotransmitter. In the central nervous system serotonin can alter mood, desire, and sleep (Filip et al., 2005). Receptors for 5-HT are classified into seven families, 5-HT_1 to 5-HT_7. Most of these receptor families couple to a G protein while one, 5-HT_3, is a ligand gated ion channel (Hoyer et al., 1994).

Of particular interest to addiction and reward is the 5-HT_2 family. They include the 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors. All members of this family are G protein-coupled receptors that complex with Gαq (Hoyer et al., 1994). When activated, these receptors activate phospholipase C second messenger pathways, one outcome of which is neuronal excitation and increased neurotransmission (Eison and Mullins, 1996). This family has been implicated as a therapeutic target for regulating anxiety, appetite,

memory, mood, and drug use (Bubar and Cunningham, 2006; Meltzer and Massey, 2011). These numerous different effects make the 5-HT_2 family of receptors an important target of study.

There are many similarities with a few important differences between the 5-HT_{2C} and 5-HT_{2A} receptors. Both receptors' mRNA is coexpressed in the VTA, NAc, prefrontal cortex and striatum (Pompeiano et al., 1994). All of these brain regions have been implicated in addiction. Both receptor subtypes are coupled to Gq, and they show a >80% sequence homology in the transmembrane region, which makes designing receptor specific drugs challenging (Bubar and Cunningham, 2006). There are several specific antagonists for both of these receptors, but there are few specific agonists. This has complicated the study of 5-HT_{2C} and 5-HT_{2A} receptor activation. To exacerbate the problem, the 5-HT_{2C} and 5-HT_{2A} receptors often have opposing behavioral effects, e.g. craving for psychostimulants (Bubar and Cunningham, 2006).

Specific drugs for this family of receptors are important because activation of 5-HT_{2A} receptor is known to cause psychotomimetic effects (Hoyer et al., 2002). Activating 5-HT_{2B} in the periphery causes valvular heart disease (Fitzgerald et al., 2000). Activation of 5-HT_{2C} receptors causes decreased appetite and ethanol consumption (Bickerdike, 2003; Tomkins et al., 2002). These results suggest that a 5-HT_{2C} agonist that does not activate 5-$HT_{2A/2B}$ receptors would have therapeutic value. Unfortunately, the most popular commercially available 5-HT_{2C} receptor agonist, Ro60-0175, demonstrates a ten-fold selectivity for the 5-HT_{2C} receptor over 5-$HT_{2A/2B}$, making the literature difficult to interpret. Thankfully, there are fairly selective antagonists for

the receptor subtypes demonstrating 100-fold preference or more (Di Matteo et al., 1999).

In the mesolimbic pathway, 5-HT receptors are known for their ability to modify dopamine release. When 5-HT_{2C} receptor agonist is administered systemically, it causes a decrease in dopamine release in the NAc that can be blocked by a selective 5-HT_{2C} receptor antagonist (Dremencov et al., 2006). Indeed, when a 5-$HT_{2B/2C}$ receptor antagonist is given systemically, there is an increase in basal dopamine neuron firing (Di Matteo et al., 2001). These studies strongly suggest that 5-HT_{2C} receptor agonists administered systemically leads to decreased dopamine in the NAc. When 5-HT_{2C} receptors agonists are administered directly to the VTA they decrease cocaine induced accumbal dopamine outflow. Low doses of 5-HT_{2C} agonist, Ro60-0175, directly administered into the NAc increase and higher doses decrease cocaine induced dopamine release (Navailles et al., 2008). This shows that there is a balance of 5-HT_{2C} receptor populations in the brain and that there are brain region dependent differences in the effect of 5-HT_{2C} receptors on accumbal dopamine release.

The 5-HT_{2A} receptor has limited effects on substance abuse. 5-HT_{2A} antagonists block while agonists enhance increased locomotor effects of cocaine (Bubar and Cunningham, 2006). However the 5-HT_{2A} antagonist M100907 did not alter operant responding for cocaine (Fletcher et al., 2002). Agonism of the 5-HT_{2A} receptor is shown to be important in the initiation of events leading to hallucinogenic activity associated with LSD and related drugs (Egan et al., 1998). The studies of the role 5-HT_{2A} receptors play in other drugs of abuse provide direction to designing an alcoholism

pharmacotherapy that does not activate 5-HT_{2A} receptors in order to avoid hallucinogenic-like effects.

The 5-HT_{2C} receptor has also been shown to be important for ethanol induced changes in neurotransmission. Agonism of this receptor increases GABA release in the VTA and blocks the ethanol induced increases in VTA dopamine neuron firing (Theile et al., 2009). 5-HT_{2C} receptor agonism reduces while antagonism increases ethanol self-administration in rats (Tomkins et al., 2002), suggesting that basal 5-HT_{2C} receptor activity can influence ethanol consumption.

Current Pharmacotherapies For Alcoholism

There are few drug treatments approved for human alcoholism and they have only been moderately effective (Edwards et al., 2011). Disulfiram, an acetaldehyde dehydrogenase inhibitor, results in large amounts of acetaldehyde produced after drinking which quickly produces headache and nausea. Disulfiram has been proven to be effective on short-term (less than 1 month) but not on long-term (more than 6 months) alcohol consumption (Wright and Moore, 1990). The mechanism of action for naltrexone, a μ and κ-opioid antagonist, is not fully understood but naltrexone has been proven to increase initial ethanol abstinence period and decrease ethanol consumption during relapse (Latt et al., 2002). In humans receiving additional cognitive behavioral therapy, naltrexone 50 mg/day demonstrates a 66.1% abstinence rate at 12 weeks (Feeney et. al., 2006). Acamprosate, an antagonist for glutaminergic N-methyl-D-aspartate (NMDA) receptors and agonist for $GABA_A$ receptors, increases abstinence in both the short and long-term when combined with psychosocial treatments (Mason, 2001). All three of these drugs have previously been shown to be effective at delaying relapse to ethanol consumption (Williams, 2005) but are only marginally effective at

treating alcoholism over placebo. For example, a meta analysis shows that naltrexone causes an increase in desirable outcomes (decreased drinks per day, decreased number of drinking days, and increased length of abstinence periods) by 12% to 19% over placebo (Kransler and Van Kirk, 2001).

There are also pharmacotherapies to treat alcoholism that are not approved for this use. They include topiramate, baclofen, and ondansetron. Topiramate is a $GABA_A$ receptor positive modulator and AMPA receptor antagonist. Topiramate is an anticonvulsant drug used to treat epilepsy but has been shown to have promise treating alcoholism. Topiramate increases the number of abstinent days, decreases drinks per day, and decreases the number of heavy drinking (more than 4 drinks) days (Johnson et al., 2003). Baclofen, a $GABA_B$ receptor agonist, was originally approved for human use to treat spasticity. It has been shown that baclofen increases the length of abstinence periods and the overall number of abstinent days (Leggio et al., 2010). Ondansetron, a 5-HT_3 receptor antagonist, is used as an antiemetic but has been shown to decrease ethanol craving and increase the number of abstinent days compared to placebo (Sellers et al., 1994). This ondansetron-induced decrease in ethanol consumption changed the basal average drinks per day from 3.8 to 2.8 while placebos lowered drinks per day to 3.3. Topiramate, baclofen, and ondansetron all show promise in treating alcoholism but display similar overall marginal effectiveness as approved pharmacotherapies for alcoholism.

The generally mild effectiveness of the current pharmacotherapies for alcoholism could be explained in several ways. Some of the treatments have been shown to be more efficacious in different subtypes of alcoholics. For example, baclofen has more

pronounced effects on alcoholics with liver cirrhosis, indicating severe alcohol dependence (Leggio et al., 2010). Another explanation for the lackluster results of the current pharmacotherapies is that the mechanism of action for ethanol addiction is not known. Without a clear target, it is difficult to intelligently design a pharmacotherapy for alcoholism. Modeling human alcoholism in animals and choosing a therapeutic target can be problematic due to different types of human alcoholics and ethanol's multiple sites of action. These difficulties in studying alcoholism carry over to the drug discovery process.

PATs

Phenylaminotetralins, PATs, represent a novel scaffold for drug discovery. Some PATs have been shown to modulate 5-HT_2 subfamily of receptors (Booth et al., 2009). (-)-trans-PAT, (2S,4R)-(-)-trans-1-phenyl-3-dimethylamino-1,2,3,4-tetrahydronaphthalene, (-)-trans m-Br-PAT, (-)-trans-(2S,4R)-N,N-dimethyl-4-(3-Bromophenyl)-1,2,3,4-tetrahydro-2-naphthalene-amine, (-)-trans-CAT, (2S,4R)-(-)-trans-cyclohexylaminotetralin, TOMCAT, (-)-trans-(2S,4R)-N,N-dimethyl-4-(3-chlorophenyl)-6-methoxy-1,2,3,4-tetrahydro-2-naphthalene-amine, and (-)-trans-p-Cl- PAT, (-)-trans-(2S,4R)-N,N-dimethyl-4-(4-Chlorophenyl)-1,2,3,4-tetrahydro-2-naphthalene-amine, have all been shown to act at 5-HT_{2C} and 5-HT_{2A} receptors. Previous studies demonstrate that tritiated PAT binding sites include the hippocampus, NAc, and striatum (Booth et al., 1999). The current understanding of affinity and function for (-)-trans-PAT and many other PAT analogues is summarized in Table 1-1 along with other established 5-HT_2 receptor modulators.

The PAT compounds are a valuable tool and potential alcoholism pharmacotherapy because some have demonstrated agonism at 5-HT_{2C} receptors and

an inverse agonism at 5-HT_{2A} receptors (Booth et al., 2009). TOMCAT, (-)-trans-p-Cl-PAT, and (-)-trans-m-Br-PAT have a similar affinity and functional profile as (-)-trans-PAT. PAT administration can separate the effect of 5-HT_{2C} receptor activation on neurotransmission and behavior from confounding influence by 5-HT_{2A} receptor activation. These receptor-specific functions also make PAT a potential addiction therapy because activation of 5-HT_{2C} receptors and blockade of 5-HT_{2A} receptors has been shown to be important for cocaine (Bubar and Cunningham, 2006) and alcoholism (Di Matteo et al., 2001).

(-)-trans-PAT also alters behavior by decreasing consumption of palatable dessert in mice, indicating anorectic effects (Rowland et al., 2008). This is important to know when studying ethanol consumption because ethanol has caloric value itself. It will be necessary to test for any non-specific decreases in ethanol consumption that are due to anorectic effects and not specifically decreasing voluntary ethanol consumption.

Together, some PATs display functional selectivity for agonism of the 5-HT_{2C} receptor. The functional profiles of these PATs suggest that they can modulate ethanol intake (Di Matteo et al., 2001). Although it will be necessary to control for non-specific decreases in overall caloric intake, select PATs represent a novel pharmacotherapy for alcoholism.

Tools to Study Ethanol Addiction

Microdialysis And CE-LIF

Microdialysis is a popular technique to measure unbound substances in any medium. Using passive diffusion, substances can be collected from a complex environment for identification or quantification. It is an invasive technique which requires a probe, subject, and sample collection method. Microdialysis samples can be

taken from a living animal that is awake and freely moving. Microdialysis is a popular method to measure extracellular neurotransmitters in the brain of because of the simplicity of the technique and the minimal damage to the surrounding tissue.

There are many types of probes commercially available and they can be highly customized. A basic probe will transport fluid from a pump into a small semipermeable membrane tip where passive diffusion can take place. The fluid is constantly mobile and pushed out of the probe tip and though a collection tube. The substance containing fluid, dialysate, is then collected for analysis. The probe can be customized by altering the volume of the transporting tubes, flow rate, and length and molecular cut off of the semipermeable membrane tip. Increasing tube size or flow rate will increase fluid turnover in the membrane tip causing more dialysate to be collected but decreasing the concentration of substances collected due to decreased time for passive diffusion. Increasing the length of the membrane tip allows for a more concentrated sample to be collected. In the brain, it is common for researchers to use the maximum length of membrane that will fit into their area of interest. Altering the molecular cutoff of the membrane determines what substances can enter the probe and what is excluded.

Once the sample is gathered, the most popular analysis method is to inject 10 to 30 µL onto a HPLC. This allows for quantification of substances with a very low limit of detection. The biggest limitation to this technique is that it requires such a large volume of dialysate. This dialysate injected represents the average concentration of neurotransmitters across 5 to 30 minutes of dialysis which results in poor temporal resolution. One way to overcome the poor temporal resolution of HPLC is to instead use capillary electrophoresis coupled to laser-induced-fluorescence (CE-LIF).

CE-LIF is a sensitive technique (Robert et al., 1998) and only requires 20 nL of dialysate to measure the quantities of multiple substances, including amino acids and catecholamines. The small sample size can be gathered from microdialysis in less than a second which gives high temporal resolution of multiple neurotransmitters (Bowser and Kennedy, 2001). The accurate measuring of multiple neurotransmitters with high temporal resolution provides a complete picture when observing how neurotransmitters change in relation to each other and determining the onset and duration of changes in neurotransmission.

Operant Conditioning

Operant conditioning is a form of learning during which the behavior of an animal is modified due to the consequences of that behavior. This includes drug self-administration setups using an operant conditioning chamber. There are many potential setups for operant conditioning chambers and their customization has been the strength of the technique. Operant chambers allow the animals to bar press in order to receive a substance. The result of pressing the bar is considered to be a positive reinforcement if the animal increases the behavior that caused the reward (Staddon and Ettenger, 1989). These rewards include substances of abuse, including ethanol. By giving the animal a novel alcoholism pharmacotherapy, one can screen drugs that reduce an animal's intake of ethanol.

In order to fully explore the effect of therapeutics on operant responding for abused substances, the operant chamber can be set up to determine the effect on dose titration or motivation alone. A fixed ratio, FR, schedule is used to examine how much of the reward the animal wants to have during the session. Typically this involves the animal pressing the bar a fixed amount of presses to receive the reward. The fixed

amount of bar presses to receive the reward can vary with easier schedules requiring a lower number of bar presses per delivery. If pressing the bar a fixed amount is fairly easy (FR5), the animals will consume as much of the abused substance as they desire. If pharmacotherapy reduces the amount of rewards the animal receives, it has altered self-titration. There are many explanations for why a potential alcoholism pharmacotherapy could result in decreases in FR responding. Self-titration of ethanol will lower if the animal finds the ethanol more potent, if the animal is being punished for ethanol consumption, or the animal finds the ethanol to be more or less rewarding.

Motivation for the reward can be measured using a progressive ratio, PR, schedule (Hoffmeister, 1979). This involves progressively more bar presses for each subsequent reward delivery. The ideal PR schedule requires enough bar presses that the animal is unable to receive the same amount of the reward as they would under a FR5 schedule. The point at which the animal stops responding for the reward is called the breakpoint. This represents the maximum amount of effort the animal will put into receiving that reward. Pharmacotherapeutic agents can alter breakpoints as well, indicating a decreased wanting of the reinforcement. Significant changes during PR responding can be explained with greater ease than FR significant changes because PR responding reflects animal desire for the reinforcement.

Measuring the effect of a pharmacotherapy on basal daily low levels of ethanol consumption may not be as relevant to human alcoholism as measuring the effect of the pharmacotherapy on escalated ethanol intake such as during the Alcohol Deprivation Effect (ADE). The ADE models alcoholic relapse. Relapse in alcoholism is characterized by compulsive alcohol seeking and consumption. Alcohol deprivation

induced craving is a tremendous difficulty for alcoholics (Martin-Fardon and Weiss, 2012). When alcohol is reintroduced following a period of abstinence, consumption temporarily increases compared to previous basal consumption (Rodd, et al. 2009). This can be tested using operant chambers by removing the ethanol from the reward for days or weeks, depending on the animal strain. When the animals are once again allowed to respond for ethanol reward, they display the increased ethanol consumption indicative of the ADE. In rats both acamprosate and naltrexone decrease aspects of the ADE singularly and when administered together (Heyser et al., 2003).

It is difficult to model voluntary ethanol consumption because rats tend not to consume pharmacologically relevant doses of ethanol (Chester and Cunningham, 2002). Researchers have addressed the low ethanol consumption problem in multiple ways including: selective rat breeding for ethanol consumption, food and water restriction, and use of sweetener in the ethanol (Samson and Czachowski, 2003). The use of sweetener in ethanol was advanced by the development of a gelatin vehicle that contains 10% ethanol and polycose. This “jello shot” method sustains fairly high self-administration levels in Sprague-Dawley rats without the need for food or water restriction (Peris et al., 2006). Consumption of ethanol-containing gelatin results in dose-related increases in brain ethanol levels. The “jello shot” procedure is easily applied to operant responding (Li et al., 2008, 2010) with plain gel serving as a control for drug effects on appetite, response rate and other confounding variables. The “jello shot” model of voluntary ethanol consumption allows for pharmacologically relevant doses of ethanol to be consumed without food or water depriving non-genetically selected rats.

Summary

Substance addiction affects many people and key brain regions have been identified that mediate the rewarding aspects. The mesolimbic pathway is composed of dopaminergic projections from the VTA to the NAc. In turn, GABA cell bodies in the NAc project back to the VTA. These projections make a loop where the VTA is sending signals to the NAc and the NAc sends signals back to the VTA. There are many other neuron projections to these brain regions which play a role in regulating the pathway. This includes serotonergic input and the 5-HT_2 family of receptors. 5-HT_{2A} and 5-HT_{2C} receptors in particular have been shown to be key modulators of the mesolimbic pathway. To this end, the 5-HT_{2A} inverse agonist and 5-HT_{2C} receptor agonist, PAT and other novel analogs are exciting new tools to investigate the role of 5-HT_2 receptors in the mesolimbic pathway and represent potential alcoholism pharmacotherapeutics.

Objectives

It is hypothesized that the 5-HT_{2C} receptor represents a potential pharmacotherapy for alcoholism (Di Matteo et al., 2001) but the 5-HT_{2C} receptor has proven difficult to study due to a lack of selective agonists. Some PAT compounds represent a novel pharmacotherapy for a poorly studied alcoholism target. (-)-trans-PAT and select analogs have been shown to have functional selectivity for the 5-HT_2 family of receptors. PAT compounds are promising in their 5-HT_{2C} agonism and 5-$HT_{2A/B}$ antagonism/inverse agonism. The goal of this thesis is to determine the effect of these PAT compounds on alcohol consumption models and establish the PAT induced changes in neurotransmission that may underlie the changes in ethanol consumption behavior. Investigating the effect of these PATs on rat voluntary ethanol consumption can illuminate the role that 5-HT_{2C} agonists play in self-titration and motivation for

ethanol. The PAT compounds will be tested on an ethanol deprivation model of alcoholism to examine their role in alcohol consumption relapse. The mechanisms of action of PAT analogs will then be studied in the NAc to determine what neuronal systems are involved by this potential pharmacotherapy.

AIM #1: Role Of 5-HT_{2C} Modulation On Voluntary Ethanol Intake Measured Using Operant Conditioning

This aim tests the hypothesis that 5-HT_{2C} agonists specifically reduce voluntary ethanol containing gelatin consumption on a FR schedule. (-)-trans-PAT along with the known 5-HT_2 receptor agonist Ro60-0175 and specific 5-HT_{2C} antagonist SB242,080 will be administered before the daily operant session responding for plain or ethanol containing gelatin. These experiments will illuminate the role that 5-HT_{2C} receptors play in titration of ethanol intake.

AIM #2: PAT Effect On The Alcohol Deprivation Effect

The ADE represents a model of ethanol consumption that parallels the tendency to increase ethanol consumption after a period of abstinence (Martin-Fardon and Weiss, 2012). This aim will both demonstrate that the ADE can be modeled using the "jello shot" model of alcoholism and test the hypothesis that (-)-trans-PAT and (-)-trans-m-Br-PAT will remove the deprivation induced increase in voluntary ethanol consumption. Pharmacotherapies that reduce the number of drinks on the reinstatement day and the subsequent high ethanol consumption days would represent a valuable treatment for alcoholism in humans.

AIM #3: Modulation Of Neurotransmission In The NAc By 5-HT_{2C} Agonists Using CE-LIF

This aim tests the hypothesis that agonism of 5-HT_{2C} receptors in the NAc will result in modulation of GABA release. The 5-HT_{2C} receptor is expressed on both

dopamine and GABA neurons in the mesolimbic pathway. The effect of systemic 5-HT_{2C} agonists on dopamine has been well studied but the effect of 5-HT_{2C} agonists on GABA is unclear. GABA release is viewed as a possible mechanism to alter ethanol induced dopamine release. Establishing the mechanism of action for pharmacotherapies that alter ethanol consumption can yield new targets for future pharmacotherapies and drive forward the understanding of alcoholism itself.

	$5\text{-}HT_{2A}$	$5\text{-}HT_{2C}$
Ro60-0175	31 nM agonist	1 nM agonist
SB242,084	160 nM antagonist	1 nM antagonist
Ketanserin	2 nM antagonist	50 nM antagonist
(-)-trans-PAT	80 nM inverse agonist	20 nM agonist
(+)-trans-PAT	520 nM inverse agonist	1300 nM agonist
(-)-trans-m-Br-PAT	15 nM antagonist	6 nM agonist
(-)-trans-p-Cl-PAT	240 nM inverse agonist	130 nM agonist
TOMCAT	35 nM inverse agonist	17 nM agonist
(-)-trans-p-Me-PAT	210 nM inverse agonist	330 nM agonist
(-)-trans-CAT	1.6 nM inverse agonist	14 nM inverse agonist

Table 1-1. List of affinities and functional activity of serotonergic drugs in the current study.

CHAPTER 2
METHODS

Animals And Housing

Female Sprague-Dawley rats (90 days old) weighing approximately 275 g at the start of the studies, were individually housed in plastic cages with food and water available *ad libitum* throughout the study except for the time spent in the operant chambers. Female rats were used for operant experiments since they maintain stable body weight over several months. For microdialysis experiments, male Sprague-Dawley rats, 300 - 400 grams, were used. All rats were maintained on a 12-h light/dark cycle in an environmentally controlled room (lights on at 6:00 am, temperature: 23±3 C, humidity: 45±25 %) and allowed a 1-week acclimatization period to the animal facilities prior to the start of the studies.

