NEURONAL MECHANISM OF INCREASED MOTIVATION FOR OPERANT ETHANOL SELF-ADMINISTRATION CAUSED BY INTERMITTENT ETHANOL BINGE EXPOSURES

By

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To my parents: Shiqiao Li and Hongji Tian and my husband Yushi Qiu
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Binge drinking is thought to be one of the most important initial steps for the development of alcoholism in the United States. In this study, we investigated the neurochemical mechanism of how binge ethanol exposure may increase motivation for ethanol. First, we characterized interactions of amino acids and dopamine (DA) release in nucleus accumbens (NAC) after optimization of detection of DA using a spiking method with capillary electrophoresis. Second, we developed an animal model of increased motivation for alcohol consumption (IMAC) using intermittent binge ethanol exposures. To determine how subsequent ethanol consumption on a progressive ratio (PR) schedule was affected, rats received four pairs of either 3 g/kg ethanol “binge” injections or saline injections during periods of plain gel reinforcement. Breakpoints (BP) and ethanol consumption rose 40% in the ethanol binge group by the fourth set of binge injections with no change in plain gel responding. Third, we measured the level of amino acids in NAC during operant responding for ethanol gel or plain gel in ethanol-naive rats. We observed a significant increase of taurine during ethanol gel responding which was well correlated with ethanol consumption. Moreover, significant increases of glycine were observed during both ethanol and plain gel responding. Characterization of this glycine increase proved that the
phenomenon is specific for NAC and is related to the amount of anticipation occurring during operant sessions. Fourth, we employed the IMAC model to determine whether a history of repeated high dose ethanol exposures influenced the neurotransmitter response to subsequent operant ethanol self-administration or to an IP alcohol binge injection. Our results demonstrated that there was greater release of taurine, DA, glycine and glutamate in the NAC of the binge rats during 10% ethanol gelatin responding and when all rats received a binge exposure of ethanol, compared to no or very little release in the control rats. Overall, binge ethanol exposure caused a long-lasting imbalance of inhibitory and excitatory neurotransmission upon subsequent ethanol intake, which may underlie the neural mechanism of increased motivation for ethanol. These changes may play a key role in understanding the development of alcoholism.
CHAPTER 1
LITERATURE REVIEW

Alcoholism

Currently, over 15 million Americans have alcohol related problems including alcohol abuse and alcohol dependence. Approximately 43% of Americans have been exposed to alcoholism in their families. The total cost of alcohol related problems is $184 billion per year, compared to $114.2 billion for other drug problems and $137 billion for smoking (Shalala, 2000). In the United States, someone is killed by an alcohol related traffic accident every 30 mins. As a total, alcohol contributes to 100,000 deaths every year and causes about half of all driving fatalities (Narconon, 2004). Therefore, alcohol is the number 1 drug problem in U.S. In order to stop this alcoholism, it is important to understand how normal drinkers develop into alcohol abusers or alcoholics via the process of addiction.

Addiction

Drug addiction has been conceptualized as a chronically relapsing disorder characterized by a compulsion to take the drug and a loss of control in limiting intake. Drug-taking behavior progresses from impulsivity to compulsivity in a three-stage cycle: preoccupation-anticipation, binge-intoxication, and withdrawal-negative affect (Koob and Le Moal, 2001, 1997). This cycle can be understood through two different theoretical perspectives: experimental psychology and neurobiology. Experimental psychology is generally considered as a branch of psychology that uses experimental methods to study psychological issues while in the case of addiction cycle, experimental psychology is also defined as positive and negative reinforcement framework. Positive reinforcement of drug taking and the pleasurable effects through which stimuli increase the probability of drug-taking behavior happens in the impulsive stage (binge-intoxication). In contrast, negative reinforcement of drug taking occurs in the compulsive stage during which
withdrawal from drug taking causes a negative affective state. Removal of the aversive state increases the probability of the response. As the beginning step of addiction, preoccupation-anticipation can be correlated with both positive and negative reinforcements (Koob and Le Moal, 2005; Koob, 2006).

On the other hand, several neurobiological hypotheses have been proposed to explain the changes in motivation for drug seeking that reflect compulsive use. One of best studied hypotheses is psychomotor sensitization. Psychomotor sensitization has been defined as increased locomotor activation following repeated drug administration. In this hypothesis, a shift in an incentive-salience state also called “drug wanting” is proposed to also be progressively increased by repeated exposure to drugs of abuse. Counteradaptation is another well studied hypothesis in which the acute effect of a drug is opposed by regulatory changes that exert aversive states. In this opponent-process theory, positive hedonic effects occur immediately after the pharmacological activation of the reward system. In contrast, the negative hedonic effects emerge later, persist, and get larger with repeated exposure (Solomon and Corbit, 1974; Siegel, 1975; Poulos and Cappell, 1991). In addition, the opponent-process theory is also able to be thought of as a part of a normal homeostatic limitation of reward function. When the opponent-process theory cannot compensate enough to return things to the original setpoint, a new setpoint is defined which is the process of allostasis.

**Binge Drinking**

Binge drinking is defined as a pattern of drinking that elevates blood alcohol concentration (BAC) to 0.8% or higher, normally consisting of five or more drinks per session for man and four or more for woman. Another definition is simply drinking to get drunk. In 2006, about 10.8 million adolescent aged 12 to 20 (28.3 percent) reported using alcohol. Approximately 7.2 million (19.0 percent) were binge drinkers and 2.4 million (6.2 percent) were heavy drinkers in
the United State (SAMHSA, 2006). In addition, about 90% of the alcohol consumed by youth (under age 21) is in the form of binge drinks, which is 15% higher than the alcohol consumed by adults in the form of binge drinks (2001 National Household Survey on drug abuse). Clinical alcohol studies indicate that the more binge exposure people have in adolescence and young adulthood, the higher chance they will develop to be alcoholics (Enoch, 2006). Therefore, binge drinking has been considered to be one of the most important beginning steps for the development of alcoholism. In current experiment, we investigated the neurochemical mechanisms caused by binge exposures and how they may effect the development of alcoholism.

**The Mechanism of CNS Effects of Ethanol**

**Ion Channels**

For many years, ethanol was thought to act nonspecifically by disrupting neuronal lipid bilayers. However, it now is believed that the primary molecular sites of action of ethanol are specific proteins particularly including ion channels and intracellular signaling cascades. A number of ion channels in the CNS are directly affected by pharmacologically relevant concentrations of ethanol, especially ligand-gated and G protein-couple receptor families and voltage-gated ion channels. A large number of studies point out that gamma-aminobutyrate type A (GABA$_A$) receptors, the primary mediators of inhibitory neurotransmission in the brain, play central roles in both short- and long-term effects of ethanol in CNS. Ethanol exposure can positively modulate GABA$_A$ function which possible act through TM2 and TM3 regions and the extracellular loop between in $\rho$ subunit (Ye et al., 1998). Research conducted over the past several years also suggests that $\delta$ subunit and $\beta$ subunit-containing GABA$_A$Rs respond in a relevant concentration range to ethanol (Santhakumar et al., 2007; Olsen et al., 2007). In addition, numerous behavior studies have shown that GABA$_A$-receptor antagonists and
antagonists at the benzodiazepine-binding site on $\text{GABA}_A$-receptors decrease alcohol consumption in animal models.

Nicotinic acetylcholine receptor is another predominant molecular target for alcohol. According to receptor subunit concentration and the concentrations of ethanol, it has been reported that ethanol can either enhance or inhibit the function of nicotinic receptors. Because many smokers are also heavy drinkers, the effect of ethanol on nicotinic receptors may be particularly important. In addition, several studies suggest that nicotine increases alcohol consumption in animal models. Furthermore, excitatory ionotropic glutamate receptors are also affected by ethanol. It has been shown that ethanol inhibits the function of N-methyl-D-aspartate (NMDA) and kainate receptors. Metabotropic glutamate receptors (mGlu) have also appeared to regulate many behavior actions of addictive drugs including ethanol. On the other hand, ethanol also inhibits N- and P/Q-type calcium channels and enhances the activity of calcium-activated potassium channels. Recent works also suggest that inwardly rectifying potassium channels are modulated by ethanol (Fleming et al., 2001; Davies, 2003). Overall, many neurotransmitter systems are affected by ethanol administration, which indicates that the neuronal mechanisms underlying ethanol action might involve mediation by multiple neurotransmitter systems rather than a single system. In the present studies, we simultaneously monitored DA and amino acid release following ethanol administration by using CE-LIFD, which may give possible mechanism for ethanol function.