Ethanol Self-Administration Training

Ethanol self-administration was established by using sweetened gelatin vehicle (jello shots) containing ethanol (10% Polycose, 10% ethanol, 0.25% gelatin, all by weight, in water). This method sustains fairly high self-administration levels in Sprague-Dawley rats without the need for food or water restriction (Peris et al., 2006). Consumption of ethanol-containing gelatin results in dose-related increases in brain ethanol levels (Peris et al., 2006). The “jello shot” procedure is easily applied to operant responding (Li et al., 2008, 2010) with plain gel serving as a control for drug effects on appetite, response rate and other confounding variables. Ethanol self-administration training consisted of two stages: free access training and operant conditioning. First during free access, the animals had 24-h home cage access to 10% ethanol-containing gel for 2 days followed by 6-h and 3-h access periods per day for 2 days each. After

this, animals had a daily one hour access period for 11 days. Next, operant training was initiated. Operant training was conducted in five operant chambers. Each chamber was housed in a sound-attenuating box equipped with a house light that illuminates with each delivery. Each chamber contained a gel dispenser calibrated to deliver 0.15 g of gel into a recessed dish positioned between the two levers. The apparatus was controlled by a microcomputer interface linked to a DELL computer using Graphic state software to record and manage the data. The rats were placed daily in the operant boxes for 30 minutes and trained to press the active lever to get the reinforcement. To encourage the association of the active lever with gel delivery, 0.3 g of gel was placed on the active lever before each rat's first operant session along with a single delivery of gel coming from the spout. Initially, the animals were trained on a fixed ratio (FR) reinforcement schedules. First, FR-1 schedule was employed, where each response on the active lever resulted in a delivery of gelatin, while responses on the inactive lever were recorded but had no scheduled consequences. When reliable responding on the FR-1 schedule was achieved (10% day to day variation), the reinforcement schedule was increased to FR-5. When stable responding and pharmacologically significant consumption levels (0.8 g/kg) had been established, the drug studies were started.

Each drug study was divided into four key days: basal, experimental, first day post experimental and second day post experimental. The basal day represented the normal consumption of the rats. Experimental day showed the consumption levels during drug administration. The first and second post experimental days determine if there were any drug-induced long lasting effects on consumption. This four day cycle was then repeated multiple times using a latin square design to test multiple drug treatments.

Before each new cycle of drug treatments we waited for the animals to reach baseline consumption.

Microdialysis Experiments

Each rat was anesthetized with isoflurane and placed in a stereotaxic instrument for implantation of a guide canulla. The guide canulla was anchored with two stainless steel screws and dental cement. The following coordinates from bregma were used for implantation: NAc +1.8 anteroposterior, +1.3 lateral, -6.2 dorsoventral, striatum +0.8 anteroposterior, +3.0 lateral, -4.0 dorsoventral. After implantation, animals were given at least 2 days to recover before either resuming operant sessions or undergoing microdialysis testing.

The technique used to gather extracellular neurotransmitter concentrations was microdialysis coupled with capillary electrophoresis separation using laser-induced fluorescence detection and has been described previously (Bowser and Kennedy, 2001; Li et al., 2008). On the experiment day, a standard calibration curve (0 to 20 μM) was first performed using a microdialysis probe with outer diameter 270 μm, 13,000 molecular weight cutoff, and variable active length (2 to 4 mm) depending on the brain region being dialysed. The probe was placed in standard solutions kept at 37 degrees Celsius. A standard curve was then produced relating the relative fluorescence to the known μM concentrations. After calibration, the probe was implanted in a non-anesthetized and freely-moving rat. The experiment began after neurotransmitter concentrations reached steady-state which was typically 2 hours after implantation. The substances measured included glutamate, aspartate, serotonin, dopamine, ornathine, GABA, taurine, glutamine, serine, and glycine.

The perfusion setup is described in Chapter 5. It allowed for determining the effect of drug reverse dialysis on both basal and stimulated neurotransmitter release. Basal effects were discovered during the 30 minute pretreatment period before the second potassium stimulation. Changes in 50 mM K+-stimulated neurotransmitter release were measured by comparing the first stimulation to the drug treated second stimulation. The third stimulation serves as a control for instrument sensitivity and should not be significantly different from the first stimulation.

Drugs

PAT compounds were synthesized at Dr. Booth's lab in the Department of Medicinal Chemistry at College of Pharmacy, University of Florida. Ro60-0175 ((S)-2-(chloro-5-fluoro-indol-1-yl)-1-methylethylamine 1:1 C4H4O4) and SB242,084 (6-chloro-5-methyl-1-[2-(2-methylpyridyl-3-oxy)-pyrid-5-yl carbomyl]indoline) were purchased from Tocris Bioscience. Ro60-0175 was dissolved in 0.9% saline. SB242,084 was prepared in 0.9% saline solution containing 8% hydroxypropyl-b-cyclodextrin and 25 mM citric acid. All drug doses are expressed as that of the salt. Gelatin and polycose were purchased from Knox brand, Kraft Foods and Abbott Laboratories respectively.

Statistical Analysis

Operant data was analyzed by one or two way repeated measures ANOVA using SPSS software. Post hoc comparisons were carried out with Bonferroni test. In all cases, the accepted level of significance was taken at $p<0.05$.

Microdialysis data analysis was performed using Lab View to determine the peak height for each measured amino acid and neurotransmitter. There was one of these data points for each analyte every 15 seconds during the experiment. The peak heights for each analyte were changed to μM concentration and then graphed over time. Data

points from specific time periods, such as during high K+ stimulations, were examined using area under the curve, AUC. Basal concentration of neurotransmission was determined by finding the average concentration for the 5 minutes before the time period of interest. AUC was determined by subtracting the basal value from the data points during the 10 minute long time period of interest. This results in the AUC value for the time period of interest. This AUC value can then be compared other AUC values in the same experiment using one-way ANOVA to determine if the AUC values are different from each other.

CHAPTER 3
ROLE OF $5HT_{2C}$ MODULATION ON VOLUNTARY ETHANOL INTAKE MEASURED USING OPERANT CONDITIONING

Rational For These Studies

Alcoholism is a disorder that involves changes of neurotransmitter release in the central nervous system. Ethanol-induced changes in dopamine in the nucleus accumbens and related brain areas have been widely studied (Salamone and Correa, 2002) and it is clear that dopamine plays a significant role in alcoholism (Sulzer, 2011). Serotonergic drive, via 5-HT_2 receptor family, is able to modulate dopamine release in brain regions associated with addiction. Agonism of the 5-HT_{2C} receptor has been shown to decrease voluntary ethanol consumption in rats (Tomkinset al., 2002). However, agonism of the 5-HT_{2A} receptor causes psychotomimetic effects (Fitzgerald et al., 2000). There are currently no specific agonists for the 5-HT_{2C} receptor available due to the high transmembrane sequence homology between the two receptor subtypes. However, the novel (-)-trans-PAT series of compounds have several drugs that exhibit functional selectivity; they act as agonists at 5-HT_{2C} and antagonists at 5-HT_{2A}. These drugs represent a possible pharmacotherapy for alcoholism.

The “jello shot” model of alcoholism is a recently established model of ethanol self-administration (Rowland et al., 2005). It models human alcohol intake through voluntary oral consumption of ethanol. Rats will bar press for a reinforcement consisting of 0.15 grams of a 10% ethanol containing gelatin. This model results in physiologically relevant doses of ethanol self-administered without the need for food/water restriction (Peris et al., 2006). The rats voluntarily consume the human equivalent of approximately 1.2 drinks or 0.8g/kg. This model is well-suited for testing drugs that affect voluntary ethanol self-administration because the large physiologically

relevant doses of ethanol self-administered allow for the visualization of pharmacotherapy induced decreases in ethanol consumption. Prior to the daily operant session, drug is administered and changes in daily consumption observed. An important strength of this model is that experiments can then be repeated using gelatin with no alcohol content as a reinforcer to determine if the drug's effect is specific for ethanol. If a drug decreased both ethanol-containing and ethanol-free gelatin consumption then it can be concluded that the drug has nonspecific effects on overall consumption (e.g. sedation, appetite suppression). The "jello shot" model of voluntary ethanol intake is a good model to test if drugs alter voluntary, physiologically relevant, ethanol consumption that is specific for the ethanol itself. Thus, this model was used to assess the effects of PAT and its analogs on voluntary ethanol consumption.

For comparison purposes, we also chose to test some commercially available 5-HT_2 ligands. Currently, the most specific 5-HT_{2C} receptor agonist commercially available is Ro60-0175. It has a modest 30-fold selectivity for activating the 5-HT_{2C} receptor over the 5-HT_{2A} receptor. This makes it difficult to attribute the behavioral effects of Ro60-0175 to just one receptor. Addition of a specific 5-HT_{2C} antagonist will be required to ensure Ro60-0175 has its action solely via agonism of 5-HT_{2C} receptors.

The 5-HT_{2C} receptor antagonist SB242,084 will be used to confirm the role that 5-HT_{2C} receptor agonists play. SB242,084 has 150 fold selectivity for the 5-HT_{2C} receptor over the related 5-HT_{2A}. By administering this compound and blocking the 5-HT_{2C} receptors before an agonist is administered, it is possible to then determine if the effects of the 5-HT_{2C} agonist are due to activity at the 5-HT_{2C} receptor.

Methods

Animals And Housing

Female Sprague-Dawley rats (90 days old) weighing approximately 275 g at the start of the studies, were individually housed in plastic cages with food and water available *ad libitum* throughout the study except for the time spent in the operant chambers. Female rats were used since they maintain stable body weight and stable ethanol consumption over several months (Li et al., 2010). They were maintained on a 12-h light/dark cycle in an environmentally controlled room (lights on at 6:00 am, temperature: 23±3 C, humidity: 45±25 %) and allowed a 1-week acclimatization period to the animal facilities prior to the start of the studies.

Ethanol Self-Administration Training

As described in Chapter 2 General Methods.

Fixed Ratio Studies

Effects of Ro60-0175 and the selective 5-HT_{2C} receptor antagonist, SB242,084 on ethanol gel self-administration and plain gel consumption

The rats (n = 10) were initially trained to consume ethanol as outlined above. Animals received once daily IP (30 min prior) and SC (50 min prior) vehicles injections prior to drug treatments to acclimate the rats to injections. When the total number of reinforcements response pattern had stabilized, the effects of the different drug treatments on ethanol self-administration behavior were evaluated. Four treatments were implemented: Ro60-0175 at two doses (0.5 and 1 mg/kg sc, 30 min pretreatment), SB242,084 (0.5 mg/kg ip, 50 min pretreatment) in combination with Ro60-0175 (0.5 mg/kg sc, 30 min pretreatment) and SB242,084 (0.5 mg/kg ip, 50 min pretreatment) alone. A Latin square design was employed such that each animal received each dose/dose combination in a balanced order. Each treatment day was

separated from the next by at least 3 days. Following the data analysis of this study, a second experiment was conducted to determine the effects of the same four treatments on plain gel consumption as a control for the first experiment. When the response pattern for plain gel had stabilized, the effects of the different drug treatments on plain gel consumption were evaluated in a similar manner.

Effect of the 5-HT_{2C} agonist and 5-HT_{2A} antagonist, PAT on ethanol gel self-administration and plain gel consumption

The rats (n=10) were switched back to ethanol gel and the consumption level was stabilized. The effect of (-)-trans PAT (0.5, 1, 5 and 10 mg/kg ip, 20 min pretreatment) and (+)-trans PAT (10 mg/kg ip, 20 min pretreatment) on ethanol self-administration behavior was evaluated. A Latin square design was employed such that each animal received each dose in a balanced order. Each treatment day was separated from the next by at least 3 days. Following the data analysis of this study, a second experiment was conducted to determine the effects of the same PAT doses on plain gel consumption as a control for the first experiment. When the response pattern had stabilized, the effects of the different drug treatments on plain gel consumption were evaluated in a similar manner.

Progressive Ratio Studies

Rats (n=10) were divided into two groups and placed on a PR-10 schedule of responding for ethanol containing gel. After a 2 week stabilization (three consecutive days of gel deliveries ± 1 from previous day), rats were administered either 5 or 10 mg/kg (-)-trans-PAT 20 minutes prior to the operant session. Breakpoint and consumption data were compared from the experimental day and the two following session days to determine the effect of (-)-trans-PAT.

Drugs

PAT compounds were synthesized in the laboratory of Dr. Raymond G. Booth in the Department of Medicinal Chemistry, College of Pharmacy, University of Florida. Ro60-0175 ((S)-2-(chloro-5-fluoro-indol-1-yl)-1-methylethylamine 1:1 C4H4O4) and SB242,084 (6-chloro-5-methyl-1-[2-(2-methylpyridyl-3-oxy)-pyrid-5-yl carbomyl]indoline) were purchased from Tocris Bioscience. Ro60-0175, (+)-trans-PAT and (-)-trans-PAT was dissolved in 0.9% saline. SB242,084 was prepared in 0.9% saline solution containing 8% hydroxypropyl-b-cyclodextrin and 25 mM citric acid. All drug doses are expressed as that of the salt. Gelatin and polycose were purchased from Kraft Foods and Abbott Laboratories respectively.

Statistical Analysis

Data were analyzed by one or two way repeated measures ANOVA using SPSS software. Post hoc comparisons were carried out using t-test with Bonferroni correction. In all cases, the accepted level of significance was taken at $p < 0.05$.

Results

Ro60-0175 Attenuates And SB242,084 Enhances Fixed Ratio Operant Responding For Ethanol-Containing Gelatin

Average basal consumption was 8.5 ± 2.1 (average ± standard error of the mean) grams of ethanol-containing gelatin per kilogram of rat (equal to 0.85 g/kg ethanol). ANOVA testing indicated a significant effect of days ($F(3,36) = 6.6$, $p<0.01$), drug treatment ($F(3,36) = 6.3$, $p<0.01$), and interaction between days and drug treatment ($F(9,144) = 9.6$, $p<0.001$). This indicates that the effect of the different drug treatments is changing over days of the experiment. Ro60-0175, the 5-HT_2 agonist, caused a significant decrease of daily voluntary ethanol containing gel consumption at both 0.5

and 1.0 mg/kg, changing ethanol-containing gelatin consumption to 6.1 ± 1.1 g/kg and 0.86 ± 0.31 g/kg respectively (Figure 3-1). The 5-HT_{2C} antagonist SB242,084 significantly increased ethanol-containing gel consumption to 13 ±1.9 g/kg. When both SB242,084 and Ro60-0175 are administered, there is no significant change, 11 ±1.7 g/kg, in ethanol-containing gelatin consumption.

Then Ro60-0175 and SB242,084 were tested on operant responding for plain gel and basal consumption was 18 ± 2.6 g/kg. ANOVA testing indicated a significant effect of days ($F(3,36) = 5.0$, $p<0.01$), drug treatment ($F(3,36) = 7.0$, $p<0.01$), and interaction between days and drug treatment ($F(9,144) = 11.5$, $p<0.001$). Ro60-0175 caused a significant decrease in plain gel consumption at both 0.5 and 1.0 mg/kg, to 10 ± 2.2 g/kg and 7.5 ± 1.5 g/kg (Figure 3-2). The 5-HT_{2C} antagonist SB242,084 had no effect on plain gel consumption with an average consumption of 23 ±3.5 g/Kg. When both SB242,084 and Ro60-0175 were administered in combination there was no significant change, 22 ±2.0 g/kg, in ethanol containing gel consumption.

(-)-trans-PAT Attenuates Fixed Ratio Operant Responding For Ethanol Containing Gelatin

The average ethanol-containing gelatin consumption for this experiment was 12 ± 2.2 g/kg. Three-way ANOVA testing showed a significant effect of days ($F(3,36) = 2.6$, $p<0.05$) and an interaction between days and drug treatment ($F(3,144) = 2.6$, $p<0.05$) but no significant effect of drug treatment alone ($F(3,36) = 2.6$, $p=0.07$). The 5-HT_{2C} agonist, 5-$HT_{2A/B}$ antagonist/inverse agonist (-)-trans-PAT caused a dose-dependent decrease in ethanol containing gel consumption (Figure 3-3). (-)-trans-PAT at 5 mg/kg was the lowest dose to cause a significant decrease from basal to 8.1 ± 1.5 g/Kg. In addition to overall ethanol consumption, the pattern of ethanol consumption was altered

by 5 mg/kg (-)-trans-PAT as well (Figure 3-4). The pattern of ethanol consumption demonstrated a significant main effect of bins ($F(10,48) = 3.2$, $p<0.001$) and a significant interaction between days and bins ($F(20,144) = 1.1$, $p<0.001$). Post hoc analysis determined (-)-trans-PAT reduced ethanol consumption during the 10-20 minute periods of the operant session when compared to basal.

Average plain gel consumption while testing (-)-trans-PAT was 22 ± 1.8 g/kg. ANOVA testing indicated no significant effects of days ($F(3,36) = 1.6$, $p=0.22$), drug treatment ($F(3,36) = 1.1$, $p=0.36$), or interaction between days and drug treatment ($F(9,144) = 0.38$, $p=0.94$). (-)-trans-PAT did not alter plain gel consumption at any dose (Figure 3-5).

When looking at the effect of (+)-trans-PAT administered at 10 mg/kg on both ethanol and plain gelatin consumption one-way ANOVA indicates there is a significant effect of days ($F(3,28) = 7.4$, $p<0.001$) during ethanol gelatin consumption while testing (+)-trans-PAT. Likewise, there was a significant effect of days ($F(3, 20) = 4.9$, $p<0.05$) during plain gel consumption in the presence of (+)-trans-PAT. (+)-trans-PAT decreased both ethanol-containing and plain gel consumption at 10 mg/kg (Figure 3-3, 3-5).

(-)-trans-PAT Alters Consumption Pattern And Total Consumption During Progressive Ratio Schedules

The average breakpoint of these rats responding for 0.15 g of ethanol gel on a PR10 schedule was 37 ± 10. ANOVA testing on breakpoints for (-)-trans-PAT at both doses indicate no significant effect of days ($F(2,12) = 1.1$, $p=0.32$), drug treatment ($F(2,12) = 0.82$, $p=0.34$), or interaction between days and drug treatment ($F(6,24) = 0.53$). (-)-trans-PAT did not alter overall consumption at either 5 or 10 mg/kg (Figure 3-6).

However, when the deliveries were binned into 5 minute intervals across the 30 minute operant session, ANOVA of 10 mg/kg (-)-trans-PAT on ethanol gelatin revealed a main effect of bins ($F(5,24) = 7.8$, $p<0.05$) and a significant interaction between days and bins ($F(10,72) = 2.1$, $p<0.05$). 10 mg/kg (-)-trans-PAT decreased basal consumption between 5 and 10 minutes from 2.2 ± 0.73 to 0.80 ± 0.22 (Figure 3-7). In the (-)-trans-PAT 5 mg/kg treated group (Figure 3-8), there was a significant main effect of bins ($F(5,24) = 8.5$ $p<0.001$), but no main effect or interaction involving treatment days. (-)-trans-PAT administered at 5 mg/kg had no effect on consumption patterns during PR10 responding for ethanol gelatin.

When the reward size was increased to 0.28 g on a PR10, average breakpoints for ethanol-containing gelatin (65.3 ± 11.6) and plain gelatin (67.1 ± 16.3) were similar (Figure 3-9, 3-10). At 1.0 mg/kg, Ro-60-0175 decreased both ethanol containing (68.3 ± 11.1% of basal) (Figure 3-9) and plain (79.2 ± 4.8% of basal) (Figure 3-10) gelatin breakpoints, though it did not have significant effects at a dose of 0.5 mg/kg. However, at 10 mg/kg, (-)-trans-PAT decreased only ethanol-containing gelatin (70.4 ± 4.1% of basal) (Figure 3-9) but not plain gelatin (106.3 ± 7.2% of basal) (Figure 3-10). ANOVA results demonstrated significant effects of days ($F(1,32) = 4.3$, $p = 0.048$), days by drug treatment ($F(3,13) = 19.0$, $p = 0.001$), days by gelatin type ($F(1,64) = 10.2$, $p = 0.004$), and days by drug treatment by gelatin type ($F(3,52) = 3.7$, $p = 0.025$).

Discussion

These results show that modulation of 5-HT_2 subfamily of receptors can alter voluntary consumption of both plain and ethanol-containing gel. The 5-HT_{2C} agonist Ro60-0175 has been shown to decrease ethanol consumption in another model of alcohol consumption (Tomkins et al., 2002) and the present experiments have

replicated that result using the “jello shot” model of alcohol consumption on a FR5 schedule. The Ro60-0175 induced decrease in plain gelatin consumption was also expected because the effective dose for decreasing ethanol consumption is close to the effective dose for decreasing food intake (Higgins and Fletcher, 2003). Ro60-0175’s nonspecific decrease in both ethanol and plain gelatin consumption makes it difficult to determine the effect that Ro60-0175 has specifically on ethanol consumption. Unfortunately, the 5-HT_{2C} antagonist SB242,084 increased ethanol gel consumption making the combination of SB242,084 and Ro60-0175 difficult to interpret. While the affinity and functional profile of Ro60-0175 and SB242,084 suggest that the decreased/increased ethanol consumption are 5-HT_{2C} mediated, this cannot be confirmed. It should be noted that while SB242,084 demonstrates >100 fold affinity preference for the 5-HT_{2C} receptor (Table 1-1), SB242,084 also acts on other 5-HT, dopamine and adrenergic receptors (Kennett et al., 1997). It is possible that the nonspecific decrease in gelatin consumption was due to Ro60-0175 acting on and SB242,084 blocking a different variant of the 5-HT_{2C} receptor. This theory is supported by the fact that (+)-trans-PAT decreased both ethanol and plain gelatin. Comparing (+)-trans-PAT to (-)-trans-PAT in terms of affinity and function at 5-HT_{2A} and 5-HT_{2C} receptors (Table 1-1) it is obvious that (-)-trans-PAT should outperform (+)-trans-PAT at these receptors. Further tests are needed to confirm what mediates the (+)-trans-PAT nonspecific effects on gelatin consumption.

However, (-)-trans-PAT caused an ethanol specific decrease in consumption on a FR5 schedule that was dose dependent. This was in agreement with previous studies observing that 5-HT_{2C} agonism leads to decreased ethanol consumption (Tomkins et

al., 2002). It was unexpected that (-)-trans-PAT did not alter plain gel consumption at any of the doses tested because of previous studies that showed (-)-trans-PAT decreasing food “treat” intake of mice starting at 4.2 mg/kg (Rowland et al., 2008). While it is unknown if higher doses of (-)-trans-PAT will alter plain gel consumption, it is possible that the dose response curves for (-)-trans-PAT on ethanol intake and food intake are farther apart than those dose response curves for Ro60-0175. This could be attributed to (-)-trans-PAT activating second messenger systems, PLC vs PLA2, in a different manner than Ro60-0175. Regardless of the mechanism of action, (-)-trans-PAT specificity to reduction of ethanol containing gelatin assures us that the treatment is specific to ethanol itself, and not due to nausea, sedation, or otherwise unable to press the bar.

(-)-trans-PAT had no effect on overall breakpoints in the PR10 studies at either 5 or 10 mg/kg but did alter the consumption pattern with 0.15 g reward size. With 0.28 g of gel as a reward, Ro60-0175 decreased both ethanol and plain gelatin breakpoints. However, (-)-trans-PAT only decreased the breakpoints for ethanol gelatin and not plain gelatin. This specific change in ethanol breakpoints signifies that (-)-trans-PAT alters the value of the ethanol to the rat. (-)-trans-PAT decreases rat’s value of ethanol while Ro60-0175 decreases the value of both plain and ethanol containing gelatin.

During PR10 with 0.15 g reward and 10 mg/kg (-)-trans-PAT there was a decrease in consumption on the experimental day between 10 and 15 minutes. This change in consumption pattern hints that (-)-trans-PAT is having an effect but it was not great enough to cause changes in overall breakpoints.

One aspect that stands out in the PR10 experiments is the relatively low basal breakpoints, 37 ± 10, for 0.15 g of gelatin compared to breakpoints previously reported with this model, 52 ± 3.2, using 0.28 g rewards (Li et al., 2010). As reinforcement size increases there is a tendency for breakpoints in increase as well (Hoffmeister, 1979). The breakpoints using 0.15 g reward may be so low that there is a floor effect and they are unable to be decreased by (-)-trans-PAT. Increasing the reward size allows the visualization of both Ro60-0175 and (-)-trans-PAT decrease in breakpoints.

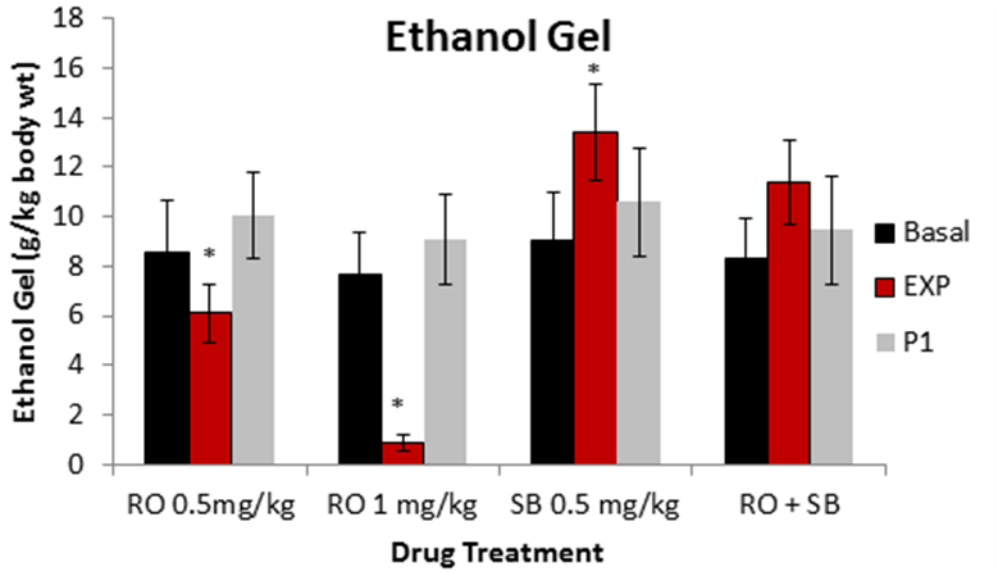

Figure 3-1. Ro60-0175 decreases and SB242,084 increases voluntary ethanol gel consumption in rats. Black columns represent the average consumption before experimental day, red represents consumption after drug administration, and grey represents consumption the day after drug administration. Shown are mean values and SEMs for N = 10. * indicates $p<0.05$ compared to basal.

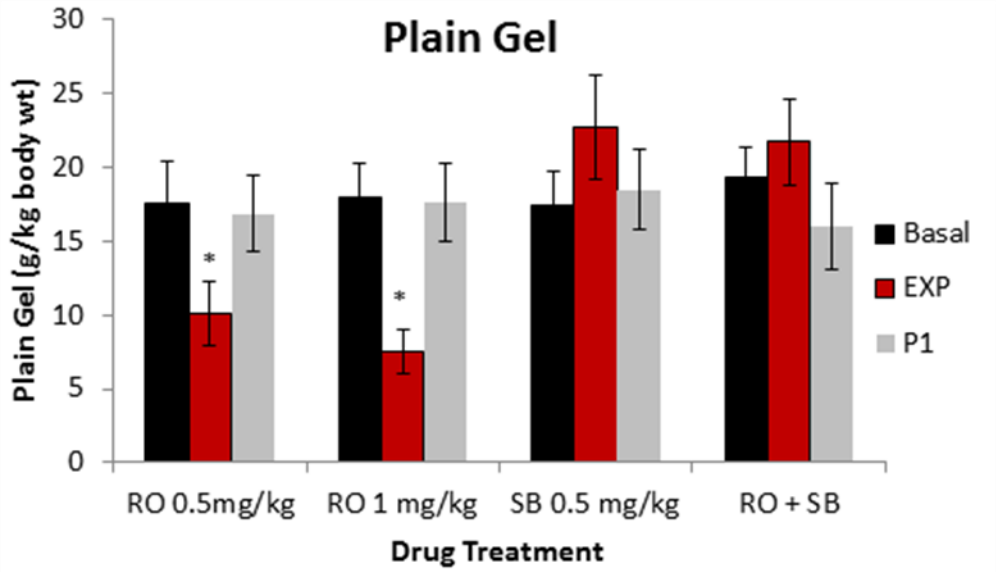

Figure 3-2. Ro60-0175 decreases voluntary plain gel consumption and SB242,084 has no significant effect. Black columns represent the average consumption before experimental day, red represents consumption after drug administration, and grey represents consumption the day after drug administration. Shown are mean values and SEMs for N = 10. * indicates $p<0.05$ compared to basal.

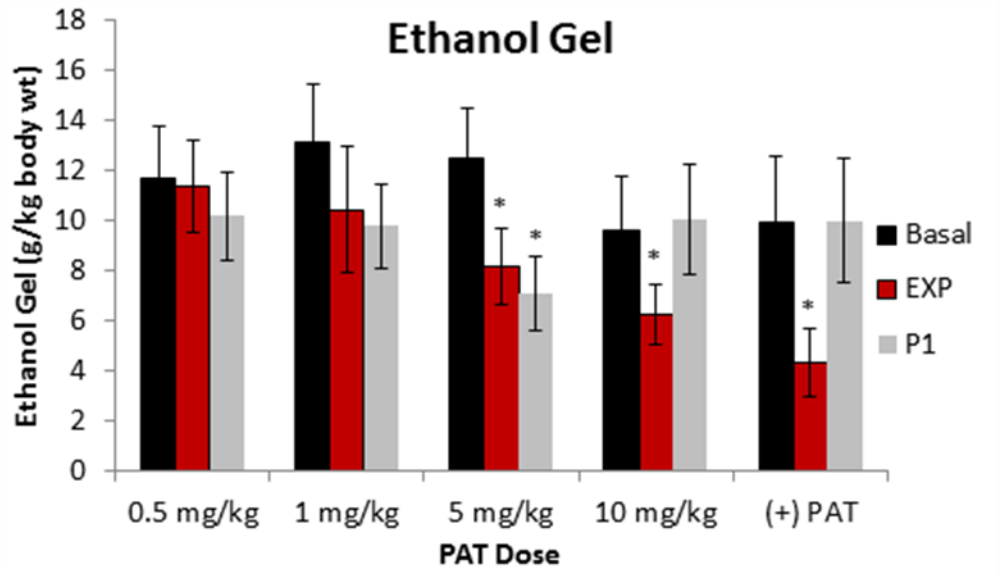

Figure 3-3. (-)-trans-PAT decreases voluntary ethanol containing gel consumption in a dose dependent manner and (+)-trans-PAT also decreases consumption at 10 mg/kg. Black columns represent the average consumption before experimental day, red represents consumption after drug administration, and grey represents consumption the day after drug administration. Shown are mean values and SEMs for N = 10. * indicates $p<0.05$ compared to basal.

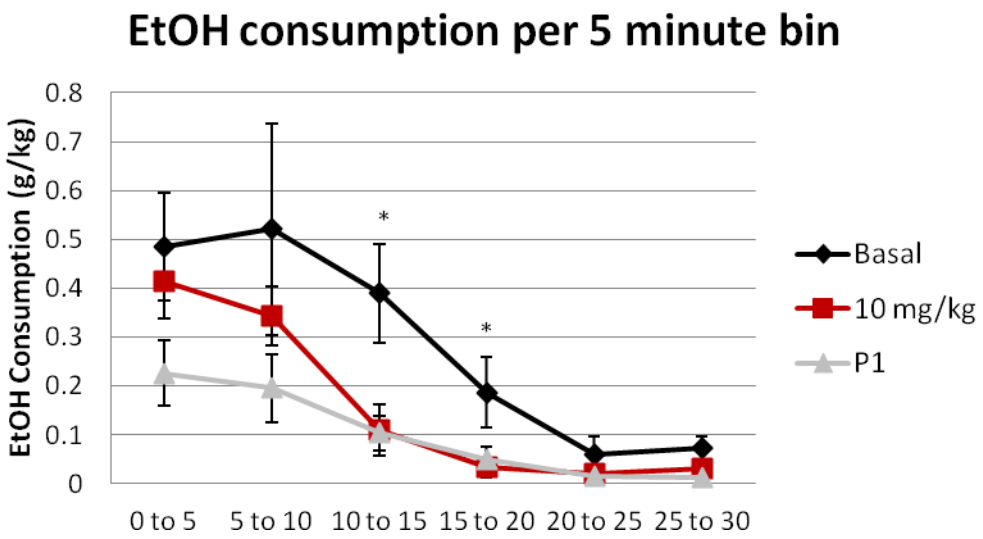

Figure 3-4. (-)-trans-PAT at 5 mg/kg reduced basal consumption patterns during a FR5 operant session. Shown are mean values and SEMs for N = 10. * indicates experimental day has $p<0.05$ compared to basal bin value.

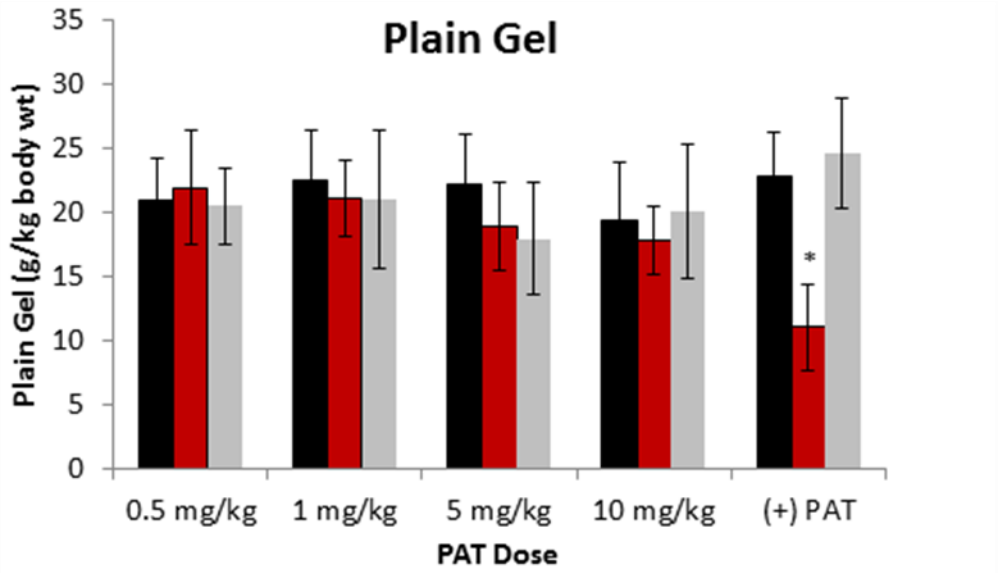

Figure 3-5. (-)-trans-PAT has no effect on voluntary plain gel consumption but (+)-trans-PAT decreases consumption at 10 mg/kg. Black columns represent the average consumption before experimental day, red represents consumption after drug administration, and grey represents consumption the day after drug administration. Shown are mean values and SEMs for N = 10. * indicates $p<0.05$ compared to basal.

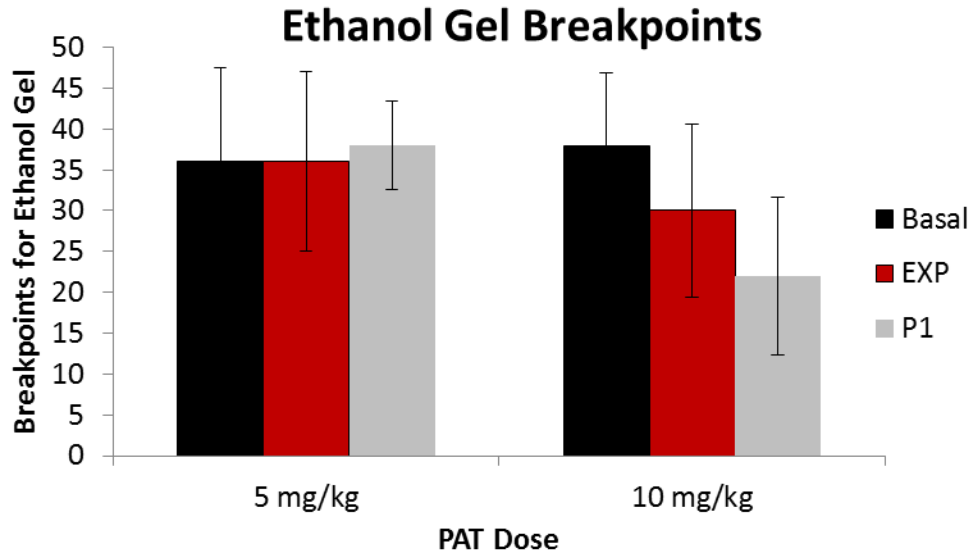

Figure 3-6. (-)-trans-PAT has no effect on breakpoints for 0.15 g gelatin under a PR10 schedule. Shown are mean values and SEMs for N = 5. * indicates $p<0.05$ compared to basal.

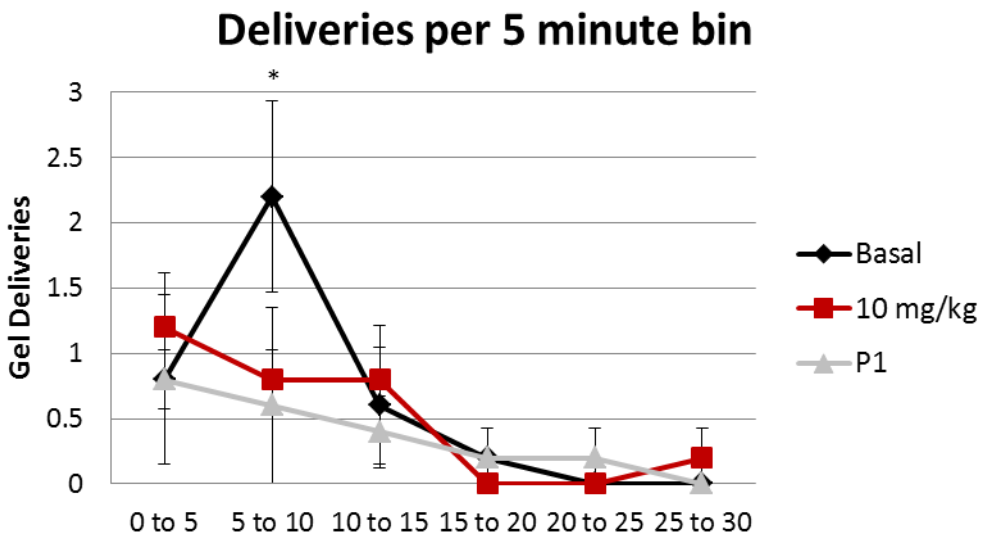

Figure 3-7. (-)-trans-PAT at 10 mg/kg alters gel deliveries between 5 and 10 minutes into the PR10 operant session for 0.15 g of ethanol containing gelatin. Shown are mean values and SEMs for N = 5. * indicates $p<0.05$ compared to basal bin value.

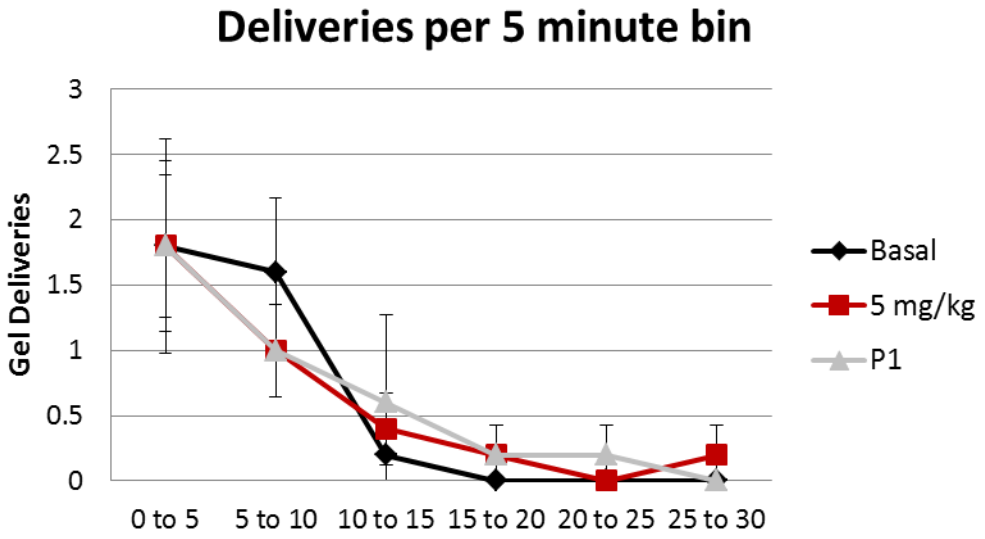

Figure 3-8. (-)-trans-PAT at 5 mg/kg has no effect on consumption patterns during a PR10 operant session for 0.15 g of ethanol containing gelatin. Shown are mean values and SEMs for N = 5. * indicates $p<0.05$ compared to basal bin value.

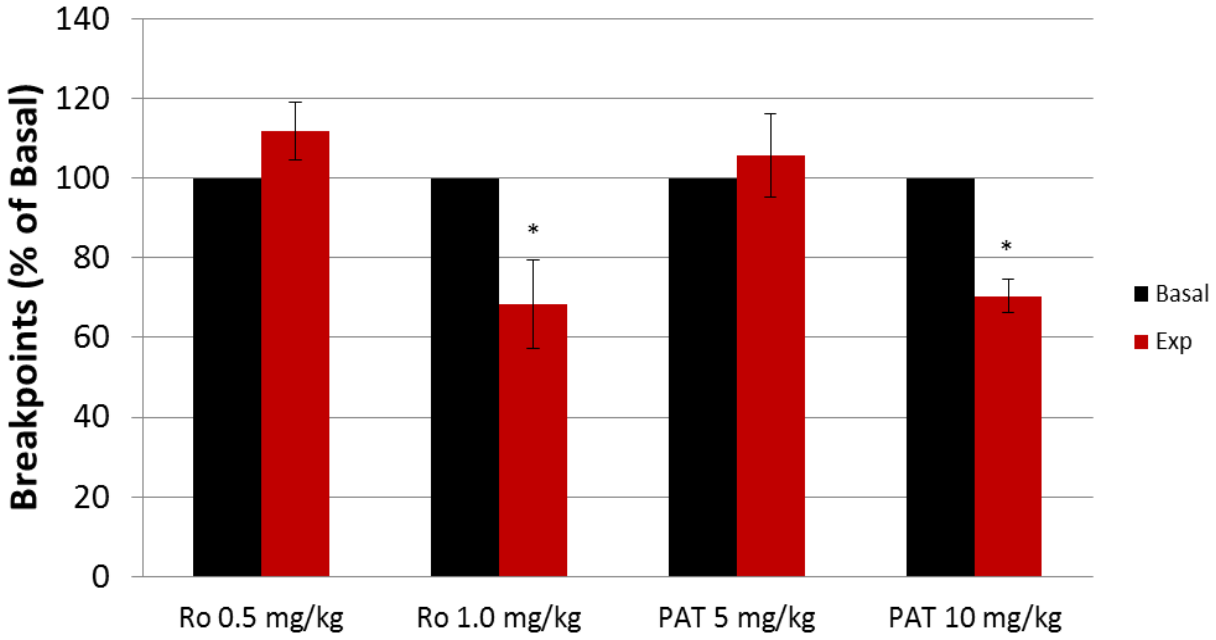

Figure 3-9. Ro60-0157 and (-)-trans-PAT at 1 and 10 mg/kg has decreased ethanol breakpoints during PR10 operant session responding for 0.28 g of gelatin reward. Shown are breakpoints expressed as a percentage of the basal breakpoint responding with N = 4-5. * indicates $p<0.05$ compared to basal.