**Protein Kinase and Signaling Enzymes**

In recent decades, many studies investigated the molecular changes in intracellular signaling cascades caused by alcohol. Generally, it is believed that pathways containing cyclic adenosine 3’,5’-monophosphate (cAMP)-dependent protein kinase A (PKA), protein kinase C (PKC), the tyrosine kinase Fyn, and phospholipase D (PLD) mediate the action of ethanol.
Ethanol modulates cAMP signaling through at least 2 mechanisms. First, ethanol inhibits type I equilibrative nucleoside transporter (ENT1), which causes the accumulation of extracellular adenosine (Nagy et al., 1990). The increased level of extracellular adenosine stimulates A2a receptors and thus activates Gs. This increases levels of intracellular cAMP and dissociates the regulatory subunits of PKA from catalytic subunits, allowing PKA to phosphorylate substrates such as CREB (Mailliard and Diamond 2004). It has been suggested that PKA phosphorylation of CREB in the nucleus accumbens may stimulate changes in gene expression that elevate ethanol self-administration. Another possible mechanism for ethanol regulation of cAMP signaling pathway involves stimulation of AC7, a specific isoform of AC. AC7 has the highest sensitivity to ethanol among the 9 known AC isoforms (Yoshimura and Tabakoff, 1995). Ethanol stimulates the phosphorylation of AC7 and enhances cAMP activity via a PKCδ-dependent mechanism (Merchenthaler et al., 1993). Then it initiates the activation of PKA and following phosphorylation of substrates.

Another important second-messenger system modulated by both acute and chronic alcohol exposure is the PI signal-transduction cascade. Previous studies demonstrate that chronic ethanol administration decreases the activity of phospholipase C (PLC) in the cerebral cortex of mice (Lin et al., 1993) and rats (Allison and Cicero, 1980), which may due to a decrease in the protein levels of the PLC-beta 1 isozyme but not of PLC-delta 1 or PLC-gamma 1 isozymes in the rat cerebral cortex (Pandey, 1996). As a key step in the PI-signaling cascade, protein kinase C (PKC) also has been shown to be modulated by acute or chronic ethanol exposure in a variety of cell systems (DePetrillo and Liou, 1993; DePetrillo, 1994; Battaini et al., 1989). Evidence from various reports indicates that PKC is an important regulator of alcohol-induced effects on many neurotransmitter receptors. The neurotransmitter receptors include GABA_A (Harris et al., 1995),
NMDA (Snell et al., 1994), serotonin2A (5-HT$_{2A}$) (Minami et al., 1997), and 5-HT$_{2C}$ (Sanna et al., 1994), and muscarinic (m1) receptors (Larsson et al., 1995).

On the other hand, many studies suggest that one of the major routes thought to produce intoxication of ethanol is via inhibition of NMDA receptors. Src family Tyrosine kinase Fyn is involved in the first mechanism that is hypothesized to modulate ethanol inhibition of NMDA receptor. Normally, when Fyn is inactive, it resides in a complex with NMDA receptors by binding to the scaffolding protein RACK1 (Yaka et al., 2003). Ethanol administration disrupts this interaction, allowing Fyn to phosphorylate NR2B (Miyakawa et al., 1997). Therefore, ethanol-induced inhibition of NMDA receptors is antagonized by the phosphorylation of NR2B by Fyn. The whole process occurs over several minutes and may contribute to acute tolerance of hippocampal NMDA receptors to inhibition by alcohol. Another mechanism involves DA- and cAMP-regulated-phosphoprotein (DARPP-32). Ethanol increases the firing rate of DA neuron in VTA (Gessa et al., 1985), thereby increasing the release of DA in NAC (Gonzales and Weiss, 1998), which activates D1 receptors, stimulating PKA and PKA-mediated phophorylation of DARPP-32 (Fienberg et al., 1998). Phospho-DARPP-32 inhibits function of serine/threonine phosphatase PP1 which normally dephosphorylate NR1 subunits. As a result, Phospho-DARPP-32 prevents NR1 dephosphorylation, diminishes the inhibitory effect of ethanol on NMDA receptor function, and stabilizes the resistance of NMDA receptors to alcohol (Maldve et al., 2002). Behavior studies show that DARPP-32 knock out mice have decreased ethanol self-administration and no conditioned place preference for ethanol (Risinger et al., 2001). In contrast to PKA and PKC, only very few studies investigate the interaction between phospholipase D (PLD) and ethanol in CNS. In general, PLD modulates the cellular function of ethanol via 2 ways: first, by preventing the generation of second messenger PA, and second, by generating a
novel phospholipid (PEth) that can affect the biophysical properties of cellular membranes and signal on its own. As a result, ethanol can affect the four main intracellular cascades which are shared by many neurotransmitter receptor systems.

**Biochemistry of Dopamine**

DA (4-(2-aminoethyl) benzene-1, 2-diol) is a member of the catecholamine family. It is a prominent neurotransmitter in the brain mediating several behavioral functions like movement, cognition, pleasure, and motivation. DA is synthesized from tyrosine which can cross the blood-brain-barrier through large neutral amino acid transporters. Tyrosine hydroxylase catalyzes tyrosine to dihydroxyphenylalanine (DOPA) requiring oxygen, iron and tetrahydrobiopterin as cofactors. DOPA can be converted to DA by DOPA decarboxylase using pyridoxal phosphate as a co-factor. There are two DA release mechanisms: vesicular release and transporter-mediated release (Raiteri et al., 1979). Vesicular release is calcium and voltage dependent while transporter-mediated release is voltage independent and only slightly calcium dependent (Pierce and Kalivas, 1997). For the vesicular release, DA is packaged after synthesis into vesicles which are then released into the synaptic left in response to a presynaptic action potential. On the other hand, transporter-mediated release is a total different story. For example, amphetamine induces DA release via reversal of the DA transporter. The possible explanation is that amphetamine, a lipophilic compound, can enter the vesicles to work as a weak base and increase the pH. The low level of acid inside of the vesicles and the low pH gradient across the vesicle membrane inhibits the uptake of DA into the vesicles and increases the cytoplasmic DA synthesis. In addition, it has also been proven that endogenous DA cannot reverse the transporters by itself in the absence of pharmacological stimulation, like application of amphetamine (Jones et al., 1998).

Released DA binds to specific DA receptors: D1, D2, D3, D4 and D5. There are two main groups of DA receptors: D1 (D1 and D5) and D2 (D2, D3 and D4). DA binds to D1 group
receptors activating Gs and then AC, increasing activity of cAMP and PKA, causing the phosphorylation of cellular proteins. In addition, activation of D1/D5 receptor may activate Gz or Go and then modulate the activity of calcium, potassium, and sodium channels. In contrast, activation of D2 receptors activates Gi and then decreases activity of AC, and decreases levels of cAMP. Additionally, activation of D2 receptors can also decrease the conductance of calcium through Go and activates potassium current through Gi.

The DA signal is terminated by reuptake to neurons via DA transporters. Then DA is enzymatically metabolized by monoamine oxidase (MAO) to homovanillic acid (HVA) or by catechol-O-methyl transferase (COMT) to 3-Methoxytyramine (3-MT). DA that is not broken down by enzymes is repackaged into vesicles for reuse.

**Mesolimbic Dopamine and Ethanol Reinforcement**

Although the exact mechanism of alcohol reinforcement is unknown, the most popular theory is that the dopaminergic system in the mesolimbic signal pathway is involved. In the past few decades, the anatomy of the mesolimbic system has been studied intensively. The mesolimbic DA system is comprised of a group of neuronal cell bodies projecting from the ventral tegmental area to several forebrain regions including the NAC, prefrontal cortex, amygdala, and hippocampus. Among those brain regions, the role of NAC in behavioral response to drug reward has been well studied. As early as 1987, Wise and Bozarth reported that mesolimbic DA plays a very important role in the rewarding effects of self-administration of addictive drugs. In addition, preclinical studies suggested that the dopaminergic system is involved in the experience of these pleasurable feelings (Wise and Bozarth, 1987). However, people switched the focus of their study from the rewarding to motivating actions of drugs since addicted and non-addicted persons could have same pleasurable feelings. Several recent studies suggest that the mesolimbic system is involved in the regulation of motivated behavior (Di Ciano
et al., 1998; Duvauchelle et al., 2000; Gratton and Wise, 1994). Moreover, the key role of NAC being the main interface for the transduction of motivation generated in the limbic system into motor responses has been emphasized since the late seventies (Mogenson et al., 1980). Some new theories have been postulated that DA may play a role in attentional signaling of reward (Schultz, 1997). Therefore, the role of mesolimbic DA in producing reinforcing qualities helps explain the addictive nature of alcohol.

About 2 decades ago, the first reports of ethanol induced-DA release in the NAC appeared in the literature (Imperato and Dichiara, 1986). Following that, a large number of laboratories replicated and extended the findings through all kinds of ethanol administration including 60% intraperitoneal administration (Imperato and Dichiara, 1986; Yoshimoto et al., 1992a, 1992b; Diana et al., 1992; Acquas et al., 1993; Tanda and Dichiara, 1998; Olive et al., 2000a; Lominac et al., 2006), 16% local perfusion of ethanol in NAC (Wozniak et al., 1990, 1991; Yoshimoto et al., 1996; Benjamin et al., 1993; Yan et al., 2003; Ericson et al., 2003), 18% ethanol self-administration (Nurmi et al., 1998; Englman et al., 2000, 2003; De Montis et al., 2004; Weiss et al., 1993, 1996; Melendez et al., 2002; Doyon et al., 2003) as well as very few intravenous administration studies (Bradberry, 2002). In addition, many studies investigated the neural mechanism of ethanol-induced DA release in NAC. The common accepted idea is ethanol may facilitate DA release in NAC by increasing the firing rate of dopaminergic neuron in VTA. There are two well studied hypotheses for the actions of ethanol involving GABA\textsubscript{A} receptors and potassium channels in VTA. GABA\textsubscript{A} receptors in VTA affect the excitation of dopaminergic neurons indirectly. It has been demonstrated that GABA\textsubscript{A} receptors are found on GABAergic interneurons in the VTA and ethanol-induced stimulation of GABA\textsubscript{A} receptors disinhibit dopaminergic neuronal activity (Stobbs et al., 2004; Klitenick et al., 1992). In contrast,
potassium channels in VTA affect the excitation of dopaminergic neurons directly which appears to be due to the reduction of potassium currents following spontaneous action potentials caused by ethanol (Brodie et al., 1999; Appel et al., 2003).

In support of the role of DA in ethanol reinforcement, behavior studies showed that ethanol self-administration is inhibited by pretreatment with D1-like and D2-like DA receptor agonists and antagonists (Zocchi et al., 2003; D’Souza et al., 2003). Accordingly, withdrawal in rats that have chronically consumed alcohol decreases DA release in the NAC, which dramatically reduces the firing of related neurons (Diana et al., 1993). However, the destruction of dopaminergic mesolimbic terminals by injecting 6-hydroxydopamine (6-OHDA) intracerebrally does not show consistent results. Some studies showed a decrease in ethanol consumption (Melchior and Myers, 1976) while others showed there is no significant effect of the lesion (Brown et al., 1977). These data suggest the existence of other alternative mechanisms independent of DA that contribute to the reinforcing properties of alcohol. One of these important mechanisms might be amino acid neurotransmitter systems in NAC.

**Biochemistry of Amino Acids**

**Taurine**

Taurine (known chemically as 2-aminoethanesulfonic acid) is the major content of bile. It can be found in almost all cells in the CNS, including neuronal cell bodies, glial cells, cerebellar granular and stellate cells, as well as in synaptosomes and synaptic vesicles (Huxtable, 1989). Taurine is synthesized through the cysteine sulfinic acid (CSA) pathway. In this pathway, CSA is synthesized from cysteine by cysteine dioxygenase (CSD) and then CSA decarboxylase produces hypotaurine. As the final product, taurine is regulated by hypotaurine dehydrogenase. Taurine is not commonly considered as a neurotransmitter since there is not a specific taurine receptor. However, there are taurine transporters on both neuronal and glial cells which function to
maintain the intracellular concentration of taurine greater than the extracellular concentration. Taurine is released by three mechanisms: neuronal depolarization, osmoregulation and the reversal of the taurine transporter. Taurine release due to the neurotransmission has several characteristics such as delay phenomena, small magnitude and influenced by animal age which have all contributed to its questionable status as a neurotransmitter. Taurine release due to osmoregulation begins with hypoosmotic conditions, which leads cells to rapidly swell. This is followed by the extrusion of osmolytes leading to cell volume recovery, a process called regulatory volume decrease (RVD) (Pasantes-Morales, 1993; Vitarella et al., 1994). Taurine has been well characterized as a preferential osmolyte released due to RVD in many cell types and in vivo (Pow et al., 2002; Estevez et al., 1999; Solis et al., 1988a).

It has been proposed that taurine has several biological roles including neuromodulation (Chepkova et al., 2002; Liljequist, 1993), antioxidation, osmoregulation (Schaffer et al., 2000), Ca2+ modulation (Huxtable, 1992), and membrane stabilization (Wright et al., 1986). Neuromodulation and osmoregulation have been considered as two most important roles of taurine in the CNS. Taurine has been suggested to increase chloride conductance (Hausser et al., 1992; Huxtable, 1992), causing the hyperpolarization of various neuronal cells. On the other hand, taurine is considered as a good osmolyte because it’s wide distribution in all the cells and poor diffusion abilities. In addition, taurine has been suggested to contribute the largest shift in osmolar equivalents caused by osmotic stress (Huxtable, 1989). For example, it has been demonstrated that osmotic stress leads to taurine release in various cells including astrocytes, erythrocytes, C6 glioma, and Ehrlich ascites tumor cells. Moreover, some studies also indicated that taurine release caused by neuronal and glial cell swelling (Kimelberg et al., 1990; Schousboe et al., 1990; Schousboe and Pasantes-Morales, 1992). The extracellular concentration of taurine
is greatly increased in vivo following perfusion of hypotonic solutions in the brain (Estevez et al., 1999; Solis et al., 1988b). One of the objectives of our current studies is to investigate the ethanol-induced taurine release during operant ethanol self-administration and the possible role of taurine in the development of alcoholism. Several previous studies have shown that taurine release increases in NAC following IP ethanol injection in a dose-dependent manner (Dahchour et al., 1994, 1996; Dahchour and De Witte, 2000; Smith et al., 2004). However, the exact mechanism of ethanol-induced taurine release is still unknown. Several studies have indicated that ethanol-induced taurine efflux is primarily due to osmoregulation (Quertemont et al., 2003).

**Glycine**

Glycine is the simplest amino acid. It is found in all mammalian body fluids and tissue proteins in substantial amounts. It is enriched in the medulla, spinal cord, and retina. Glycine can be formed from serine by reversible folate-dependent reaction catalyzed by the enzyme serine trans-hydroxymethylase. Serine itself can also be formed in nerve tissue from glucose via the intermediates 3-phosphoglycerate and 3-phosphoserine. In addition, glycine might be formed from glyoxylate via a transaminase reaction with glutamate. Overall, serine serves as the major precursor of glycine in the CNS. As an inhibitory neurotransmitter, glycine is stored into synaptic vesicles after synthesis. In response to depolarization, glycine releases into the synaptic cleft and binds to a specific ligand-gated chloride channel receptor on the post synaptic cell, increasing chloride conductance and causing IPSPs. The synaptic action of glycine ends by decreasing extracellular concentration of glycine via the reuptake process by specific high-affinity transporters located in neuronal and glial plasma membranes. Additionally, as a required co-agonist with glutamate for the NMDA receptor, glycine can allosterically regulate the NMDA receptor complex via a distinct glycine-binding site. Glycine is metabolized to serine through serine hydroxymethyltransferase and then to the glycolytic intermediates, 3-phosphoglycerate and
pyruvate by dehydratase. The glycine system has also recently emerged to be important for investigation of neural mechanism of ethanol. Most previous studies focused on the relationship between the glycine receptor (GlyR) or transporter (GlyT1) and ethanol. It is suggested that glycine and strychnine alter extracellular DA levels in the NAC, probably via GlyR stimulation and blockade, respectively (Molander et. al., 2005). Strychnine-sensitive GlyR in the NAC regulate both basal and ethanol-induced mesolimbic DA activity (Molander and Söderpalm, 2005a, 2005b). In addition, the taurine-induced DA increase, observed after ethanol administration, is completely blocked by perfusion of the competitive GlyR antagonist strychnine in the NAC (Ericson et. al., 2006). Therefore, GlyRs in the NAC might constitute targets for EtOH in its mesolimbic DA-activating effect. However, few studies have investigated the release of glycine in NAC during alcohol consumption. Our current studies investigated glycine release in NAC during ethanol self-administration and the possible role of glycine transmission in positive reinforcement and anticipation of reward.