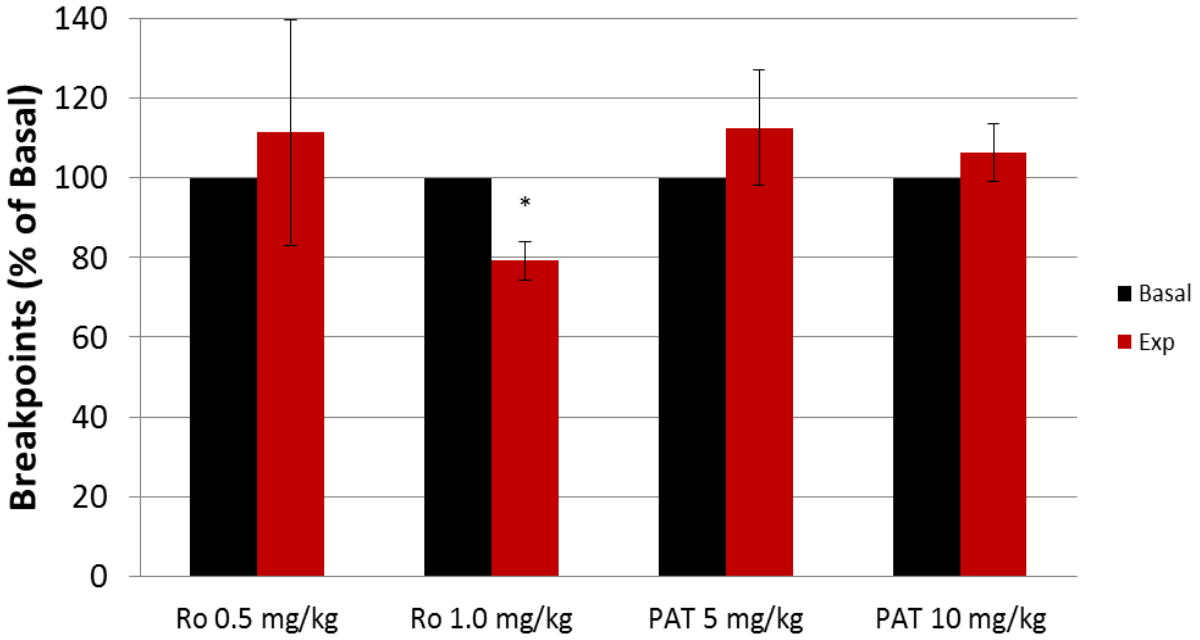

Figure 3-10. Ro60-0157 at 1 mg/kg but not (-)-trans-PAT decreased plain gelatin breakpoints during PR10 operant session responding for 0.28 g of gelatin reward. Shown are breakpoints expressed as a percentage of the basal breakpoint responding with N = 4-5. * indicates $p<0.05$ compared to basal.

CHAPTER 4
PAT INHIBITS THE ALCOHOL DEPRIVATION EFFECT

Rationale For Experiment

The Alcohol Deprivation Effect (ADE) is a model of relapse in recovering alcoholics. Relapse in alcoholism is characterized by compulsive alcohol seeking and consumption. Alcohol deprivation induced craving is a tremendous difficulty for alcoholics (Martin-Fardon and Weiss, 2012). In animals, when alcohol is reintroduced following a period of abstinence, consumption temporarily increases compared to previous basal consumption (Rodd, et al. 2009). This can be tested using operant chambers by removing the ethanol from the reward for days or weeks. When the animals are once again allowed to respond for ethanol reward, they display the increased ethanol consumption indicative of the ADE. In rats both acamprosate and naltrexone decrease aspects of the ADE singularly and when administered together (Heyser et al., 2003). The aim of this study was to test novel 5-HT_{2C} agonists that could decrease the severity and duration of the ADE.

Methods

Animals And Housing

Female Sprague-Dawley rats (90 days old) weighing approximately 275 g at the start of the studies, were individually housed in plastic cages with food and water available *ad libitum* throughout the study except for the time spent in the operant chambers. Female rats were used since they maintain stable body weight over several months. They were maintained on a 12-h light/dark cycle in an environmentally controlled room (lights on at 6:00 am, temperature: 23±3 C, humidity: 45±25 %) and allowed a 1-week acclimatization period to the animal facilities prior to the start of the studies.

Ethanol Self-Administration Training

Ethanol self-administration was established by using sweetened gelatin vehicle (jello shots) containing ethanol (10% Polycose, 10% ethanol, 0.25% gelatin, all by weight, in water). This method sustains fairly high self-administration levels in Sprague-Dawley rats without the need for food or water restriction (Peris et al., 2006). Ethanol self-administration training consisted of two stages: free access training and operant conditioning. First during free access, the animals had 24-h home cage access to 10% ethanol-containing gel for 2 days followed by 6-h and 3-h access periods per day for 2 days each. After this, animals had a daily one hour access period for 11 days. Next, operant training was initiated. Operant training was conducted in five operant chambers. Each chamber was housed in a sound-attenuating box equipped with a house light that illuminates with each delivery. Each chamber contained a gel dispenser calibrated to deliver 0.15 g of gel into a recessed dish positioned between the two levers. The apparatus was controlled by a microcomputer interface linked to a DELL computer using Graphic state software to record and manage the data. The rats were placed daily in the operant boxes for 30 minutes and trained to press the active lever to get the reinforcement. To encourage the association of the active lever with gel delivery, 0.3 g of gel was placed on the active lever before each rat's first operant session along with a single delivery of gel coming from the spout. Initially, the animals were trained on a fixed ratio (FR) reinforcement schedules. First, FR-1 schedule was employed, where each response on the active lever resulted in a delivery of gelatin, while responses on the inactive lever were recorded but had no scheduled consequences. When reliable responding on the FR-1 schedule was achieved (10% day to day variation), the reinforcement schedule was increased to FR-5. When stable

responding and pharmacologically significant consumption levels (0.8 g/kg) had been established, the drug studies were started.

Drug Studies

The “jello shot” model of inducing voluntary ethanol self-administration (Peris, et al. 2006) was used to habituate alcohol-naïve female Sprague-Dawley rats to freely consume ethanol without need for food or water restrictions. The “jello shot” contains 10% ethanol in gelatin with a glucose-polymer caloric source (polycose). After tapering of free-access sessions from 24-hr to 1-hr (Peris et al., 2006), animals were introduced to an operant-responding environment and incrementally trained to bar-press for gel deliveries during a 30-min session. A fixed-ratio schedule, increased from FR-1 to FR-5, regulated the amount of effort required for each delivery. This mechanism allowed for accurate determination of consumption levels surrounding periods of alcohol availability and deprivation.

Animals were then divided into 4 groups (N = 6 for each group): the non-deprived saline control group, the non-deprived 5 mg/kg PAT group, the alcohol deprived saline control group and the alcohol deprived 5 mg/kg PAT group. Animals were habituated to once daily saline injections prior to operant responding to control for the effect of injections on operant responding. The experimental timeline contained 10 days of all groups responding for ethanol gelatin to establish baseline consumption for each group. Then the alcohol deprived animals responded for plain gelatin for 3 weeks while the non-deprived animals continued to respond for ethanol gelatin. After the 3 week deprivation (or continued daily ethanol access), all animals responded for ethanol gelatin again and this day is called the reinstatement day. The reinstatement day also marks the day where two groups of animals received PAT injection 20 min prior to the

start of the operant session while the other two groups continued to receive daily saline. All four groups continued responding for ethanol gel for 15 days after reinstatement to observe the ADE induced changes in ethanol consumption return to basal. After that the 5-week long experiment was repeated again with (-)-trans-m-Br-PAT instead of (-)-trans-PAT. Animals that were non-deprived in the (-)-trans-PAT experiment remained non-deprived during the (-)-trans-m-Br-PAT experiment and likewise for the deprived animals. However, to not bias our study to the animals' drug history, half of all animals in each group had previous 5 mg/kg (-)-trans-PAT administration and the other half were drug naive.

Drugs

PAT compounds were synthesized at Dr. Booth's lab in the department of Medicinal Chemistry at College of Pharmacy, University of Florida. All drug doses are expressed as that of the salt. Gelatin and polycose were purchased from Kraft Foods and Abbott Laboratories respectively.

Statistical Analysis

Data were analyzed by one or two way repeated measures ANOVA using SPSS software. Post hoc comparisons were carried out using T-test with Bonferroni correction. In all cases, the accepted level of significance was taken at $p < 0.05$.

Results

(-)-trans-PAT Alters The ADE

The "jello shot" model of alcoholism produced a stable baseline of ethanol containing gelatin consumption 1.2 ± 0.37 g/Kg. Animals were then divided into four groups, N = 6, and half were deprived of daily ethanol by switching the deprived animals to operant responding for plain gelatin. These animals were deprived for 3 weeks while

the other two groups received daily ethanol gelatin operant sessions. After 3 weeks, the deprived animals were switched back to ethanol gelatin. On that day of reinstatement, one of the deprived and one of the non-deprived groups were administered 5 m/kg (-)-trans-PAT. Both the ethanol deprivation and administration of (-)-trans-PAT influenced the consumption of ethanol. Three-way ANOVA indicates that there were significant main effects of deprivation ($F(1,11) = 24$, $p<0.05$), drug treatment ($F(1,11) = 1800$, $p<0.001$), days ($F(4,25) = 36$, $p<0.05$). All interactions were also significant, including deprivation and drug ($F(3,20) = 34$, $p<0.05$), deprivation and days ($F(9,110) = 21$, $p<0.05$), drug treatment and days ($F(9,110) = 23$, $p<0.05$), and deprivation and drug treatment and days ($F(19,230)$ 7.1, $p<0.05$).

(-)-trans-PAT decreases voluntary consumption of ethanol containing gel in non-deprived rats

Looking at the non-deprived rats, (-)-trans-PAT decreased ethanol consumption to 0.69 ± 0.03 g/kg on the experimental day while saline control animals consumed 1.3 ± 0.32 g/kg (Figure 4-1). Follow up post-hoc comparisons indicated that among the non-deprived animals, there was a significant effect of drug treatment ($F(1,5) = 190$, $p<0.001$) and interaction between days and drug treatment ($F(9,110) = 9.2$, $p<0.05$), but not days alone ($F(4,19) = 3.2$, $p=0.054$). Bonferroni post-hoc tests showed that the only significant difference between non-deprived (-)-trans-PAT and saline treated animals was on the experimental day.

(-)-trans-PAT prevents the ethanol deprivation effect in deprived rats

The ADE is an increase in alcohol consumption after a period of abstinence. ANOVA testing of the ethanol deprived ethanol consumption showed a significant effect of drug treatment ($F(1,5) = 100$, $p<0.001$), days ($F(4,19) = 30$, $p<0.001$), and

interaction between days and drug treatment ($F(9,110) = 8.0$, $p<0.05$). The "jello shot" model of alcoholism demonstrated an alcohol deprivation effect that started one day after ethanol reinstatement and returns to basal levels, 1.2 ± 0.37 g/kg, six days after reinstatement (Figure 4-2). When (-)-trans-PAT is injected on the day of reinstatement, there is a decrease in experimental day consumption, 0.69 ± 0.16 g/kg, similar to experimental day effect of non-deprived (-)-trans-PAT in Figure 4-1. The ethanol deprived (-)-trans-PAT group then returns to basal consumption and does not display the increased consumption found in the ethanol deprived saline injected rats.

(-)-trans-m-Br-PAT Does Not Alter Non-deprived Or Deprived Ethanol Consumption

Non-deprived animals demonstrated a lower basal consumption, 0.93 ± 0.28 g/kg, than deprived animals, 1.2 ± 0.23 g/kg (Figure 4-3 and 4-4). Three-way ANOVA comparing main effect of deprivation, drug treatment and days found that only deprivation was significant ($F(1,11) = 35$, $p<0.05$). No interactions between main variables were found to be significant. In the non-deprived animals, there was no difference in consumption between control and (-)-trans-m-Br-PAT (Figure 4-3). Deprived saline injected control animals displayed the ADE as indicated by their increasing consumption to 1.8 ± 0.21 g/kg. Deprived (-)-m-Br-trans-PAT treated animals were not significantly different from control deprived animals throughout the experiment (Figure 4-4).

Ro60-0175 Alters Non-deprived But Not Deprived Ethanol Consumption

When rats underwent a three-week period of responding for plain gelatin, the level of ethanol self-administration was higher upon reaccess to ethanol gelatin (51.1 ± 5.2% increase on Post 1) compared to non-deprived rats (Figure 4-5, 4-6), which lasted for

three to five days. Systemic administration of the 5-HT_{2C} agonist, Ro60-0175, on the first day of ethanol gelatin reaccess produced differing effects on consumption, depending on ethanol deprivation. In non-deprived rats, Ro60-0175 reduced ethanol consumption by about 30% (Figure 4-5) compared to non-deprived rats that received saline (0.69 ± 0.12 g/kg vs 0.98 ± 0.13 g/kg, respectively). Ethanol self-administration returned to normal levels in drug-treated rats on subsequent days.

In the deprived rats, Ro60-0175 did not significantly alter ethanol consumption compared to saline controls on experimental day (0.93 ± 0.17 g/kg vs 1.13 ± 0.21 g/kg, respectively) (Figure 4-6). Three-way ANOVA showed a significant main effect of ethanol deprivation ($F(1,5) = 10.9$, $p<0.05$) but no other main effects or interactions were significant.

Discussion

This data shows that the ADE can be modeled using the “jello shot” model of alcoholism and it produces a five day long increase in ethanol consumption after a three week deprivation. There is evidence in the literature that the ADE can be observed after shorter deprivations (2 days) using P-rats, a strain of rat genetically selected to consume ethanol (Bell et al., 2006). Other models of the ADE use a 2 week deprivation to produce an ADE that lasts for only 24 hours (Theilen et al., 2004). The “jello shot” model of alcoholism produced a 47% increase in ethanol consumption on the second day which is comparable to the increases observed in other models (Martin-Fardon and Weiss, 2012).

These results also show that (-)-trans-PAT reduces both daily basal consumption and ADE induced increases in ethanol consumption. This indicates that (-)-trans-PAT is capable of preventing the changes that result in the ADE because (-)-trans-PAT

significantly decreases consumption in deprived animals for multiple days compared to the one day in non-deprived rats. This suggests that functionally selective 5-HT_{2C} agonists both decrease basal consumption and decrease the elevated consumption seen after deprivation in human alcoholics.

Surprisingly, (-)-trans-m-Br-PAT had no effect on daily or ADE consumption. Previously we observed that strong 5-HT_{2C} receptor agonism causes a decrease in voluntary ethanol containing gelatin consumption. When comparing available affinity and functional data, (-)-trans-m-Br-PAT is very similar to (-)-trans-PAT (Table 1-1). These unexpected results may be due to insufficient dose, 5 mg/kg, of the (-)-trans-m-Br-PAT. Unlike (-)-trans-PAT, a dose response curve was not established for (-)-trans-m-Br-PAT. There may also be differences in the human 5-HT_{2C} receptor that is used for affinity studies and the rat 5-HT_{2C} receptor that mediates the drugs ability to alter voluntary ethanol consumption. Possible pharmacokinetic reasons for this seem unlikely because of the structural similarity of (-)-trans-m-Br-PAT and (-)-trans-PAT. (-)-trans-m-Br-PAT remains a powerful potential drug to reduce alcohol intake that requires further testing to ascertain the full effects on voluntary ethanol consumption.

Ro60-0175 decreased non-deprived but not deprivation induced increase in consumption. This was expected because of the overlapping dose effect curves for Ro60-0175 explained in the previous chapter. Briefly, Ro60-0175 decreases both plain and ethanol containing gelatin consumption on FR5 schedules. This makes Ro60-0175 seem to have a more potent effect on ethanol containing gelatin consumption (Ro60-0175 decreases consumption for the vehicle gelatin and ethanol together) than the

specific effect of Ro60-0175 has on ethanol consumption. Therefore it was unsurprising that the dose of Ro60-0175 selected was too low to alter the ADE.

Rats that were deprived of ethanol in the Ro60-0175 and (-)-trans-m-Br-PAT experiment were the same rats deprived in the (-)-trans-PAT experiment. While the basal values for the (-)-trans-m-Br-PAT experiment of the deprived and non-deprived rats were not significantly different from each other, there does seem to be variation in basal consumption between repeated deprivations in this animal model.

In summary, the ADE is a model of alcoholism relapse that is important to study the pharmacotheraputic value of a novel drug (Lê and Shaham, 2002). The 5-HT_{2C} agonist (-)-trans-PAT, administered once before reinstatement to ethanol consumption, completely prevents the ADE from occurring over the next five days. Another 5-HT_{2C} agonist, (-)-trans-m-Br-PAT, however, did not alter ethanol consumption or prevent the ADE from occurring. This was most likely due to using too low of a dose of (-)-trans-m-Br-PAT because of differences in (-)-trans-m-Br-PAT affinity between the human and rat receptor. The 5-HT2 family agonist, Ro60-0175, did not alter the ADE either due to difficulty in determining the proper dose from the confounding effects on plain gel consumption. Functionally selective 5-HT_{2C} receptor agonists show promise as a novel pharmacotherapy for alcoholism that decrease daily ethanol consumption and prevent ethanol deprivation induced increases in ethanol consumption.

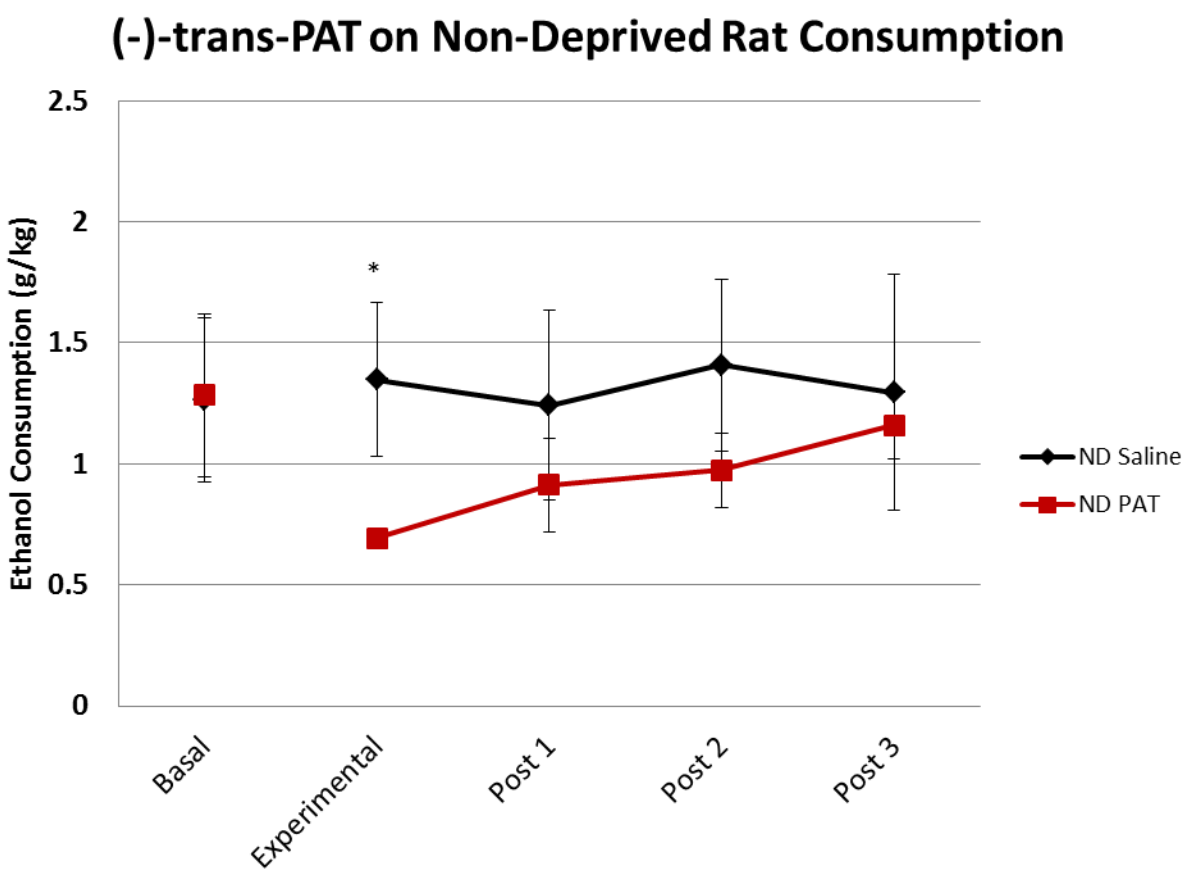

Figure 4-1. (-)-trans-PAT administration causes a temporary decrease in voluntary ethanol consumption. Basal consumption values were taken 3 weeks before Experimental day with Post 1-3 showing the consumption the days immediately following the Experimental day. N = 6 * indicates a significant difference between groups on that day.