**Glutamate**

Glutamate is the best known member of the excitatory neurotransmitter family. Glutamatergic neurons are particularly abundant in the cerebral cortex. They project to different subcortical parts of the brain, such as the nucleus accumbens, the hippocampus, and the substantia nigra. Because glutamate cannot cross the blood-brain-barrier, it is formed locally. Glutamate is synthesized in the nerve terminals from glutamine by the activity of glutamate dehydrogenase or by the transmutation of α-ketoglutarate through the Krebs cycle. Glutamate is released from a variety of sources through two different mechanisms, vesicular and non-vesicular mechanisms. The vesicular mechanism is thought to be similar to that described above. In the glutamate-containing nerve terminals, Glutamate is stored in synaptic vesicles and upon depolarization of the nerve terminal. It is released by a calcium-dependent exocytotic process.
The action of synaptic Glutamate is terminated by a high-affinity uptake process via the plasma membrane Glutamate transporter on the presynaptic membrane or on the glial cells. The Glutamate taken up into glial cells is converted by glutamine synthetase into glutamine, which is then transported via a low-affinity process into the neighboring nerve terminals, where it serves as a precursor for Glutamate. Glutamate binds to four types of glutamate receptors: NMDA receptor, kainate receptor, AMPA receptor and metabotropic receptor. Activation of non-NMDA receptors results in the immediate opening of an ion channel which allows more permeable to Na and K\(^+\) than Ca\(^{2+}\), causing fast excitatory synaptic transmission. On the other hand, activation of NMDA receptors requires the co-agonist glycine and causes excitatory synaptic transmission but not fast. Activation of metabotropic glutamate receptor causes the mobilization of intracellular calcium via the activation of phospholipase C.

The non-vesicular mechanism of glutamate release includes glial release and cystine/glutamate exchange (Danbolt, 2001). The glial release is due to reversal of glutamate transporters (Fillenz, 1995). Although the current studies mostly focus on vesicular release, the primary source of extracellular glutamate detected in the synaptic cleft seems to result from cystine/glutamate exchange. In microdialysis studies of nucleus accumbens, 50~70% of basal extracellular glutamate is released by cystine/glutamate exchange (Baker et al., 2002). In the present studies, we investigated glutamate release following intermittent ethanol binge exposures. Previous studies showed that ethanol inhibits NMDA (Lovinger and Zieglgansberger, 1996; Lovinger et al., 1989, Wirkner et al., 2000), kainate (Dildy-Mayfield and Harris, 1995) and metabotropic (Minami et al., 2003) glutamate receptors. Depending on the dose of ethanol administration, the change of glutamate release in NAC is biphasic. Acute low dose ethanol administration has been reported to increase glutamate release but high dose ethanol
administration decreases it (Moghaddam and Bolinao, 1994; Dahchour et al., 1994, 1996; Dahchour and De Witte, 2000; Selim and Bradberry, 1996; Piepponen et al., 2002; Szumlinski et al., 2005; Lominac et al., 2006). Moreover, with repeated ethanol injections, a sensitized glutamate release has also been observed in the NAC (Szumlinski et al., 2005, 2007).

**Neurotransmitter Interactions and Ethanol Function in NAC**

**Taurine, Dopamine and Glycine**

The taurine uptake inhibitor guanidinoethyl sulfonate produced a significant decrease in extracellular levels of aspartate, glutamate and glycine with no effect on extracellular DA levels (Olive et al., 2000b). However, acamprosate, an analog of taurine, has been shown to provoke a significant increase of extracellular DA in nucleus accumbens (Cano-Cebrian et al., 2003) and delay or suppress ethanol stimulated increases in NAC DA release (Olive et al., 2002). Behavioral studies further show that acamprosate dose-dependently reduces ethanol intake and preference (Cowen et al., 2005; Olive et al., 2002). On the other hand, glycine decreases accumbal DA levels in some rats but not others. As a result, GlyRs in NAC are tonically activated and of importance for regulating extracellular DA levels (Molander and Söderpalm, 2005a, 2005b). Recent electrophysiology work indicates that taurine binds mainly to glycine receptors in NAC (Jiang et al., 2004). Taurine can elevate DA levels in the rat nucleus accumbens and the elevation of taurine-induced DA is antagonized by strychnine (Ericson et al., 2006).

**DA and Glutamate**

NAC neurons receive convergent inputs from ventral tegmental area DA neurons and glutamate-containing neurons originating from medial prefrontal cortex, amygdala and hippocampus. Evidence exists for an interaction between DA and excitatory synaptic
transmission in NAC (Pennartz et al., 1992; Li and Kauer, 2004). Several studies suggest that DA receptors may presynaptically regulate the release of glutamate in NAC. Electrophysiological studies demonstrate D2-regulation of excitation of cortical-accumbens neurons (O’Donnell et al., 1994) and hippocampal-accumbens neurons (Yang et al., 1986) recorded in vitro. In contrast to D2 receptors, little evidence indicates that presynaptic DA D1 receptors attenuate excitatory limbic inputs to the rat nucleus accumbens (Pennartz et al., 1992). On the other hand, postsynaptic interaction between DA and glutamate in the NAC are critical for motivation, reward, and other behavioral functions that are disrupted after chronic exposure of drugs of abuse (Everitt and Wolf, 2002). For example, behavior studies indicate that simultaneous DA and glutamate receptor activation is required for the acquisition of lever press behavior to obtain food reinforcement (Smith-Roe and Kelley, 2000). Yet, little is known about the mechanisms that enable DA receptors to modulate glutamate transmission or glutamate-dependent forms of plasticity and mixed results have been reported to date in NAC on the DA-glutamate interaction. Pennartz et al. (1994) reported that there is no effect of DA on synaptic plasticity in the core subregion of NAC. While Li and Kauer (2004) found that DA inhibits long-term potentiation in vitro in NAC. These discrepancies may be due to different in vitro preparation because Floresco et al. (2001) found DA and NMDA receptor-dependent synaptic plasticity in NAC in vivo. In addition, Wolf et al. (2003) suggest that D1 receptors can control the fundamental mechanisms for regulating excitatory synaptic transmission and glutamate-dependent forms of plasticity in NAC through the investigation of D1 and AMPA receptor interactions.

**Taurine and Glutamate**

The interaction between taurine and glutamate has been barely studied in NAC but well investigated in other brain regions including hippocampus (Magnusson et al., 1991; Oja and
Saransaari, 1992; Shibanoki et al., 1993; Colivicchi et al., 1998) and striatum (Shibanoki et al., 1993; Segovia et al., 1997). These studies showed that the excitatory amino acid neurotransmitter glutamate can stimulate the release of taurine. In addition, one of the reasons why glutamate-taurine interactions are thought to be important is because taurine also can regulate excitatory ability of glutamate. Taurine can inhibit NMDA and kainate receptors (Kurachi and Aihara, 1985a, 1985b; Kurachi et al., 1983; Lehmann et al., 1984) and the agonists of NMDA and kainate receptors have been shown to induce taurine release in vivo (Del Arco and Mora, 1999; Del Arco et al., 2000; Menendez et al., 1989). The underlying mechanism of taurine release caused by stimulation of glutamate receptor agonists is unknown. One of most possible explanations is that the release of taurine is due to swelling of cells which to prevent excitotoxicity effect of glutamate receptor agonist. However, several studies demonstrated that the release of taurine is due to the nitric oxide cascade (Scheller et al., 2000) or NMDA dependent Ca2+ mobilization (Menendez et al., 1993; Rogers et al., 1990), but is not osmotically dependent (Shibanoki et al., 1993). In conclusion, there is a mutual interaction of taurine with excitatory amino acid function. Moreover, the interactions among all of these neurotransmitter systems may help us to explore the time course of different neurotransmitter release caused by ethanol action.

**Operant Conditioning**

One of the most valid preclinical/basic models of drug abuse is self-administration-based methods which can parallel various aspects of human conditions during drug consumption. Application of the methods allows insight of common neural mechanisms and therefore helps to identify strategies useful in the intervention regarding human drug consumption. According to behavioral criteria, self-administration procedures can be classified as operant and non-operant procedures. The non-operant procedures are restricted to oral self-administration procedures.
Operant or, more precisely operant conditioning, is defined as the use of consequences to modify
the occurrence and form of behavior.

Theoretically, the whole process of drug self-administration has been divided into two
components “motivation” and "consumption". The motivation part is the initiation of drug
consumption. It controls drug-seeking behavior and directs behavior toward sources of drug. In
contrast, consumption is directly correlated to maintenance and termination of drug intake.
Although it is presumed that both portions affect non-operant drug self-administration, the most
common dependent variables in the non-operant procedure are the amount of drug intake.
Therefore, motivation is not normally considered in the non-operant condition. On the other
hand, the operant procedure provides environmental and temporal cues for animals by using
well-defined sessions in different cages. In operant procedures, experimenter can vary how hard
the animal must work to obtain drug, frequency of drug presentation, and dose of drug per
reinforcement. In this way, it separates drug-seeking behavior from drug consumption.

The great majority of operant self-administration studies use fixed ratio (FR) schedules to
exploring patterns of rate of drug intake and preliminary screening of drugs with abuse liability.
However, the rate of drug self-administration in the simple FR schedule is insensitive to changes
in reinforcement efficacy of the drug. In order to circumvent this difficulty, a number of different
procedures have been introduced from 1973 to 1993. They include variable interval schedules
(Balster and Schuster, 1973), variable dose (Gerber and Wise, 1989) and discrete trials (Fitch
and Robert, 1993). The most widely used of these new methodologies has been the progressive
ratio (PR) schedule.

**Progressive Ratio**

PR schedule was firstly developed by Hodos and Valenstein to examine the reinforcing
efficacy of sweetened milk solutions and then has been adapted to study drug self-administration
The objective for the PR schedule is to increase the response requirement until the animal stops responding. Therefore, under this schedule, the requirement of response gradually increases after the delivery of each reinforcement and the final ratio of responses successfully completed is labeled the breaking point (BP). BP reflects the maximum effort that an animal will expend to receive a reinforcement, in another word, the motivation of the animal to self-administer a drug. For example, in the study of 6-OHDA lesions of NAC, rats that had stopped self-administering cocaine were expected to have some changes on self-administration of apomorphine. The FR schedule showed that the rate of apomorphine self-administration continued at prelesion rates (Roberts et al., 1989). In contrast, it was found that there was a significant increase of BP using PR schedule which reflected the development of DA receptor supersensitivity (Roberts, 1989). In addition, PR schedule can be useful to evaluate the effect of a variety of pretreatments. However, the PR schedule is not without limitations. The biggest problem is that PR schedules yield only a single measure from a whole session. In the present study, the feasibility of using this procedure for establishing operant self-administration of ethanol was determined. We chose fixed ratio (FR) schedules to establish baseline responding for ethanol reinforcement and progressive ratio (PR) schedules to explore subsequent maintenance of intake as a function of motivation.

**Microdialysis and CE-LIF**

Microdialysis is a technique used to monitor the chemistry of the extracellular space but can also be used to introduce a constant amount of biologically active substance into the extracellular cerebro-spinal fluid allowing for direct manipulation of neurotransmission. Prior to the development of microdialysis, many techniques have been applied to get accurate and continuous *in vivo* measurement of neurotransmitter concentrations in the brain, e.g., cortical cup and push-pull cannula. Compared to these methods, microdialysis is the only technique that can
collect any substance continuously from remote brain regions with a limited amount of tissue damage. Microdialysis has been used in all kinds of neurobiological science studies, such as monitoring levels of drugs, detecting basal level of neurotransmitters or changes induced by drugs, and determining the relationship between different behavior changes and neurotransmission. There are two main pitfalls of this technique. First, we can only collect a very small amount of sample and measurement of analytes in the sample may be limited by separation and detection techniques that require larger volumes. Second, the lesion of brain tissue caused by the insertion of the dialysis probe may change the original composition of the extracellular fluid in the brain as might constant perfusion with artificial cerebrospinal fluid (aCSF).

The microdialysis probe consists of a side-by-side arrangement of two glass capillaries, one of which is slightly shorter than the other, inserted into a semi-permeable membrane sack. Perfusion with a low rate of aCSF enters the sack from one end of the long glass capillary and flows out from the other end close to the bottom of the sack. Neurotransmitters diffuse from the extracellular fluid into the probe and are transferred for sample collection by the short capillary. The direction of the analyte flow across the membrane depends mainly on the concentration gradient which is correlated not only with the differences in concentration between sample and extracellular fluid but also with the velocity of flow inside the microdialysis sack.

Microdialysis samples can be analyzed in a number of ways: the most common one is high-performance liquid chromatography (HPLC); the most recent one is capillary electrophoresis with laser-induced fluorescence detection (CE-LIFD). Although HPLC is the most common technique employed to analyze the microdialysis samples, it requires different columns, mobile phases and separation conditions to detect catecholamine vs. amino acids. It also requires large injection volumes (10-20µL) and therefore a long dialysis sampling time,
usually 10 to 20 minutes. Each sample represents an average concentration value obtained during this 10 to 20 minutes. Therefore, this method cannot monitor rapid changes of extracellular chemicals associated with neurotransmission. On the other hand, CE-LIFD possesses a high mass sensitivity (Zhou et al., 1995; Robert et al., 1998), so it requires a very low-volume of sample (≈20nL). This decreases the sampling time from 10-20 minutes to 1s, which allows monitoring of rapid changes that may be correlated with neuronal events. Second, CE-LIFD allows separation of both amino acids and catecholamines simultaneously. In this point, CE-LIFD shows a particular advantage to analyze and monitor many neurotransmitters and related substances in extracellular fluid (Bert et al., 2002; Bowser and Kennedy, 2001). However, the validity of this method to differentiate neuronally relevant neurotransmitter release is still being evaluated.

**Summary**

Alcoholism is a severe disease that affects millions of American each year. As a common pattern of excessive alcohol use, binge drinking affects the highest percentage of drinking people, especially young people. Recent work suggests that people who had more binge exposure in adolescence and young adulthood may have a greater chance of developing alcoholism. Therefore, binge drinking is commonly considered as one of the beginning steps for the development of alcohol dependence. Alcohol dependence is a very complicated process that involves the regulation of numerous cellular functions. In general, the DA mesolimbic pathway is believed to be involved such that ethanol alters DA transmission. Moreover, either acute or chronic ethanol administration has been shown to affect a number of transmissions of amino acids in the CNS, including taurine, glutamate and glycine. Very few studies investigate the neuronal mechanism of binge exposure. However, examination into the changes of neuronal
transmissions of DA and amino acids may give insight into the neurochemical mechanisms of binge exposure contributing to the development of alcohol dependence. Therefore the studies in this dissertation were design to investigate the possible neuronal mechanism of increased motivation caused by binge exposures.

**Objectives**

The neuronal mechanism of development of alcoholism is far from clear. Due to a ubiquitous effect of ethanol on different neural systems, it is necessary to monitor transmission of both catecholamine and amino acids following binge exposures. In vivo microdialysis was employed to gain insight into the binge exposure-induced changes of catecholamine and amino acid levels.