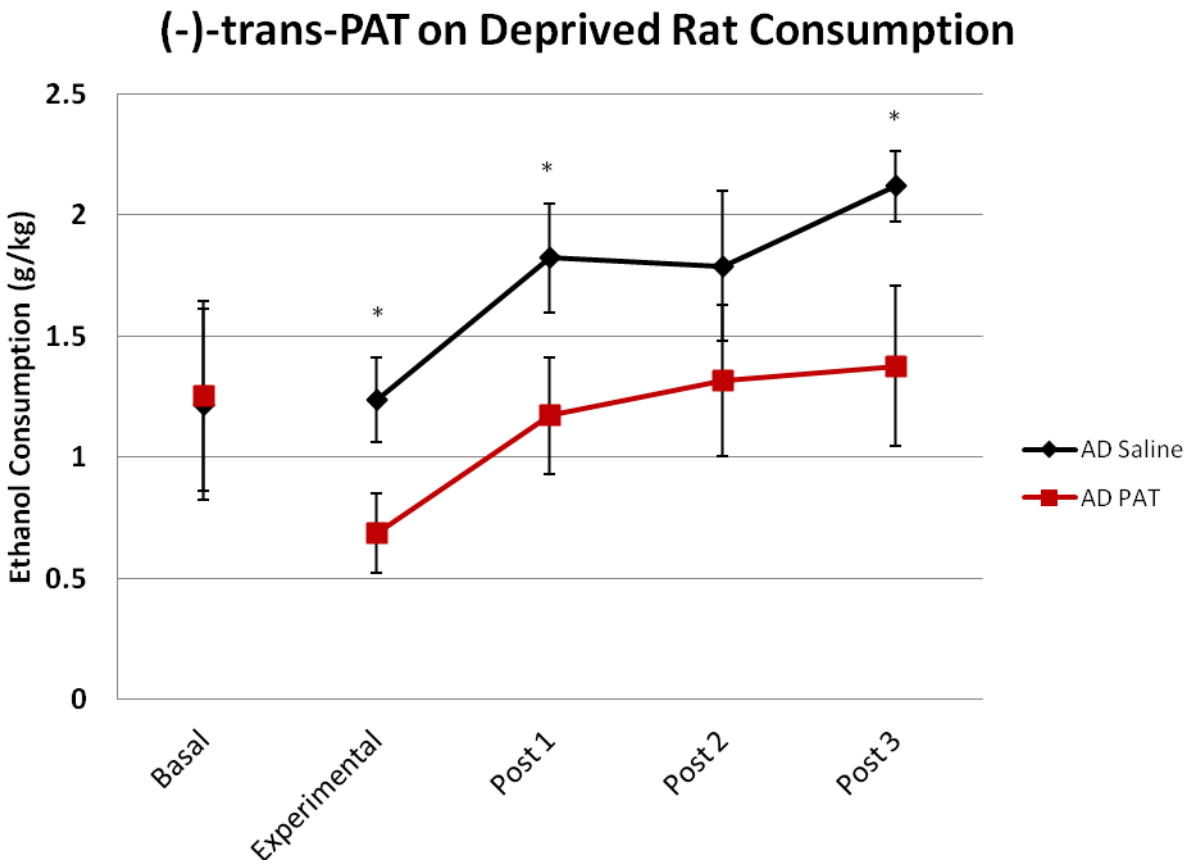

Figure 4-2. (-)-trans-PAT administration prevents the ADE. Ethanol deprived rats demonstrate increased consumption of ethanol after reinstatement. Basal consumption values were taken 3 weeks before Experimental day with Post 1-3 showing the consumption the days immediately following the Experimental day. N = 6 * indicates a significant difference between groups on that day.

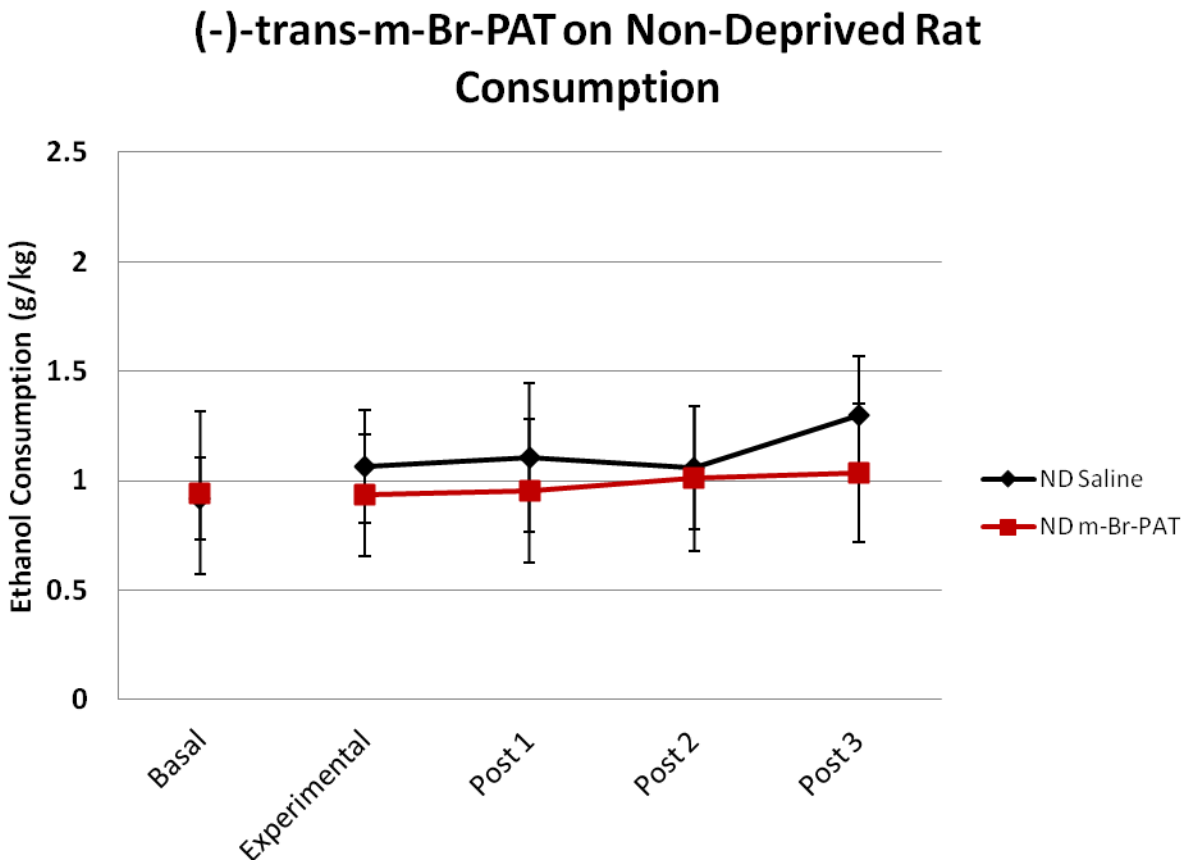

Figure 4-3. (-)-trans-m-Br-PAT administration had no effect on voluntary ethanol consumption. Ethanol non-deprived rats demonstrate stable baseline of ethanol consumption. Basal consumption values were taken 3 weeks before Experimental day with Post 1-3 showing the consumption the days immediately following the Experimental day. N = 6 * indicates a significant difference between groups on that day.

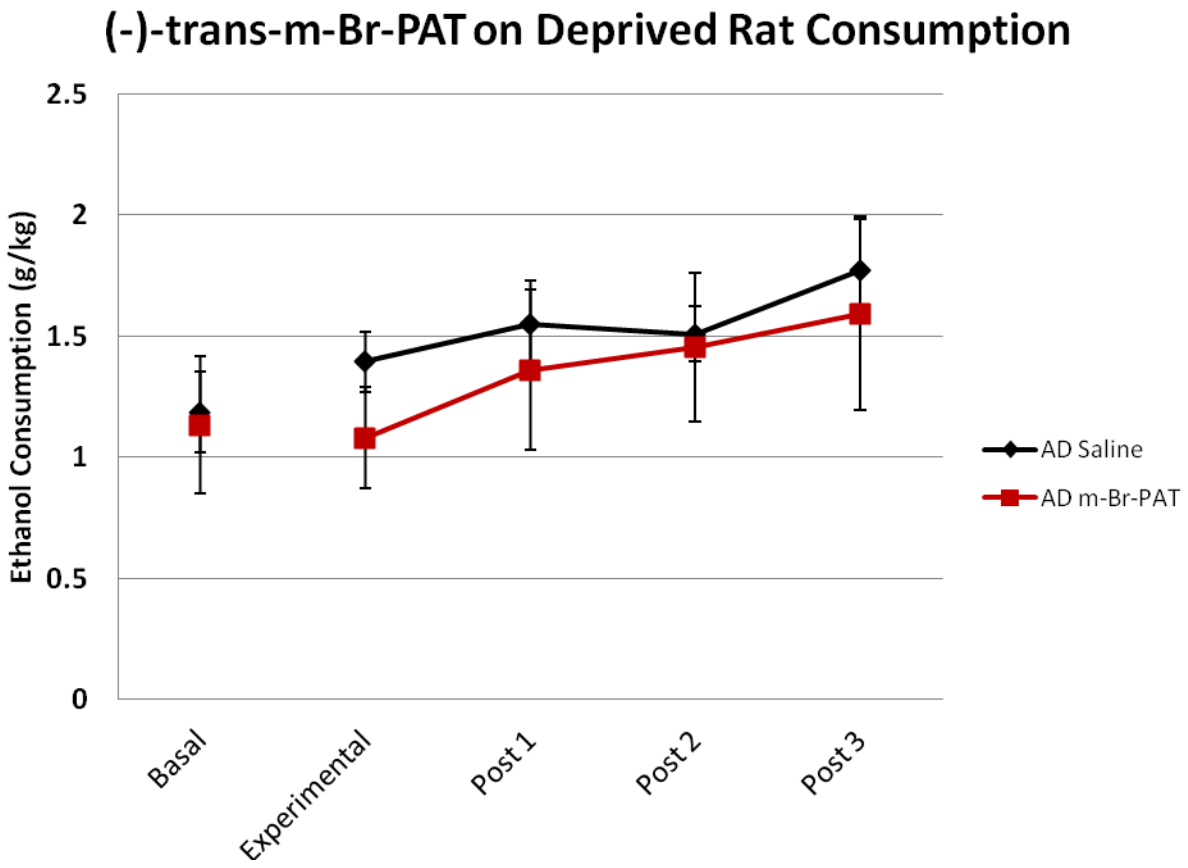

Figure 4-4. (-)-trans-m-Br-PAT administration had no effect on ADE. Ethanol deprived rats demonstrate increased consumption of ethanol after reinstatement. Basal consumption values were taken 3 weeks before Experimental day with Post 1-3 showing the consumption the days immediately following the Experimental day. N = 6 * indicates a significant difference between groups on that day.

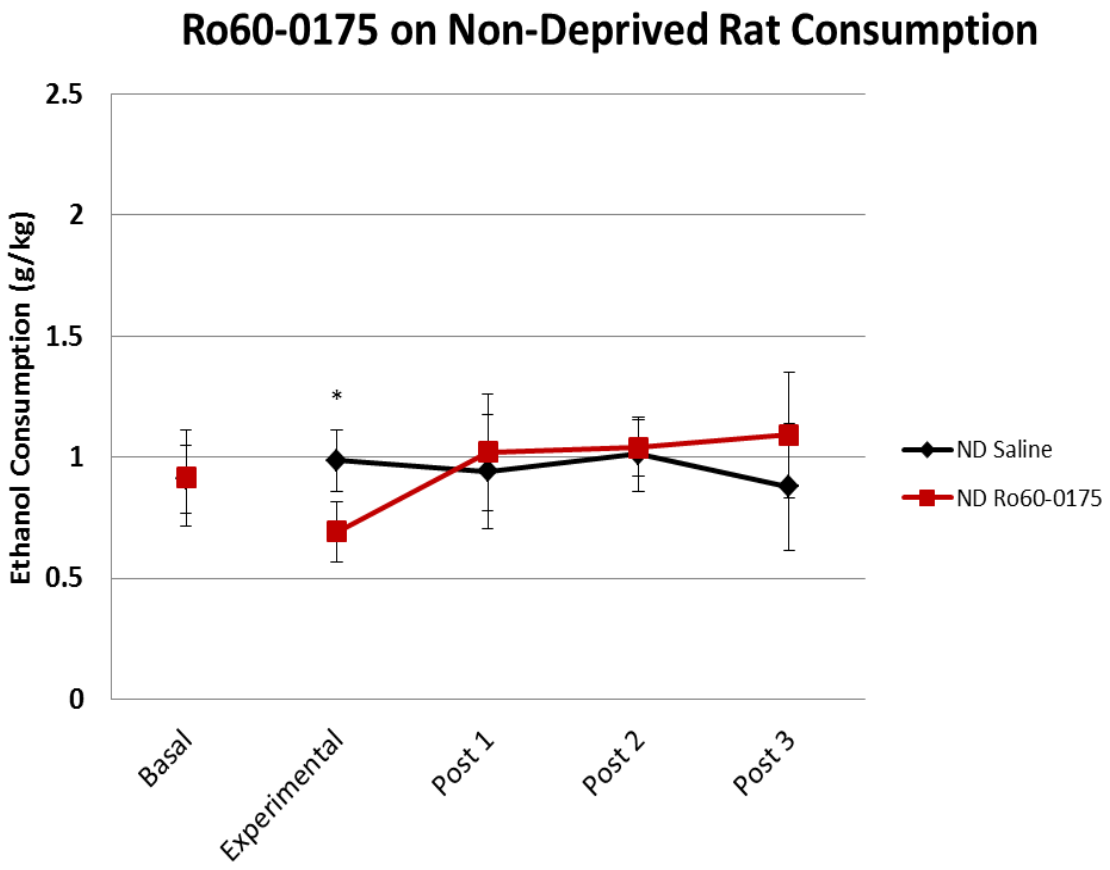

Figure 4-5. Ro60-0175 administration decreases experimental day voluntary ethanol consumption. Ethanol non-deprived rats demonstrate stable baseline of ethanol consumption. Basal consumption values were taken 3 weeks before Experimental day with Post 1-3 showing the consumption the days immediately following the Experimental day. N = 6 * indicates a significant difference between groups on that day.

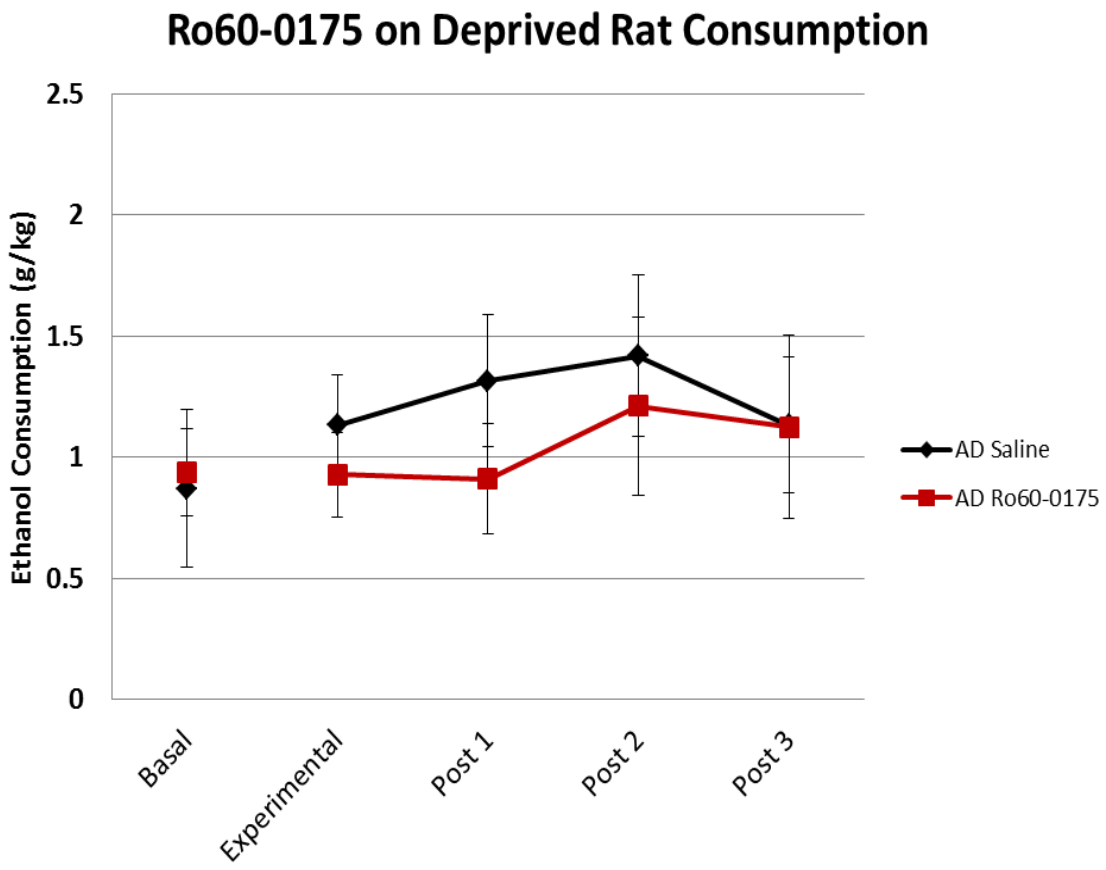

Figure 4-6. Ro60-0175 administration does not alter the ADE. Ethanol deprived rats demonstrate increased consumption of ethanol after reinstatement. Basal consumption values were taken 3 weeks before Experimental day with Post 1-3 showing the consumption the days immediately following the Experimental day. N = 6 * indicates a significant difference between groups on that day.

CHAPTER 5
MODULATION OF NEUROTRANSMISSION IN THE NAC BY 5-HT_{2C} AGONISTS USING CAPILLARY ELECTROPHORESES WITH LASER INDUCED FLUORESCENCE DETECTION

Alcoholism is a complex disorder that has a wide range of effects on neurotransmitters, including GABA (Koob et al., 1998). While there are conflicting reports about ethanol-induced changes of GABA concentrations in the mesolimbic pathway (Theile et al., 2009; Xaio and Ye, 2008), it is clear that 5-HT_{2C} receptors can modulate both GABA and dopamine release (Bubar et al., 2011). This makes the 5-HT_{2C} receptor a promising therapeutic target for alcoholism.

Unfortunately, the 5-HT_{2C} receptor is difficult to study because it shares a high transmembrane sequence homology with the rest of the 5-HT_2 family (Julius et al., 1990), making it difficult to find agonists that activate only one out of the three members of this family. This is problematic because 5-HT_{2C} receptor agonists are thought to have therapeutic properties while activating 5-HT_{2A} receptors causes psychotomimetic effects. These opposing effects of 5-$HT_{2A/C}$ agonists make it difficult to study the role these receptors play. The current most popular 5-HT_{2C} agonist, Ro60-0175, reports having good binding specificity for 5-HT_{2C} (pKi = 9.0) compared to 5-HT_{2A} (pKi = 7.5) (Damjanoska et al., 2003). The EC50 values for Ro60-0175 have only a 10 fold favoring for 5-HT_{2C} over 5-HT_{2A} (Porter et al., 1999). Recently (-)-trans-PAT has demonstrated functional selectivity for this receptor family. It acts as a high affinity 5-HT_{2C} agonist while being an inverse agonist at 5-$HT_{2A/B}$ receptors (Table 1-1). This compound inspired the creation of PAT analogs, some of which share this functional specificity. Using these compounds, it is now possible to investigate the effects of 5-HT_{2C} agonists without confounding 5-HT_{2A} agonism.

There are two conditions to test a drugs effect on neurotransmitter release in microdialysis experiments, basal or stimulated. Administering the drug during basal conditions will determine the effect of the drug on basal neurotransmission. This can prove difficult to detect decreases in neurotransmitter concentrations if the neurotransmitter is found in low nM range. Stimulated release results in an increase in extracellular neurotransmitter concentrations and the effects of drugs can be detected. There are many chemicals to increase neurotransmitter concentrations including cocaine, ethanol, and high potassium (K+). K+ stimulation results in all neurons being brought to threshold and causes calcium dependent release of neurotransmitters (Sellström and Hamberger, 1977). By testing PAT compounds during K+-stimulated conditions, it is possible to observe decreases in neurotransmitters found in low nM concentrations (e.g. GABA) using CE-LIF.

The 5-HT_{2C} receptor agonists are notorious for binding to multiple different receptor families and subtypes. The PAT analogs are no exception, binding with high affinity to 5-HT_{2A}, 5-HT_{2C}, and H_1 receptors. This makes it problematic to determine which action at which receptor causes PAT analog induced changes. Follow up studies must be performed to determine the site of action for PAT induced changes in neurotransmission.

Mepyramine is a high affinity antagonist that is specific for the H_1 receptor and has been widely used in research for decades. The PAT analogs have the highest affinity for H_1 receptors, so it is possible that their ability to alter K+-induced changes in neurotransmission is mediated by H_1 receptor and not through 5-HT_{2C}. H_1 receptors have been shown to alter K+-stimulated 5-HT release (Son et al., 2001) but the role of

H1 receptors on K+-stimulated GABA release remains unclear. To this end, mepyramine was tested alone on K+-stimulation induced increases in neurotransmitter release. Mepyramine and (-)-trans-PAT share similar affinities for the H1 receptor so equivalent doses can be tested. Once the effects of mepyramine on basal and K+-stimulated GABA release was determined, its ability to block any effects of PAT analogs mediated via H_1 receptors was assessed.

Ketanserin is a high affinity antagonist for the 5-HT_{2A}, 5-HT_{2C}, H_1 and alpha-1 adrenergic receptors. It is commercially available and has been widely used in research for the last 25 years. Using this compound, it is possible to block the 5-HT_{2C} agonism from PAT analogs. When comparing the functional profile of these PAT analogs to ketanserin, there is overlapping antagonism/inverse agonism of 5-HT_{2A} and H_1 receptors. The largest difference between these PATs and ketanserin is the function at the 5-HT_{2C} receptor. If ketanserin blocks PAT analogs from attenuating K+-induced GABA release, then this effect is mediated by the 5-HT_{2C} receptors.

Methods

Animals

Male Sprague-Dawley rats 60 days of age (Harlan, Indianapolis, IN), weighing 225 to 250 g, were singly housed in a temperature- and humidity-controlled environment with a 12 hour normal phase light/dark cycle (06:00-18:00). All tests were conducted during the light phase. Rats were acclimated to our housing facilities for at least 1 week prior to experimentation. The subjects had *ad libitum* access to food and water throughout the experiment. Rat use was approved by the Institutional Animal Care and Use Committee and was consistent with the NIH Guide for the Care and Use of Laboratory Animals.