The first aim of my research was to methodologically optimize CE-LIFD technique to simultaneously monitor DA and amino acids in NAC. Changes in neurotransmitter release may occur over a short time period during behavioral studies. Thus, in vivo detection of these transmitters should have a high temporal resolution. To date only fast-scan cyclic voltammetry (FSCV) and high-performance liquid chromatography (HPLC) have been used most often. However, both methods can not detect amino acids and catecholamine simultaneously. A newer method, CE has been recently adapted to measure in vivo the extracellular levels of amino acid with much high temporal resolution (15sec) which provides the means to exam the neural process. This aim included two steps: first, we applied various agents to increase DA level to the same range of other amino acids (see chapter 3). Although we successfully detected the drug-induced DA simultaneously with amino acids, this method was not feasible for behavior studies. Therefore, the second step was to bring the DA peak within the same range of amino acids by the spiking method which was demonstrated in chapter 6.
The second aim of my research was to develop a good rat model with increasing motivation of operant ethanol self-administration caused by binge exposures (CHAPTER 4). Sensitization phenomena have been implicated in the neuronal mechanisms involved in the development of drug dependence and abuse. Behavioral sensitization to ethanol has been widely studied. However, sensitization to ethanol indicated by either increases of drug intake or increases of motivation has barely been studied. Only a few previous studies give evidence for previous ethanol exposure leading to an increase of ethanol intake. For example, intermittent high dose exposure to alcohol leads to long-lasting increases in voluntary ethanol consumption in rats (Rimondini et al., 2002) and ethanol drinking is increased after repeated chronic ethanol exposure and withdrawal experience in C57BL/6 mice (Becker and Lopez, 2004). However, it is still unknown whether tolerance to reinforcement or sensitization of craving (and the corresponding neurochemical changes) causes the increase of ethanol intake following binge exposure. Therefore, our rat model termed as Increased Motivation of Alcohol Consumption (IMAC) provides a good tool to investigate the neurobiological consequences of repeated binge alcohol exposures in rodents.

The third aim of my research was to monitor the extracellular concentration of amino acids during operant ethanol self-administration (CHAPTER 5). Most studies investigating operant ethanol self-administration indicated that the dopaminergic system in the mesolimbic pathway is the most important mechanism. Previous studies suggested that operant self-administration of ethanol is associated with increases in extracellular levels of DA in the NAC (Weiss et al., 1993; Doyon et al., 2003). Naltrexone attenuates the rewarding properties of ethanol by interfering with ethanol-induced stimulation of DA activity in the NAC (Gonzales and Weiss, 1998). However, a recent study pointed out that a transient increase of DA level in NAC may be related to the
stimulus of ethanol presentation rather than its pharmacological action (Doyon et al., 2004). On the other hand, nondopaminergic mechanisms for regulation of operant ethanol self-administration are barely studied. However, there are a few studies reporting amino acid changes following nonoperant ethanol self-administration. For example, withdrawal from repeated ethanol administration causes increased basal levels of extracellular glutamate as measured by microdialysis (Dahchour and De Witte, 2003). An escalation of taurine level was observed to significantly correlate with the ethanol dose (Dahchour et al., 2000). Therefore, it is very important to know the changes of other neurotransmitters in the mesolimbic system, like glutamate, glycine and taurine, since they may be involved in the mechanisms underlying ethanol self-administration as well as those involved in positive reinforcement and anticipation of reward.

The fourth aim of my research was to investigate the possible neuronal mechanism of increasing motivation of operant ethanol self-administration caused by binge exposures (CHAPTER 6). Most behavior sensitization studies indicated that the extracellular concentration of DA increases following pretreatment of amphetamine, cocaine, nicotine, and ethanol (Pontieri et al., 1995). However, other studies suggest that DA level is decreased in NAC. For example, DA terminal function is reduced after cocaine binge self administration and deprivation (Mateo et al., 2005). Recent work demonstrates that binge-like ethanol consumption has no effect on DA release in NAC but sensitizes the release of glutamate (Szumlinski et al., 2007). The discrepancy on DA release following binge exposure may be due to the different ethanol administration and different subregion of NAC investigated. The present study employed the IMAC model to determine whether a history of repeated high dose ethanol exposures influences the
neurotransmitter response to subsequent operant ethanol self-administration or to an IP alcohol binge injection.
CHAPTER 2
GENERAL METHODS

Animals

Male and female Sprague-Dawley rats were used in this study. Male rats used in chapter 4 were approximately 6 months of age and weighed 485±11 g at the beginning of the study. Female rats used in chapter 3, 4 and 5 were approximately 6 weeks of age and weighed 200±8 g at the beginning of the study. Body weight was recorded weekly. All rats were conventionally individually housed in a vivarium with a 12:12 light dark cycle (lights off at 0900) and an ambient temperature maintained at 23±2 °C. Rat use was approved by the IACUC and was consistent with the NIH Guide for the Care and Use of Laboratory Animals. Purina 5001 Rodent Chow and tap water was available ad libitum at all times except during daily operant sessions.

Chemicals

NaCl, CaCl₂, KCl, MgSO₄, o-phthaldialdehyde (OPA), β-mercaptoethanol (BME), sodium tetraborate, and hydroxypropyl-β-cyclodextrin (HPBCD), Ethylene-bis (oxyethylenenitrilo) tetraacetic acid (EGTA), amino acids, sodium borate, pargyline, sulpiride, quinelorane and 2-phenylethylamine hydrochloride were obtained from Sigma-Aldrich (St. Louis, MO). Ethanol (95%) was obtained from Aaper Alcohols and Chemicals, Shelbyville KY. Saline was purchased from Baxter Healthcare Corporation, Deerfield, IL. Sodium phosphate and potassium cyanide were from Fisher Scientific (Pittsburgh, PA). All aqueous solutions were prepared with deionized water (Milli-Q system, Millipore, Milford, MA) and filtered through a 0.2 µm nylon membrane filter.

Behavioral Apparatus for CHAPTER 4, 5 and 6

Each chamber consisted of a test cage housed in a sound attenuating isolation cubicle. Test cages measured approximately 50.8 cm W × 25.4 cm D × 30.5 cm H with a grid non shock floor.
and solid walls. Two levers protruded through one wall of the chamber, placed symmetrically on either side of an optical lickometer. The lickometer was equipped with a photobeam to detect licks. A standard drinking spout was affixed into the lickometer, such that the spout protruded into the chamber. A 20 mL syringe filled with 10% gelatin was placed into a syringe pump inside the cubicle, outside of the test cage before each session. The syringe was connected to tubing with an inside diameter of 3.2 mm and an outside diameter of 4.8 mm by Parafilm. The tubing led into the drinking spout. The drinking spouts were cleaned daily with hot water. Syringes with gelatin were refrigerated between sessions. The test cages and accessories were modular units purchased from Coulbourn Instruments (Allentown, PA). L2T2 v.4.00 operating software (Coulbourn Instruments, Allentown, PA) for chapter 4 and Graphic State 5.2 operating software (Coulbourn Instruments, Allentown, PA) for chapter 5 and 6 were used to control gelatin delivery and collect behavioral data.

**Blood Ethanol Determinations for CHAPTER 4**

The ethanol content of 10 µL blood samples taken from the tail at the end of the operant session was determined by the alcohol dehydrogenase assay (Kristoffersen and Smith-Kielland, 2005) adapted for small volume samples (Peris et al., 2006). Each sample was stored in 100 µL 0.6 M glycine buffer (pH 9.2) on ice until all samples were collected. A standard curve was prepared containing 10 µL of 0, 0.32, 0.63, 1.25, 2.5, 5, 10 and 20 mM ethanol added to 100 µL glycine buffer both before and after sample collection, to ensure that ethanol evaporation was minimal during the sampling period. After all samples were collected, 100 µL of enzyme solution (0.88 mg NAD and 0.29 mg ADH per mL of 0.6 M glycine buffer) was added to each sample and then all samples were incubated at 37°C in a shaking water bath for 20 minutes. Samples were then kept on ice for at least 5 minutes, before transfer to a 96-well plate (Costar, Corning NY) on ice. Absorbance was read at 340 nm using a Synergy HT plate reader (Bio-Tek, 41
Absorbance was converted to mM ethanol by the use of a polynomial equation since at these low volumes and concentrations. The standard curve is not linear. Fits were generally better than $R^2 = 0.98$, with less than 10% error when the absorbance of standard curve values are recalculated back to mM concentrations.

**Microdialysis and Surgeries for CHAPTER 3**

The method for microdialysis is that described previously (Smith et al., 2004). Each rat was anesthetized with ketamine /xylazine (approximately 86 mg/kg ketamine and 13 mg/kg xylazine) and placed in a stereotaxic apparatus for surgical implantation of a guide cannula. The guide cannula (21 gauge; Plastics One, Roanoke, VA) was positioned above the left side of brain using the following coordinates: +1.6 anteroposterior, +1.7 lateral for the shell subregion and +1.8 lateral for the core subregion, -6.2 dorsoventral. The guide cannula was secured to the skull with dental cement anchored by two stainless steel screws. After surgery, each rat was housed separately and allowed to recover for at least 7 days with daily handling to facilitate probe insertion.

Microdialysis probes (o.d. 270 µm; active length 2 mm for NAC; cellulose membrane, 13,000 molecular weight cut-off) was constructed by the method of Pettit and Justice (1989). The probe was perfused with artificial cerebrospinal fluid (aCSF; 145 mM NaCl, 2.7 mM KCl, 1.0 mM MgCl$_2$, 1.2 mM CaCl$_2$, 0.45mM monobasic phosphate, 1.55 dibasic phosphate, pH 7.0-7.4) at 1 µL/min (CMA 100 microinjection pump; 1 mL Exmire syringe) and flow was accurately calibrated prior to standards testing (see below). On the morning of the experiment, each rat was moved into the dialysis cage (40 cm X 40 cm X 30 cm high) provided with bedding, water bottle and food, and the dialysis probe was inserted under light halothane anesthesia. The probe was perfused with aCSF for 2 hours to allow neurotransmitter concentrations to stabilize.
The amino acid concentrations in dialysate samples were analyzed on-line by CE-LIF as described below. A calibration curve was generated before the experiment by collecting 30 samples each after placing the tip of the dialysis probe in three different concentrations of a 37°C, well-stirred standard solution containing glutamate, aspartate, DA, serotonin, GABA, taurine, serine, glutamine, cystine, and glycine. The mean relative fluorescence of these samples was converted to concentration by plotting peak height versus standard concentration. Thus, extraction fraction was automatically accounted for except for minor differences in diffusion based on analytes in solution versus in a matrix.

**Microdialysis and Surgeries for Freely-moving Animals in CHAPTER 5 and 6**

Each rat was anesthetized with isoflurane and placed in a stereotaxic apparatus for surgical implantation of a guide cannula. The guide cannula (21 gauge; Plastics One, Roanoke, VA) was positioned to end within 1 mm above the left side of NAC or hippocampus using the following flat skull coordinates from bregma: +1.8 anteroposterior, +1.3 lateral, -6.2 dorsoventral for NAC, -5.0 anteroposterior, +5.0 lateral, -6.2 dorsoventral for hippocampus. The guide cannula was secured to the skull with dental cement anchored by two stainless steel screws. After surgery, each rat was individually housed and allowed to recover for at least 2 days before daily ethanol gel or pain gel operant session was restarted. Rats then resumed operant sessions but were now tethered to a swivel in the top of the operant chamber by a spring attached to a clip embedded in the dental cement on their skulls. Habituation to the tethering procedure continued for 3~7 daily sessions.

Microdialysis probes (o.d. 270 µm; active length 2 mm; cellulose membrane, 13,000 molecular weight cut-off) were constructed by the method of Pettit and Justice (1989). The probe was perfused with artificial cerebrospinal fluid (aCSF; 145 mM NaCl, 2.7 mM KCl, 1.0 mM...
MgCl₂, 1.2 mM CaCl₂, 0.45 mM monobasic phosphate, 1.55 mM dibasic phosphate, pH 7.0-7.4) at 1 µL/min (CMA 100 microinjection pump; 1 mL Exmire syringe). A calibration curve was generated for each probe by collecting a total of 175 samples after placing the tip of the dialysis probe in seven different concentrations (0.63 - 20 µM) of a 37°C, well-stirred standard solution containing glutamate, aspartate, GABA, taurine, serine, glutamine and glycine. The mean relative fluorescence of these samples was converted to concentration by plotting peak height versus standard concentration. Thus, extraction fraction in brain dialysate samples was automatically accounted for except for possible minor differences in diffusion based on analytes in solution versus in a matrix.

At this point, each rat (in the Chapter 5) was moved into the dialysis cage which was either an operant chamber without the sound attenuating chamber or a home cage provided with bedding, water bottle and food. The dialysis probe was inserted into NAC or hippocampus without anesthesia 2 hrs before the start of the 50 min operant session or 1 hr free access period. The levels of amino acids were analyzed on-line every 15 sec during the 2 hr baseline period, the operant session or 1 hr gel presentation, and for 1~2 additional hrs after.

In Chapter 6, each rat was moved into the dialysis cage which was an operant chamber without the sound attenuating chamber provided with bedding, water bottle and food. The dialysis probe was inserted into NAC without anesthesia 2 hrs before the start of the 30 min operant session. The levels of amino acids were analyzed on-line every 15 sec during the 2 hr baseline period; the 30-min operant ethanol responding session and 1 additional hr after; 30-min operant plain gel responding session and 1 additional hr after; and 2 hr following 3g/kg ethanol ip injection.
Amino acids and GABA were measured by CE-LIF utilizing the procedure established by Lada & Kennedy (1996) and then further refined by Bowser and Kennedy (2001). Briefly, analytes in the dialysate containing primary amine moieties were derivatized on-line by mixing dialysate flow with a 1 μL/min stream of OPA derivatization solution (10 mM OPA, 40 mM BME, 36 mM borate, 0.81 mM HPBCD, 10% MeOH (v/v), pH 10.5), as well as a 5 μM solution of 2-aminoadipic acid (internal standard), with a reaction time of approximately 30 sec. Again, ongoing optimization experiments may lead to modifications in the derivatization and/or separation conditions. The reaction time does not limit the temporal resolution of the measurement, although it does induce about a 4-5 minute delay between changes at the dialysis probe and their detection by the CE system. The exact length of this delay time was accurately determined for each day of experimentation during the calibration procedure. Samples of the dialysate were injected onto the capillary using a flow-gate interface as follows. The outlet of the reaction capillary were aligned with the inlet of the CE separation capillary (10 cm long, 10 μm i.d., 150 μm o.d. fused silica) in a Plexiglas block, leaving a gap of approximately 30 μm. A 1mL/min cross-flow was applied between the capillaries using a syringe pump (Pump 22, Harvard Apparatus, Holliston, MA), which carries 90% of the sample solution to waste. To inject a plug of sample, the cross-flow was stopped for 1 sec allowing the sample to fill the gap between capillaries and the voltage then was raised to -2 kV for 100 msec. After injection, the gating cross-flow was resumed, and the separation voltage was ramped from –2 kV to -20 kV over 500 msec. The flow-gate was held at ground to isolate the animal from the voltage applied to the separation capillary. The solenoid valve and voltage supply was controlled by software
written in-house using LabView 7.0 (National Instruments, Austin, TX), a NI PCI-6030E data
acquisition board (National Instruments), and a PC (Dell Computer Corporation, Austin, TX).

The OPA labeled analytes was detected using LIF in a sheath-flow detector cell. The outlet
of the separation capillary was inserted into a 2 mm square o.d., 200 µm square i.d. quartz
cuvette (Mindrum Precision, Inc., Rancho Cucamonga, CA). Sheath buffer (40 mM borate, pH
9.5) was siphoned around the outside of the separation capillary outlet using gravity flow (15-cm
height difference between buffer reservoir levels). The use of sheath flow and grinding the
capillary outlet to a point allowed analytes to migrate off the end of the separation capillary
while maintaining their velocity thereby allowing for efficient detection using LIF. This
arrangement reduced the background signal caused by laser scatter as well as background
fluorescence caused by impurities in the fused silica. Fluorescence was excited using the 351 nm
line (20 mW total UV) of an argon-ion laser (Enterprise II 622; Coherent Laser Group, Santa
Clara, CA) focused onto the analytes using a 1x fused silica lens (Newport Corp., Irvine, CA).
Emission was collected at 90° using a 60X, 0.7 N.A. long-working distance objective (Universe
Kogaku Inc., Oyster Bay, NY), spatially filtered using an iris, passed through a 450 ± 25 nm
bandpass filter (Melles Groit, Irvine, CA), and collected on a photomultiplier tube (PMT, R9220;
Hamamatsu, Bridgewater, NJ). Current from the PMT was amplified and filtered (10 ms rise-
time) using a current amplifier (Keithley Instruments, Cleveland, OH) and sampled with the
same software and hardware used for controlling the injections.