Chemicals

NaCl, CaCl2, KCl, MgSO4, monobasic borate, dibasic borate, o-phthaldialdehyde (OPA), β-mercaptoethanol (BME), hydroxypropyl-β-cyclodextrin (HPBCD), sodium metaboisulfate, citric acid, RO60-0175, mepyramine, and ketanserin were supplied by Sigma, St. Louis (USA).

PAT analogs were supplied by the laboratory of Dr. Raymond Booth, Department of Medicinal Chemistry at College of Pharmacy, University of Florida.

Microdialysis And Surgeries

As described in Chapter 2. Each rat will be anesthetized with isoflurane and placed in a stereotaxic instrument for implantation of a guide canulla. The guide canulla will be anchored with two stainless steel screws and dental cement. The following coordinates from bregma will be used for implantation: Nucleus Accumbens +1.8 anteroposterior, +1.3 lateral, -6.2 dorsoventral, Striatum +0.8 anteroposterior, +3.0 lateral, -4.0 dorsoventral. After implantation, animals are given at least 2 days to recover before microdialysis testing.

Experiment Procedures

Experimental procedures are described as following. (1) When measuring the effect of novel PATs on basal neurotransmission, aCSF was constantly perfused throughout the experiment. The animal was then switched from plain aCSF to drug in an aCSF vehicle for 15 minutes. Lastly the animal was switched back to plain aCSF to investigate if any drug induced changes dissipated.

(2) In the K+ stimulation experiment, aCSF was first perfused for 5 minutes. Then high K+ aCSF (95 mM NaCl, 50 mM KCl, 1.0 mM MgCl2, 1.2 mM CaCl2, 0.45 mM monobasic phosphate, 1.55 mM dibasic phosphate, pH 7.4) was perfused into the NAc

or striatum for 10 minutes. The animal was then switched back to plain aCSF for 40 minutes. The perfusion and washing procedures were repeated three times with the second stimulation paired with drug perfusion.

(3) K+ stimulation experiments with constant ketanserin or mepyramine are performed similar to Experimental Procedure 2. This experiment includes three 10 minute high K+ stimulations with drug perfusion paired to the second stimulation. In addition to this, there is a constant perfusion throughout the entire experiment of either 50 µM mepyramine or 50 µM ketanserin.

CE-LIF

As described in Chapter 2. The technique used to gather data will be microdialysis coupled with capillary electrophoresis with laser-induced fluorescence detection and has been described previously (Bowser and Kennedy 2001). On the experiment day, a standard calibration curve (0 to 20 µM) will first be performed using a microdialysis probe with outer diameter 270 µm, 13,000 molecular weight cutoff, and variable active length (2 to 4 mm) depending on the brain region being dialysed. After calibration, the probe will be implanted in the non-anesthetized and free moving rat. The experiment begins after neurotransmitter concentrations reach steady-state which is typically 2 hours after implantation. The substances measured will include glutamate, aspartate, serotonin, dopamine, ornathine, GABA, taurine, glutamine, serine, and glycine.

Data Analysis And Histology

Data output was converted into concentrations of neurotransmitters over time as described in Chapter 2. AUC was then determined for each K+-induced increase in neurotransmitter release. The three AUC values were then converted to a percent of

the first (control) AUC. One-way ANOVA with Tukey post-hoc tests with significance level $p < 0.05$ were performed using SPSS on the AUC values to determine significant changes.

A Bright Instruments cryostat was used to section the frozen brains after the experiment. Coronal sections 40 µm thick were removed until probe tract is observed. Probe placement was determined by tract location in relation to brain arcetecture described with a brain atlas (Paxinos and Watson, 2005). Only animals with the majority (over 50%) of the 2 or 4 mm long probe in the targeted brain region were included in the study.

Results

Effect Of (-)-trans-PAT Perfusion On Basal Neurotransmitter Release In NAc

In our study, the microdialysis experiment started with a 15 minute basal period. We then directly perfused 5 µM of (-)-trans-PAT directly into the NAc for 15 minutes. The GABA concentrations before, during, and after are plotted in Figure 5-2. The PAT compounds had no measureable effect on basal accumbal neurotransmitter concentration in rats ($F(2,8) = 2.8$, $p>0.05$).

Effect Of (-)-trans-PAT On K+-Induced Neurotransmitter Release In The NAc.

The effects of (-)-trans-PAT on K+-stimulated increases in accumbal GABA and taurine were examined using the procedure described in Experimental Procedure 2. (-)-trans-PAT reduced K+-stimulated GABA release ($F(2,8) = 180$, $p<0.0001$) (Figure 5-3) but not taurine ($t(2) = 0.69$, $p>0.05$) (Figure 5-4). To determine what receptor is mediating this effect, ketanserin or mepyramine were constantly perfused as in Experimental Procedure 3 to block 5-HT_{2C} and H_1 receptors respectively. Ketanserin (Figure 5-5) and mepyramine (Figure 5-7) had no effect on stimulated GABA release (F

(2,8) = 0.60, p>0.05) (F (2,8) = 3.7, p>0.05). However when ketanserin is perfused throughout the experiment, it blocks (-)-trans-PAT ability to decrease stimulated GABA release (Figure 5-6) (F (2,8) = 1.6, p>0.05). Mepyramine, however, was unable to alter the decrease in stimulated GABA levels that was caused by (-)-trans-PAT (Figure 5-8) (F (2,8) = 180, p<0.0001).

Effect Of Additional 5-HT_{2C} Receptor Agonists On K+-Induced Neurotransmitter Release In The NAc.

Other novel and established 5-HT_{2C} agonists were then tested for the ability to reduce K+-stimulated GABA release. This includes (-)-trans-p-Cl-PAT, TOMCAT, and Ro60-0175. All three compounds were able to reduce stimulated GABA release as shown in Figure 5-9 (F (2,8) = 220, p<0.0001), Figure 5-11 (F (2,8) = 39, p<0.01), and Figure 5-13 (F (2,8) = 75, p<0.001). When ketanserin was perfused throughout the experiment to block 5-HT_{2C} agonism, both (-)-trans-p-Cl-PAT (Figure 5-10) and TOMCAT (Figure 5-12) were unable to change stimulated GABA concentrations (F (2,8) = 0.72, p>0.05) (F (2,8) = 2.2, p>0.05).

Effect Of 5-HT_2 Receptor Modulation On K+-Induced Neurotransmitter Release In The NAc.

To fully explore the pharmacology behind the 5-HT_{2C} receptor effects described above, 5-HT_{2C} agonists with poor affinity, partial efficacy, or 5-HT_{2C} antagonist were tested. The (-)-trans-p-Me-PAT is a 5-HT_{2C} agonist that has 10-fold lower affinity for the 5-HT_{2C} receptor compared to (-)-trans-PAT. When (-)-trans-p-Me-PAT was administered during K+ stimulation, there was no significant change in K+-stimulated GABA release (Figure 5-14) (F (2,8) = 0.28, p>0.05). A partial agonist, (+)-trans-PAT was also tested and showed no effect on stimulated GABA release (Figure 5-15) (F (2,8) = 2.4, p>0.05). Lastly, the 5-HT_{2C} receptor antagonist, (-)-trans-CAT was tested

but caused no change in stimulated GABA release (Figure 5-16) ($F(2,8) = 0.55$, $p>0.05$). Together, this shows that high affinity full agonists for the 5-HT_{2C} receptor are required for reduction of K+-stimulated GABA release in the NAc.

Effect Of Novel (-)-trans-PAT Perfusion On K+-Induced Neurotransmitter Release In The Striatum.

(-)-trans-PAT was then tested for its ability to alter stimulated GABA release in the striatum, another brain region receiving large amounts of dopaminergic input. K+ stimulation caused a robust increase in GABA and taurine similar to what was observed in the NAc, however when (-)-trans-PAT was perfused during the second stimulation, K+'s ability to increase GABA is significantly reduced (Figure 5-17) ($F(2,11) = 120$, $p<0.001$). Taurine increases remain unaffected by (-)-trans-PAT (Figure 5-18) ($t(2) =$ 076, $p>0.05$).

Discussion

This study examined the effect of multiple novel and established 5-HT_2 receptor modulators on both basal and stimulated neurotransmission in the NAc and striatum. Reverse dialysis of (-)-trans-PAT had no observable effect on basal neurotransmission in the NAc. However, novel PAT analogs with 5-HT_{2C} agonism reduced K+-stimulated GABA release. K+-stimulated taurine concentrations were unaffected by the presence of PAT analogs. Analogs that are 5-HT_{2C} antagonist/inverse agonists had no effect on extracellular neurotransmitter concentrations. Although we were able to reliably measure K+-stimulated GABA release, our ability to detect hypothesized decreases in basal GABA release were limited by our detection sensitivity.

PATs Effect On K+-Stimulated Release In NAc And Striatum

This study replicated previous work with K+ stimulation (Sellström and Hamberger, 1977) and advanced it into an *in vivo* screening procedure for 5-HT_2 receptor modulators. Drugs with good affinity and agonism for the 5-HT_{2C} receptor, e.g., (-)-trans-PAT, (-)-trans-p-Cl -PAT, TOMCAT, and Ro60-0175, caused a decrease of K+-stimulated GABA release in the NAc. Taurine release remained unaffected. The only 5-HT_{2C} agonist tested that did not decrease GABA release was the (-)-trans-p-Me-PAT and this can be attributed to its poor affinity for the 5-HT_{2C} receptor.

Drugs that are 5-HT_{2C} antagonists/inverse agonists did not alter K+-stimulated GABA or taurine release in the NAc. Although it was originally hypothesized that the inverse agonists should have the opposite effect of the agonists, this was not observed. The K+-stimulation might be so powerful that potentiating more release is impossible due to a ceiling effect. It is also possible there was not enough endogenous drive on the 5-HT_{2C} receptor for an antagonist to block. Either of these could explain why 5-HT_{2C} receptor antagonists/inverse agonists were unable to alter stimulated GABA release.

In the striatum, 5-HT_{2C} agonism caused a decrease in K+-stimulated GABA release but not taurine. The effects of both the K+-stimulation and 5-HT_{2C} agonism are the same that was observed in the NAc. Both the striatum and NAc both have a large amount of dopamine neurons projecting to them and both express 5-HT receptors (Alex and Pehek, 2007).

H1 Receptors Do Not Alter GABA During High K+-Induced GABA Release.

Mepyramine at 50 µM directly perfused into the NAc had no effect on basal or high K+-stimulated GABA release. This supports the hypothesis that the PAT analog's ability to decrease high K+-stimulated GABA release is mediated by their action on 5-HT_{2C}

receptors and not through their action on H_1 receptors. Mepyramine locally perfused into the NAc did not influence basal or stimulated release of GABA, therefore it is fit to be used to block H_1 receptors such that PAT analogs cannot act on them.

5-HT_{2C} Receptors Mediate Decreases In High K+-Induced GABA Release.

The experiments in which ketanserin was constantly perfused throughout demonstrated that (-)-trans-PAT, TOMCAT, and (-)-trans-p-Cl-PAT were mediating their effects through a receptor that is common to the binding profile of the PAT analogs and ketanserin. These receptors include the 5-$HT_{2A/C}$ and H_1 receptors. From the results of the mepyramine studies, we can rule out H_1 receptors as having a role in attenuating K+-induced GABA release. This leaves only the 5-$HT_{2A/C}$ receptors.

To determine if the (-)-trans-PAT, TOMCAT, and (-)-trans-p-Cl-PAT mediate their effect through 5-HT_{2A} or 5-HT_{2C} receptors, the function of these drugs at both receptors was examined. These three PAT analogs are all 5-HT_{2A} antagonist/inverse agonist and 5-HT_{2C} agonists. From the drugs studied, it is apparent that the only drugs that altered GABA release had strong affinity and agonism at 5-HT_{2C} receptors. 5-HT_{2A} receptor antagonism/inverse agonism by (-)-trans-CAT or ketanserin did not decrease K+-stimulated GABA release. Together, this indicates that 5-HT_{2A} antagonism/inverse agonism does not play a role in the attenuation of K+-stimulated GABA release, and 5-HT_{2C} agonism does.

Previous electrophysiological studies have shown that 5-HT_{2C} agonism causes an increase in local GABA release onto dopaminergic neurons in the VTA (Theile et al., 2009). These results conflict with the results of the present study where we observed 5-HT_{2C} agonists had no measurable effect on basal GABA levels and decreased stimulated GABA release in both the striatum and NAc. However it should be pointed

out that the electrophysiological studies were performed in different brain regions than the current dialysis experiments. This apparent disagreement is rectified in light of previous studies (Navailles et al., 2008) where 5-HT_{2C} receptors have been shown to differentially regulate cocaine-induced effects depending on brain region (VTA vs NAc). Taken together, these findings demonstrate that 5-HT_{2C} receptor agonists modulate GABAergic drive in the mesolimbic pathway differently in the NAc than in the VTA.

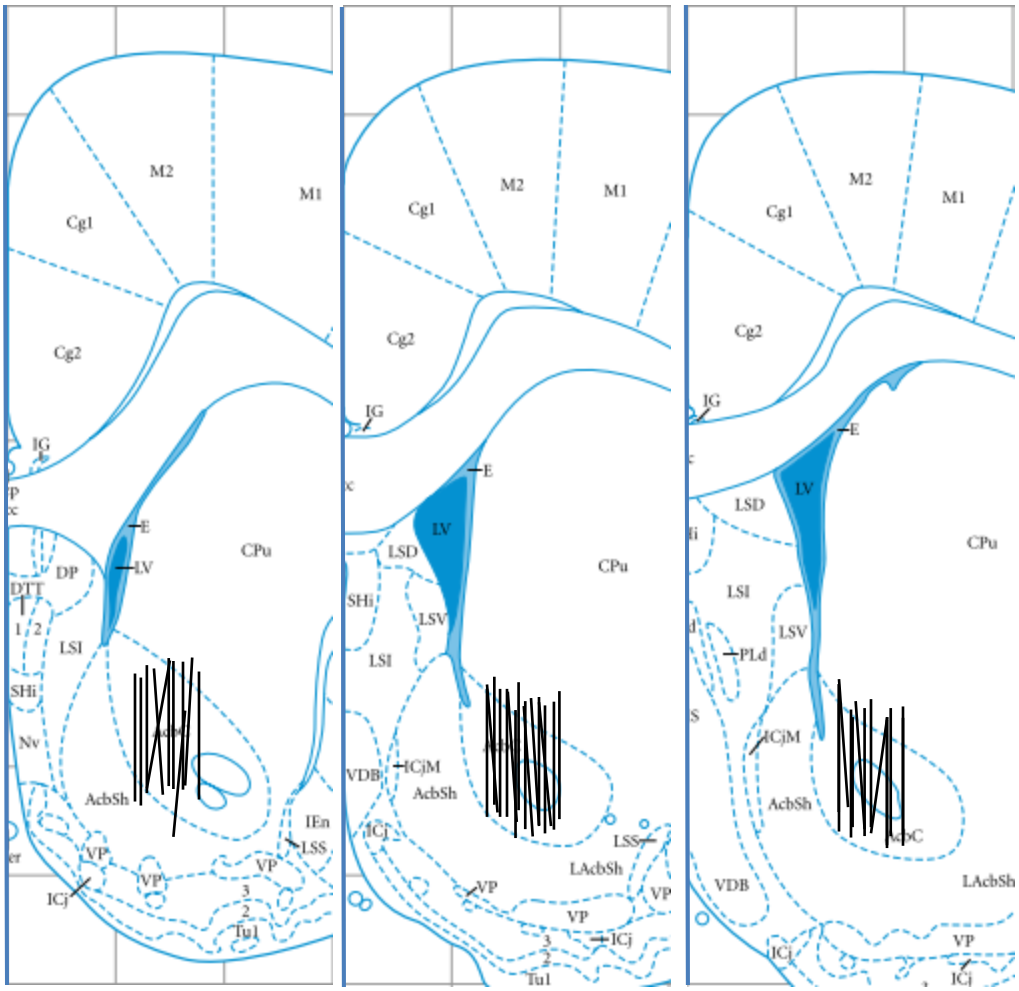

Figure 5-1. Coronal sections showing microdialysis probe placement within the NAc. Lines indicate the active dialysis regions. Numbers below the figure represent the position of the slice relative to bregma. Figure was adapted from Paxinos and Watson, 2005.

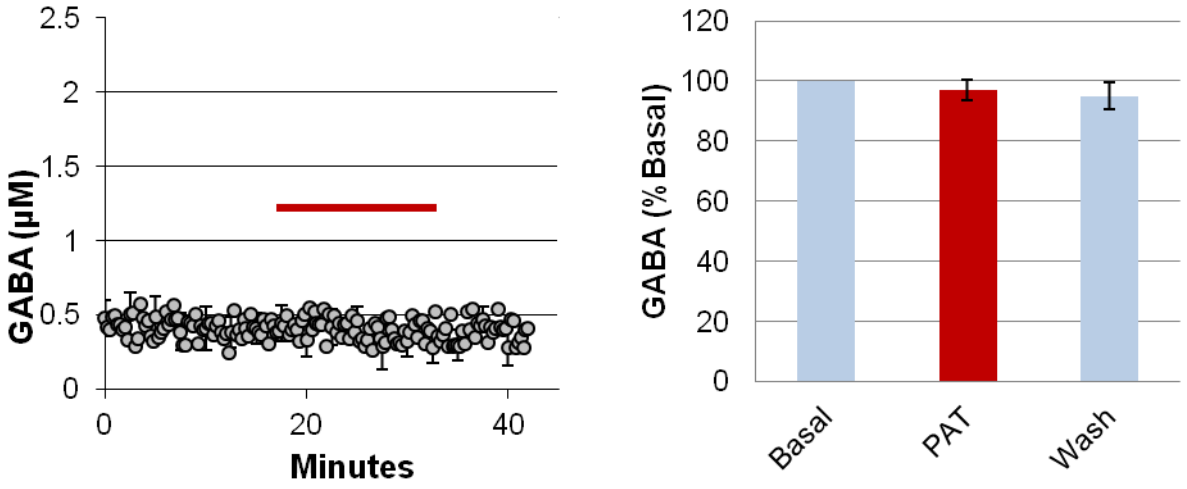

Figure 5-2. (-)-trans-PAT does not alter basal GABA concentrations in the NAc. (A) The concentration of GABA over time. Red line indicates when 5 µM (-)-trans-PAT was added to the aCSF. (B) AUC values for the data presented in Panel A. Data shown are mean values ± SEMs for N = 3. * indicates $p < 0.05$. ($F(2,8) = 2.8$, $p>0.05$)

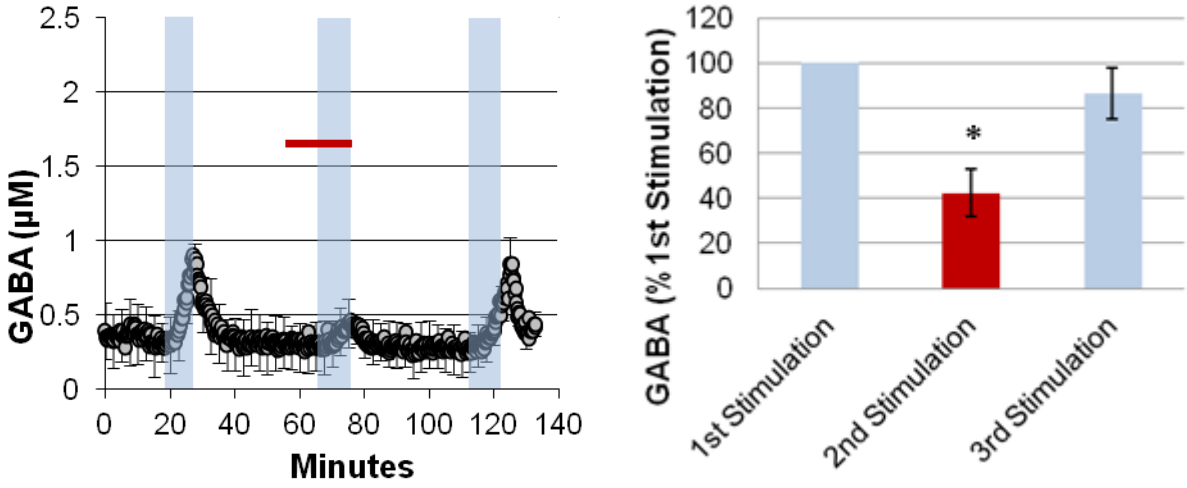

Figure 5-3. (-)-trans-PAT decreased K+-stimulated GABA release in the NAc. (A) The concentration of GABA over time. Blue shaded areas represent perfusion with 50 mM K+ containing aCSF. Red line indicates when 50 µM (-)-trans-PAT was added to the aCSF. (B) AUC values for the data presented in Panel A. Data shown are mean values ± SEMs for N = 3. * indicates $p < 0.05$. ($F(2,8) = 180$, $p<0.0001$)

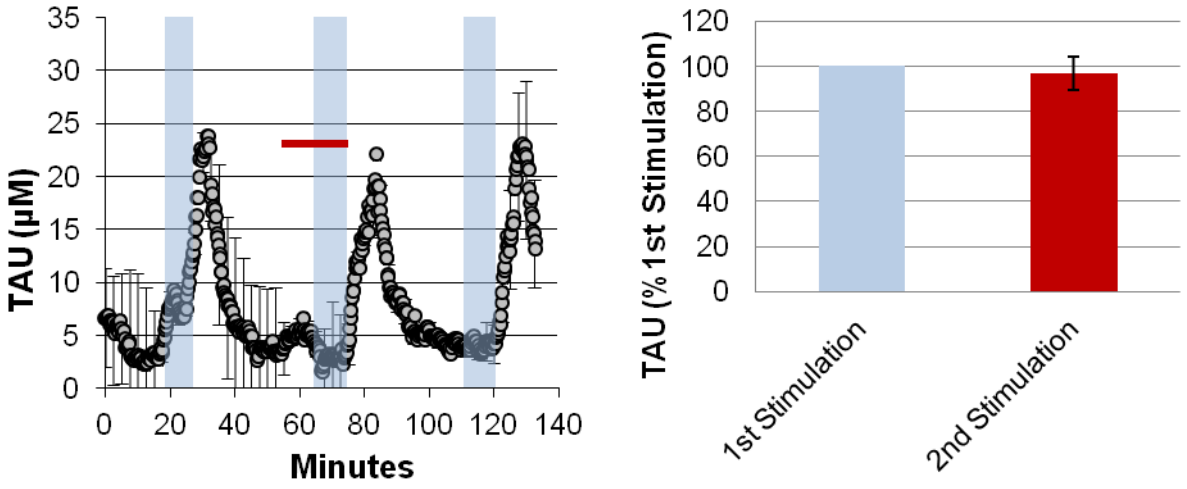

Figure 5-4. (-)-trans-PAT did not alter K+-stimulated taurine release in the NAc. (A) The concentration of taurine over time. Blue shaded areas represent perfusion with 50 mM K+ containing aCSF. Red line indicates when 50 µM (-)-trans-PAT was added to the aCSF. (B) AUC values for the data presented in Panel A. Data shown are mean values ± SEMs for N = 3. * indicates $p < 0.05$. ($t(2) = 0.69$, $p>0.05$)

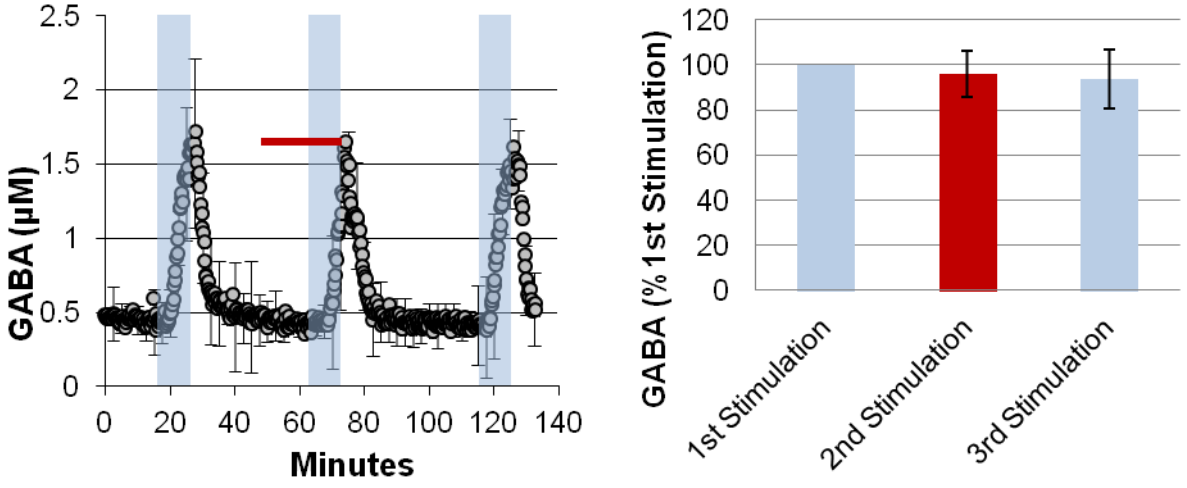

Figure 5-5. Ketanserin had no effect on K+-stimulated GABA release in the NAc. (A) The concentration of GABA over time. Blue shaded areas represent perfusion with 50 mM K+ containing aCSF. Red line indicates when 50 µM ketanserin was added to the aCSF. (B) AUC values for the data presented in Panel A. Data shown are mean values ± SEMs for N = 3. * indicates $p < 0.05$. ($F(2,8) = 0.60$, $p>0.05$)

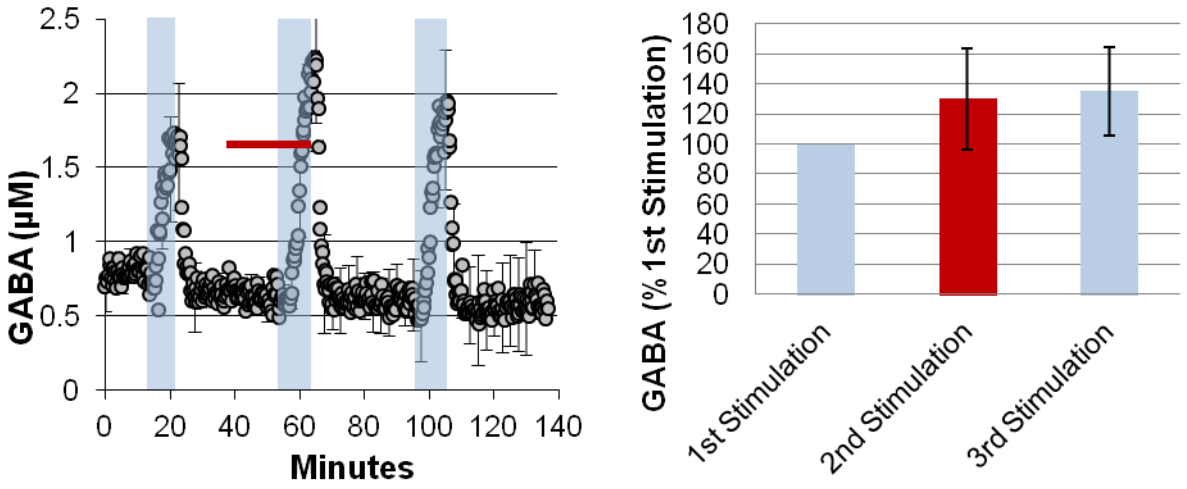

Figure 5-6. (-)-trans-PAT had no effect on K+-stimulated GABA release in the NAc in the presence of ketanserin. (A) The concentration of GABA over time. Blue shaded areas represent perfusion with 50 mM K+ containing aCSF. Red line indicates when 50 µM (-)-trans-PAT was added to the aCSF. Ketanserin was perfused throughout the whole experiment. (B) AUC values for the data presented in Panel A. Data shown are mean values ± SEMs for N = 3. * indicates $p < 0.05$. ($F(2,8) = 1.6$, $p>0.05$)

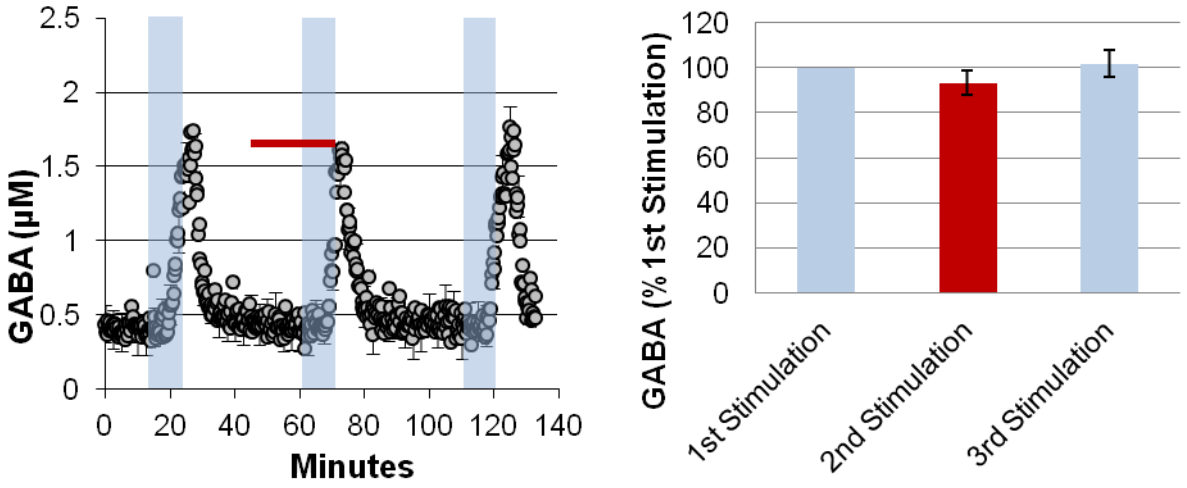

Figure 5-7. Mepyramine had no effect on K+-stimulated GABA release in the NAc. (A) The concentration of GABA over time. Blue shaded areas represent perfusion with 50 mM K+ containing aCSF. Red line indicates when 50 µM mepyramine was added to the aCSF. (B) AUC values for the data presented in Panel A. Data shown are mean values ± SEMs for N = 3. * indicates $p < 0.05$. ($F(2,8) = 3.7$, $p>0.05$)

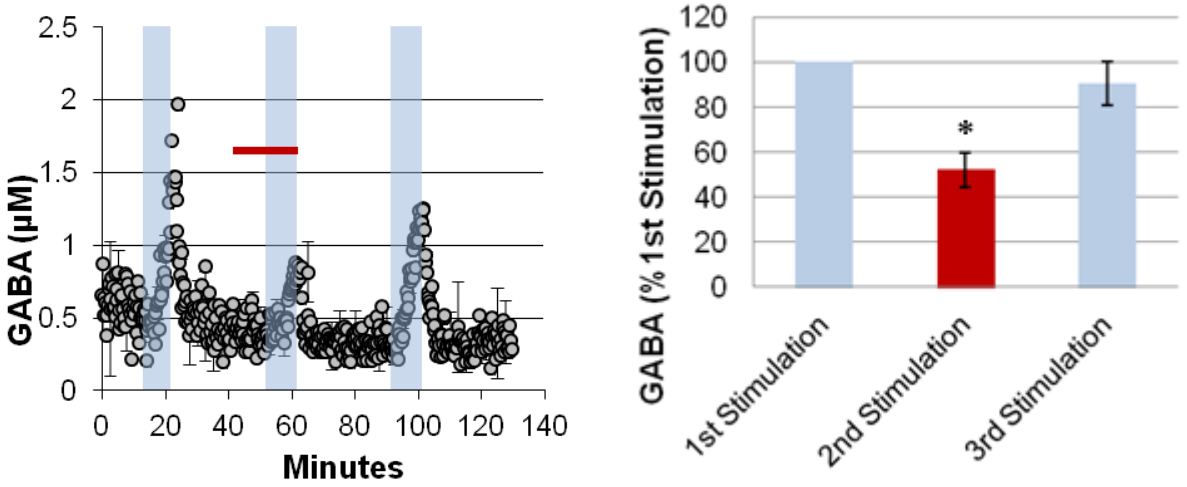

Figure 5-8. (-)-trans-PAT decreased K+-stimulated GABA release in the NAc in the presence of mepyramine. (A) The concentration of GABA over time. Blue shaded areas represent perfusion with 50 mM K+ containing aCSF. Red line indicates when 50 µM (-)-trans-PAT was added to the aCSF. Mepyramine was perfused throughout the experiment. (B) AUC values for the data presented in Panel A. Data shown are mean values ± SEMs for N = 3. * indicates $p < 0.05$. ($F(2,8) = 180$, $p<0.0001$)

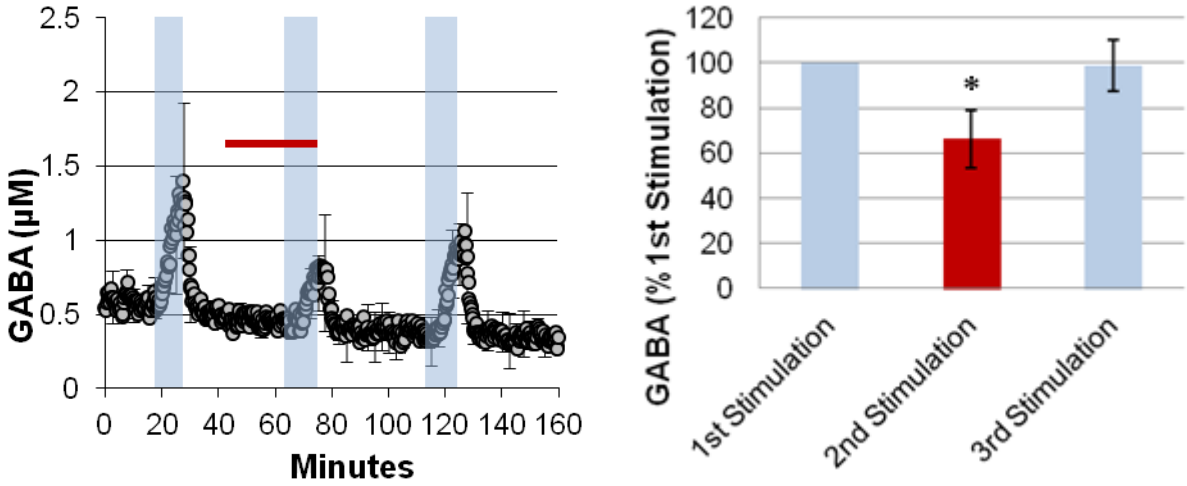

Figure 5-9. (-)-trans-p-Cl-PAT decreased K+-stimulated GABA release in the NAc. (A) The concentration of GABA over time. Blue shaded areas represent perfusion with 50 mM K+ containing aCSF. Red line indicates when 50 µM (-)-trans-p-Cl-PAT was added to the aCSF. (B) AUC values for the data presented in Panel A. Data shown are mean values ± SEMs for N = 3. * indicates $p < 0.05$. ($F(2,8) = 220$, $p<0.0001$)

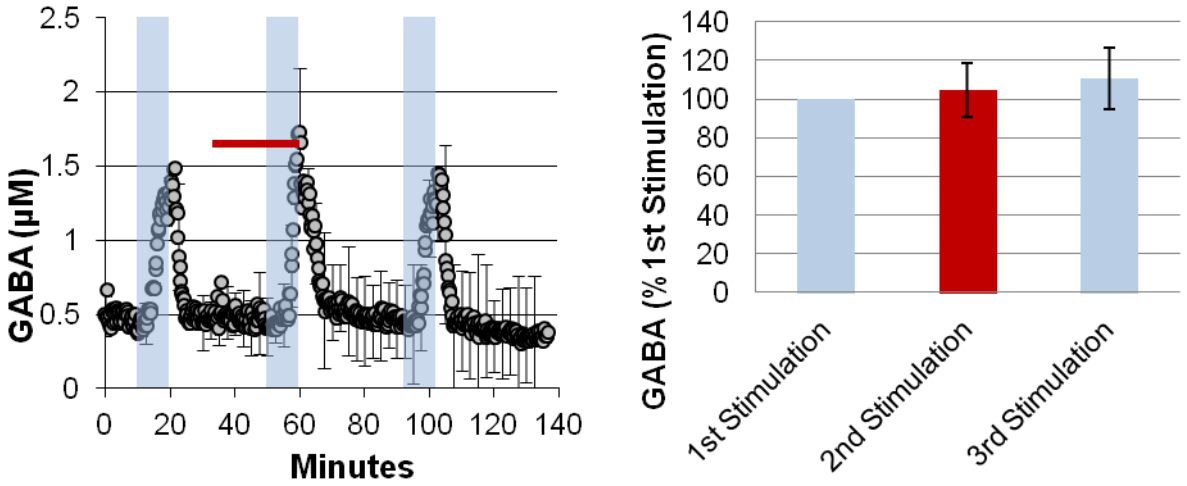

Figure 5-10. (-)-trans-p-Cl-PAT had no effect on K+-stimulated GABA release in the NAc in the presence of ketanserin. (A) The concentration of GABA over time. Blue shaded areas represent perfusion with 50 mM K+ containing aCSF. Red line indicates when 50 µM (-)-trans-p-Cl-PAT was added to the aCSF. Ketanserin was perfused throughout the whole experiment. (B) AUC values for the data presented in Panel A. Data shown are mean values ± SEMs for N = 3. * indicates $p < 0.05$. ($F(2,8) = 0.72$, $p>0.05$)

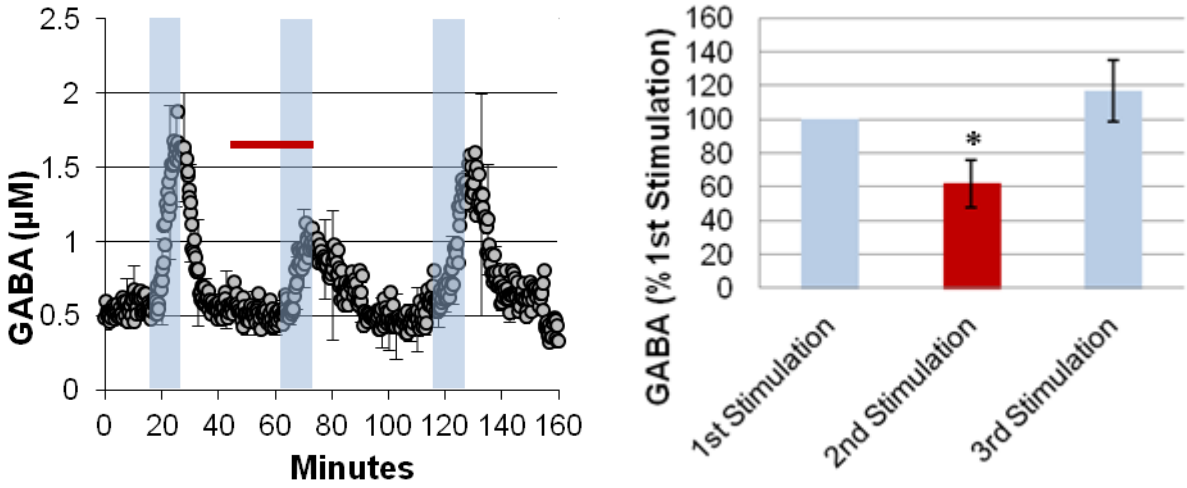

Figure 5-11. TOMCAT decreased K+-stimulated GABA release in the NAc. (A) The concentration of GABA over time. Blue shaded areas represent perfusion with 50 mM K+ containing aCSF. Red line indicates when 50 µM TOMCAT was added to the aCSF. (B) AUC values for the data presented in Panel A. Data shown are mean values ± SEMs for N = 3. * indicates $p < 0.05$. ($F(2,8) = 39$, $p<0.01$)

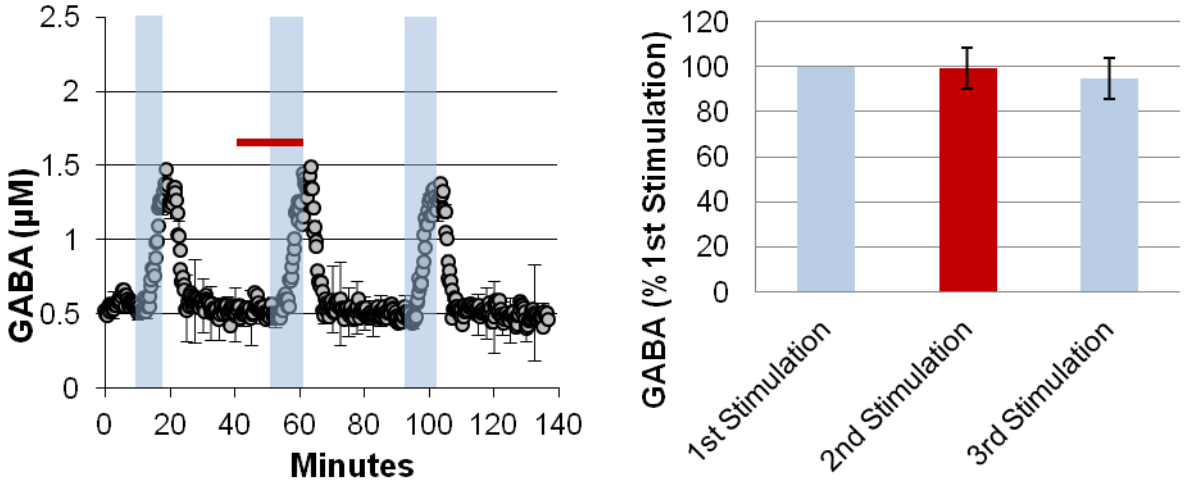

Figure 5-12. TOMCAT had no effect on K+-stimulated GABA release in the NAc in the presence of ketanserin. (A) The concentration of GABA over time. Blue shaded areas represent perfusion with 50 mM K+ containing aCSF. Red line indicates when 50 µM TOMCAT was added to the aCSF. Ketanserin was perfused throughout the whole experiment. (B) AUC values for the data presented in Panel A. Data shown are mean values ± SEMs for N = 3. * indicates $p < 0.05$. ($F(2,8) = 2.2$, $p>0.05$)

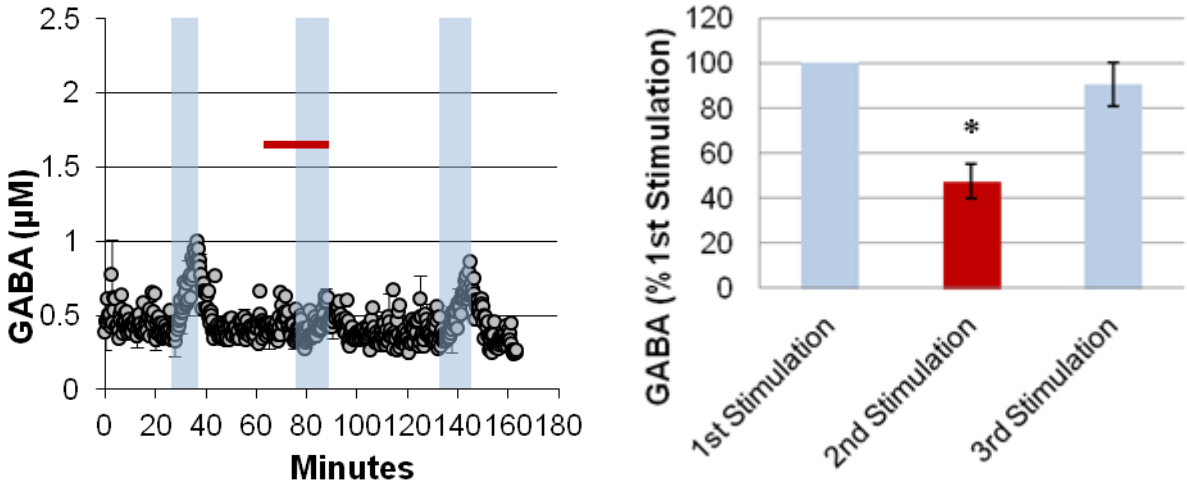

Figure 5-13. Ro60-0175 decreased K+-stimulated GABA release in the NAc. (A) The concentration of GABA over time. Blue shaded areas represent perfusion with 50 mM K+ containing aCSF. Red line indicates when 50 µM Ro60-0175 was added to the aCSF. (B) AUC values for the data presented in Panel A. Data shown are mean values ± SEMs for N = 3. * indicates $p < 0.05$. ($F(2,8) = 75$, $p<0.001$)

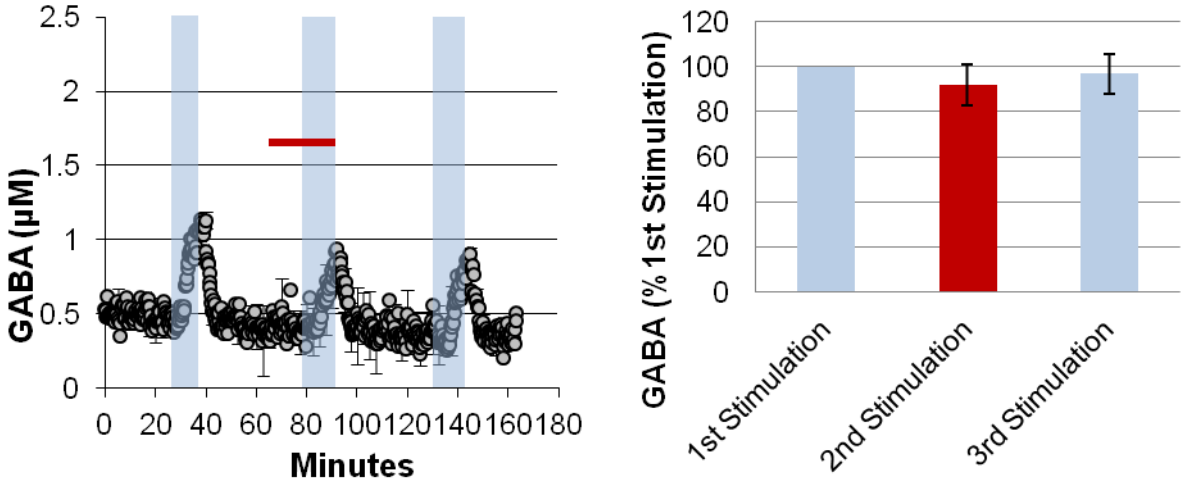

Figure 5-14. (-)-trans-p-Me-PAT had no effect on K+-stimulated GABA release in the NAc. (A) The concentration of GABA over time. Blue shaded areas represent perfusion with 50 mM K+ containing aCSF. Red line indicates when 50 µM (-)-trans-p-Me-PAT was added to the aCSF. (B) AUC values for the data presented in Panel A. Data shown are mean values ± SEMs for N = 3. * indicates $p < 0.05$. ($F(2,8) = 0.28$, $p>0.05$)

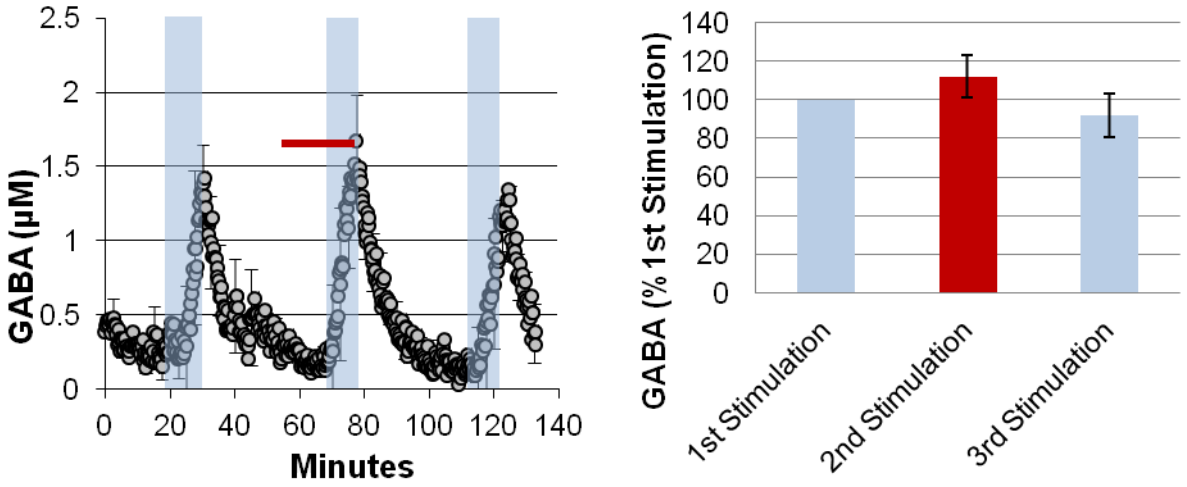

Figure 5-15. (+)-trans-PAT had no effect on K+-stimulated GABA release in the NAc. (A) The concentration of GABA over time. Blue shaded areas represent perfusion with 50 mM K+ containing aCSF. Red line indicates when 50 µM (+)-trans-PAT was added to the aCSF. (B) AUC values for the data presented in Panel A. Data shown are mean values ± SEMs for N = 3. * indicates $p < 0.05$. ($F(2,8) = 2.4$, $p>0.05$)

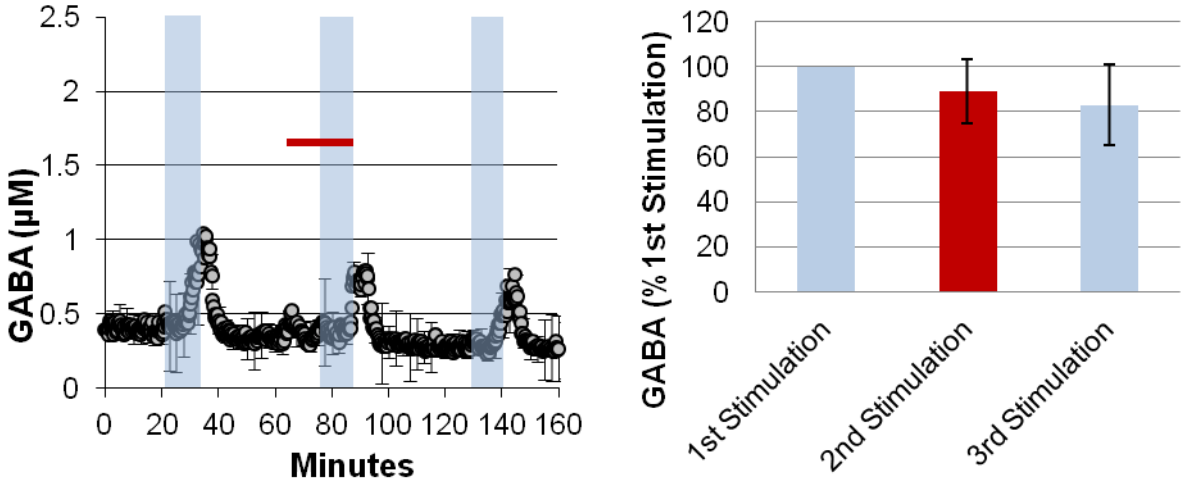

Figure 5-16. (-)-trans-CAT had no effect on K+-stimulated GABA release in the NAc. (A) The concentration of GABA over time. Blue shaded areas represent perfusion with 50 mM K+ containing aCSF. Red line indicates when 50 µM (-)-trans-CAT was added to the aCSF. (B) AUC values for the data presented in Panel A. Data shown are mean values ± SEMs for N = 3. * indicates $p < 0.05$. ($F(2,8) = 0.55$, $p>0.05$)

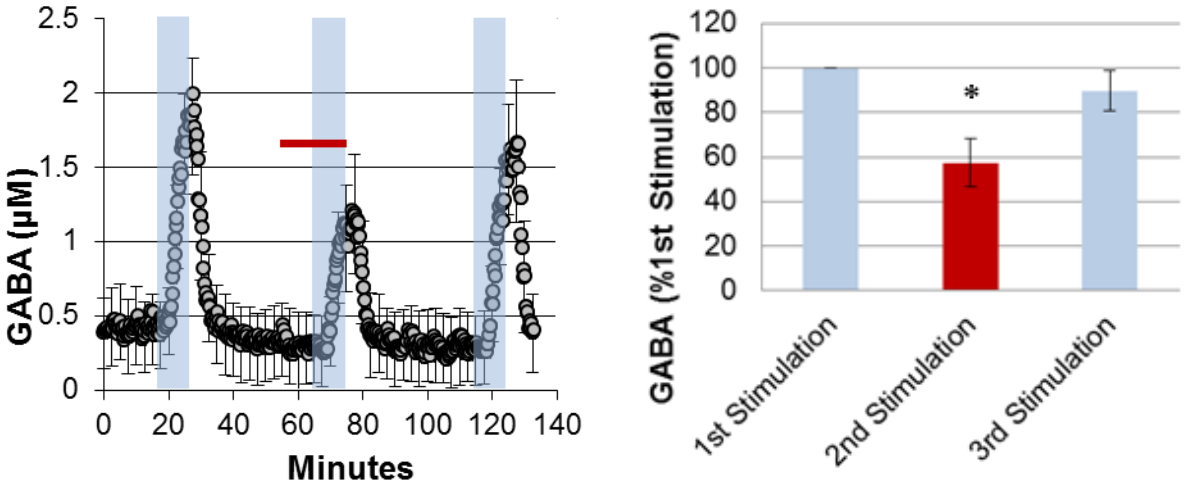

Figure 5-17. (-)-trans-PAT decreased K+-stimulated GABA release in the striatum. (A) The concentration of GABA over time. Blue shaded areas represent perfusion with 50 mM K+ containing aCSF. Red line indicates when 50 µM (-)-trans-PAT was added to the aCSF. (B) AUC values for the data presented in Panel A. Data shown are mean values ± SEMs for N = 4. * indicates $p < 0.05$. ($F(2,11) = 120$, $p<0.001$)

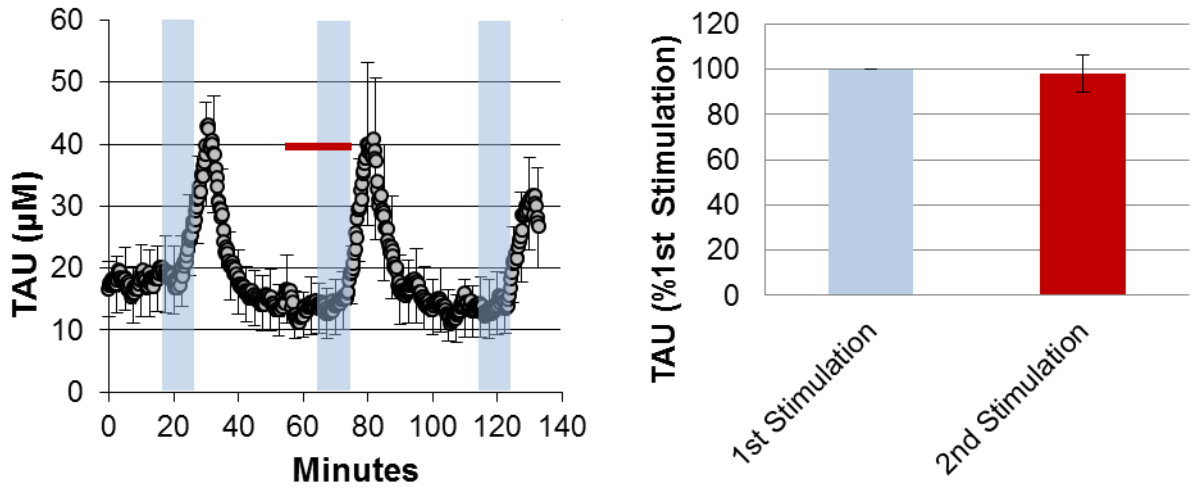

Figure 5-18. (-)-trans-PAT had no effect on K+-stimulated taurine release in the striatum. (A) The concentration of taurine over time. Blue shaded areas represent perfusion with 50 mM K+ containing aCSF. Red line indicates when 50 µM (-)-trans-PAT was added to the aCSF. (B) AUC values for the data presented in Panel A. Data shown are mean values ± SEMs for N = 4. * indicates $p < 0.05$. ($t(2) = 076$, $p>0.05$)

CHAPTER 6
GENERAL DISCUSSION

Discussion

Alcoholism is a complex disorder in humans that lacks highly effective pharmacotherapies (Edwards et al., 2011). An estimated 17.8% of Americans have abused alcohol and 12.5% of Americans have demonstrated alcohol dependence sometime during their life (Hasin et al., 2007). In the current study, we investigated the pharmacotheraputic potential of 5-HT_{2C} agonists using the "jello shot" model of alcoholism and examined the mechanism of action of these drugs in reward centers of the brain. This dissertation in total provides encouraging evidence for functionally selective agonists of the 5-HT_{2C} receptor acting as pharmacotherapy for alcoholism.

"Jello Shot" Model Of Alcoholism

The "jello shot" model of alcoholism demonstrates physiologically relevant self-administration of ethanol and shows an ADE after 3 week deprivation. An ideal model of alcoholism should have the following 7 attributes (Cicero et. al., 1971):

- oral self-administration of ethanol
- ethanol consumed should result in pharmacologically relevant dose
- ethanol should be consumed for post-ingestive pharmacological effects
- ethanol should be positively reinforcing
- chronic ethanol consumption should lead to metabolic and functional tolerance
- chronic ethanol consumption should lead to dependence
- animals should display characteristics associated with relapse

The "jello shot" model of alcoholism clearly demonstrates some of the above attributes of an ideal model of alcoholism. The rats voluntarily self-administer ethanol in a sweetened vehicle (Rowland et. al., 2005). Pharmacologically relevant dose of ethanol has been observed previously (Peris et. al., 2006), and the current study demonstrated pharmacologically relevant doses with 0.85 ± 0.21 g/kg basal ethanol consumption

during a 30 minute operant session. Additionally, for the first time using this model, the characteristics associated with relapse were explored when looking at the ADE. Animals deprived of ethanol for 3 weeks were shown to greatly increase consumption after reinstatement. The other 4 attributes are not covered in this dissertation though there are previous studies examining aspects of positive reinforcement such as motivation for ethanol reinforcements (Li et. al., 2009). Overall, the ADE experiments add support to this animal model mirroring the beginning pattern of human alcohol consumption.

Mesolimbic Pathway

The mesolimbic pathway is widely known to be important in the development of addiction. Changes in neurotransmission in this pathway mediate the rewarding effects of many substances of abuse including alcohol. One of the most prevalent modulators of neurotransmission in the mesolimbic pathway is GABA. Modulating GABA release in the mesolimbic pathway represents a potential target to reduce the rewarding value of ethanol.

Ethanol has complex effects on GABA release in the mesolimbic pathway. Microdialysis studies have shown that systemic ethanol administration does not change GABA concentrations in the NAc (Smith et. al, 2004) or VTA (Kemppainen et. al., 2010). This conflicts with the electrophysiology studies which demonstrate that ethanol increases spontaneous and evoked GABA release in the VTA and numerous other brain regions (Kelm et. al., 2011). Apparently, the net effect of ethanol on GABA concentration is negligible but ethanol causes significant effects on individual GABA neurons. This supports the hypothesis of addictive substances increasing GABA release onto inhibitory neurons that then project onto dopaminergic neurons. To put it

simply, ethanol causes GABA to inhibit neurons that would otherwise inhibit dopamine release. Thus an increase in DA concentrations is seen and the reward value of the drug is perceived. This explanation was first used to explain the addictive properties of benzodiazepines (Tan et al., 2010) and quickly applied to alcoholism (Kelm et. al., 2011).

The above mechanism of action for ethanol is problematic in terms of finding a singular target for potential pharmacotherapy. This is because both of the receptors in the pathway are for the same ligand, GABA, and treatment would require antagonism of the first and agonism of the second. When looking at the current available pharmacotherapies, topiramate directly and naltrexone indirectly have the ability to modulate $GABA_A$ receptors. Naltrexone acts as an indirect GABA agonist (Johnson and North, 1992) and naltrexone's mechanism of action for decreasing ethanol consumption is partially mediated by the chloride channel associated with $GABA_A$ receptors (Gewiss et al., 1994). Topiramate, a $GABA_A$ receptor positive modulator and AMPA receptor antagonist, also has therapeutic effects for alcoholics (Johnson et al., 2003). Baclofen, a $GABA_B$ agonist, has demonstrated the ability to reduce ethanol withdrawal, craving, and intake (Colombo et al., 2004). $GABA_{A/B}$ receptors are expressed throughout the mesolimbic pathway (Chester et al., 2002; Li et al., 2004) and because both GABA and dopamine neurons express both $GABA_{A/B}$ receptors it is difficult to design a pharmacotherapy that inhibits the GABA receptors on GABA neurons but not GABA receptors on dopamine neurons. These three drugs provide strong evidence that modulating GABA in the mesolimbic pathway represents a pharmacotherapeutic target but improved results may be observed with the ability to selectively modulate the GABA

neurons in the reward pathway. Specifically, an alcoholism treatment could decrease GABAergic drive onto GABA neurons that then project onto DA neurons.

5-HT_{2C} receptors represent an elusive target for alcoholism pharmacotherapy because of their ability to modulate dopamine in the mesolimbic pathway (Di Matteo et al., 2001). Agonism of this receptor increases GABA release in the VTA and blocks the ethanol induced increases in VTA dopamine neuron firing (Theile et al., 2009). The current study demonstrates that 5-HT_{2C} agonism decreases stimulated GABA release in the NAc. Together, 5-HT_{2C} receptor agonism decreases NAc and increases VTA GABA concentrations. These brain region specific modulations of GABA in the mesolimbic pathway via 5-HT_{2C} agonists represent a powerful pharmacotherapy for alcoholism.

PAT Analogs

Several novel PAT analogs have demonstrated the ability to decrease K+-stimulated GABA release in the NAc. This decrease has been shown to be mediated specifically by 5-HT_{2C} receptors. Another 5-HT_{2C} receptor agonist, Ro60-0175 showed the same decrease in K+-stimulated GABA release. It is evident that agonists for the 5-HT_{2C} receptor are able to modulate GABA in the NAc. The 5-HT_{2C} agonists (-)-trans-PAT and Ro60-0175 tested in both microdialysis and operant responding experiments, reduced K+-stimulated GABA in the NAc and consumption of ethanol containing gelatin. The importance of 5-HT_{2C} agonists in decreasing ethanol consumption is clear and there is strong evidence that the mechanism by which these drugs alter behavior is through GABA.

Future Studies

This dissertation clearly demonstrated the importance of the 5-HT_{2C} receptor in treating alcoholism. There are a few aspects of this work that remain unclear or

untested. This includes experiments surrounding the inability of (-)-m-Br-trans-PAT to alter the ADE, and the lack of effect of (-)-trans-PAT on progressive ratio responding for ethanol gel.

To clarify the results of testing (-)-m-Br-trans-PAT in Chapter 4, the affinity and functional studies for both (-)-m-Br-trans-PAT and (-)-trans-PAT should be performed using rat 5-HT_{2C} receptors as opposed to human. This may clarify why these two compounds had similar human affinity values and functional profile but only (-)-trans-PAT was able to reduce voluntary ethanol consumption and the ADE. To further explore (-)-m-Br-trans-PAT, a full dose response curve on voluntary FR5 consumption of both ethanol and plain gelatin similar to the experiments performed with (-)-trans-PAT (Figure 3-3 and Figure 3-5) should be performed. Then, knowing the lowest effective dose, repeat the ADE experiment to determine if (-)-trans-m-Br-PAT alters ethanol consumption after a deprivation.

There was a significant difference in breakpoints observed during PR10 operant sessions using (-)-trans-PAT responding for 0.28 g of ethanol containing gelatin but not with 0.15 g. Obviously if the reinforcer is larger, the animals will work harder for another administration resulting in a larger breakpoint. With the breakpoints so low responding for 0.15 g of gelatin there may have been a floor effect where the breakpoints couldn't go lower. Using 0.28 g of gelatin as a reward solved this problem and allowed the visualization of (-)-trans-PAT reducing the rewarding value of ethanol specifically. (-)-trans-PAT decreases motivation for ethanol but not the plain gelatin vehicle.

The current work went into great detail about the role of 5-HT_{2C} receptor agonism and K+-stimulated GABA concentrations in the NAc. While this setup yields very

consistent information about stimulated release of GABA and taurine, a more alcoholism focused project would be to use ethanol as the GABA stimulating agent. Ethanol (1 mol/L) reverse dialyzed into the central nucleus of the amygdala has been shown to stimulate GABA release measured by microdialysis (Roberto et. al., 2010). A literature search did not show any experiments using this ethanol reverse dialyzation technique in the NAc. After a study to determine if reverse dialyzed ethanol will significantly stimulate GABA release in the NAc, it would then be possible to test novel PAT on ethanol stimulated GABA release. The ethanol induced GABA release would be more directly related to alcoholism than the K+-stimulated GABA release.

Lastly, there are other neurotransmitters involved in alcoholism besides GABA and DA and they are currently understudied. For example, increases in glycine are associated with anticipation before an operant session responding for ethanol containing gelatin (Li et. al., 2008). Administering PAT before an operant session may decrease this anticipatory glycine and therefore demonstrate that PAT decreases anticipation for ethanol. This would further bolster PAT as a pharmacotherapy that decreases voluntary ethanol consumption, prevents the ADE seen in relapsing alcoholics, and reduces daily anticipation for ethanol.

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BIOGRAPHICAL SKETCH

James was born to Dan and Peggy Kasper. He grew up in Lisbon, Wisconsin and graduated from Hamilton High School in 2001. He then attended University of Wisconsin-Eau Claire and graduated in 2006 with a Bachelor's of Science degree. After college he attended University of Florida under the guidance of Dr. Joanna Peris where he received his Ph.D in the summer of 2012.