**CE-LIF for CHAPTER 6**

In CHAPTER 6 the instrument used to detect primary amino acids in aCSF was slightly
different from the instrument used in CHAPTER 3 and 5. They both offer on-line real-time
analysis of amino acids with a high temporal resolution and they are similar in principle: primary
amino acids are derivatized and those fluorescent products are detected. In addition, they both utilize a flow-gate system that allows for homogenous sample injections and finally have similar limits of detection. The instrument in CHAPTER 6 includes modifications to allow for the use of the DA spiking technique which basically combines 50 nM DA standards with dialysate prior to derivitization and separation.

**Histology**

A cryostat was used to make 40µm coronal sections. Probe placement was verified by the comparison of these sections with a brain atlas (Paxinos and Watson, 2005). The criterion for probe selection is that the majority (i.e. more than 50%) of the 2mm probe length must be contained within the NAC. A similar criterion was utilized for the hippocampus study (2mm probe length must be contained within the hippocampus). In addition, upon sectioning for probe placement if any pathology was observed the subject was not included in the study.

**Data Analysis**

Each hour of data collection using the CE-LIF instrument results in about 300 electropherogram data files. Each file contains over 11,000 data points. The detection of all peaks and the calculation of peak heights and peak areas were performed by “Cutter”, a LabView-constructed VI in which electropherograms are batch post-processed. The first twenty or more samples collected for each hour (prior to different stimulations or drug treatments) were used to calculate a baseline. If there was considerable individual variability in the baseline values of analytes across animals, subsequent data were expressed as a percent of this baseline and calculated across animals as the mean ± SEM. In chapter 3, we calculated the “relative ratio” to evaluate the change caused by different drug effects on the K+-induced GABA and taurine release. Basically, we calculated the sum of 100 points of the percent basal level. The 100 points definitely included the stimulated peak. The sum of the 100 points was subtracted by 10,000.
This result presented the area of the stimulated peak. Finally, we divided the second and the third stimulated peak values by the first stimulated peak value to give the relative ratio. In chapter 3, 5 and 6, the area under the curve (AUC) was calculated for each amino acid by summing the % baseline value of each amino acid calculated over time.

As an internal standard, we used a peak that is comprised of all the non-charged analytes that are labeled by OPA. This peak (which we will refer to as the OPA peak) has been shown to be very consistent from 1 to 6 hrs after probe implantation since the only factor which might alter its size is the injection efficiency of the flow gate. Thus, it is a good indicator of instrument variability and can be used as a control for the levels of the amino acids. In some cases, we present the OPA data as a reference peak and in some cases, in order to reduce sample to sample variability caused by the instrument, we present the data as a ratio of the analyte of interest to the OPA peak. We also calculated the area under the curve for each time-response curve for every analyte of interest as well as the OPA peak. To summarize these data, \( \Delta \text{AUC} \) was calculated for each amino acid by subtracting the OPA AUC from the amino acid AUC. The AUC ratio for each amino acid was calculated by dividing amino acid \( \Delta \text{AUC} \) by the OPA \( \Delta \text{AUC} \).

Relative ratio values and AUC ratio values are applied to a JMP data table to perform statistical analysis. Statistic significance within and among the groups were determined using one-way ANOVA with post hoc Tukey-Kramer or Student-Newman-Keuls Multiple Comparison Test. Numerical data in the text and error bars in figures are expressed as the mean ± SEM (standard error of the mean). Statistical significance was considered to be p<0.05.
CHAPTER 3
DOPAMINE INHIBITORY REGULATION OF POTASSIUM-INDUCED GABA AND GLUTAMATE RELEASE IN NAC USING CAPILLARY ELECTROPHORESIS LASER INDUCED FLUORESCENCE DETECTION

Introduction

In the past several decades, the physiological and behavioral function of the mesolimbic DA system has been the subject of intensive research. Most of these studies focus on the NAC, which is a brain region involved in functions ranging from motivation and reward to feeding and drug addiction. The neural mechanisms underlying how food reward and drug addiction develop in NAC are far from clear. One of objectives for current study is to investigate the interaction of neurotransmission systems in NAC including DA, GABA and glutamate. It may provide possible targets for studying development of food reward and drug addiction.

In the NAC, glutamatergic input from its corticolimbic afferents and dopaminergic input from the ventral tegmental area converge onto common dendrites of the medium spiny neurons that populate the accumbens (Totterdell and Smith, 1989, Sesack and Pickel, 1990, Smith and Bolam, 1990). DA input to nucleus accumbens has been shown to modulate processing of concurrent glutamate input from limbic structures carrying motor and motivational information (Pennartz et al., 1994; Schmidt, 1998; Horvitz, 2002). These DA/glutamate interactions are fundamentally involved in the response selection process (Steiniger-Brach and Kretschmer, 2005). Previous studies showed that glutamate release in the NAC is modulated by presynaptic D₂ receptors (Kalivas and Duffy, 1997). In contrast to D₂ receptors, little evidence supports D₁ receptor mediated reduction in excitatory input to the NAC (Pennartz, 1992). On the other hand, GABAergic circuitry descending from the NAC to the pedunculopontine tegmental nucleus via the ventral pallidum appears to be especially important in directing the behavioral sequelae associated with reward produced by various drugs of abuse. In the NAC, over eighty percent of
neurons use GABA for signal transmission (Smith and Sharp, 1994b). It has been demonstrated that D2 receptors are colocalized with GABAnergic neurons in NAC. Therefore, a dopaminergic inhibitory regulation of GABA release has been observed in NAC (Hemmati et al., 2001).

Changes in neurotransmitter release are thought to occur over a short time period during most behavioral events. Thus, in vivo detection of neurotransmitters under these conditions should have a high temporal resolution. To date, fast-scan cyclic voltammetry (FSCV) and high-performance liquid chromatography (HPLC) have been used most often. FSCV provides good chemical selectivity as well as subsecond temporal resolution (Robinson et al., 2003). However, it is not able to determine the basal concentrations or to detect DA changes for periods longer than 90 seconds (Heien et al., 2005). In contrast to FSCV, HPLC can estimate basal concentrations and detect both DA and amino acid changes over longer times, but it requires longer sampling times, usually 5 to 20 minutes per sample. Each sample represents an average concentration during this period and so precludes detection of more rapid changes of extracellular chemicals associated with neurotransmission. A newer method, capillary electrophoresis (CE), possesses a high mass sensitivity (Zhou et al., 1995; Robert et al., 1998; Shou et al., 2006) and so requires only a very low volume of sample ($\approx 20$ nl). This decreases the sampling time to 1 sec. Coupled with a separation time of about 14 sec, this brings the sampling frequency within the range to allow monitoring of rapid changes that may correlate with behavioral episodes. CE also may be used to detect both catecholamines and amino acids simultaneously in dialysates of extracellular fluid (Bowser et al., 2001).

The present study employed microdialysis coupled with CE-LIF detection to instigate the interaction among DA, GABA and glutamate. The first aim is to characterize the GABA and glutamate release in NAC by multiple high potassium stimulations and no calcium perfusion
administration. Then, in order to determine whether there is dopaminergic inhibition of potassium-induced GABA and glutamate release in NAC, pargyline (a monoamine oxidase B inhibitor), sulpiride (a D2/D3 receptor antagonist), quinelorane (a D2/D3 receptor agonist) and 2-phenylethylamine hydrochloride (PEA) (a lipophilic amine increasing extracellular concentration of DA by reversing transporter mechanism) were directly perfused into NAC. Following that, the study was extended to determine whether the dopaminergic regulation of the GABAergic and glutamatergic transmissions is regulated by D2 receptor.

**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 hr normal phase light/dark cycle (06:00-18:00). All testing were conducted during the light phase. Rats were acclimated to our colony 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, CaCl$_2$, KCl, MgSO$_4$, monobasic borate, dibasic borate, o-phthaldialdehyde (OPA), β-mercaptoethanol (BME), hydroxypropyl-β-cyclodextrin (HPBCD), sodium metabisulfate, citric acid, sodium citrate acid, pargyline, sulpiride, quinelorane and 2-phenylethylamine hydrochloride were supplied by Sigma, St. Louis (USA).

**Microdialysis and Surgeries**

As described in Chapter 2.
Experiment Procedures

Different experiment procedures are described as following. (1) In the four-time high potassium stimulation experiment, we first perfused high potassium 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 7.0-7.4) into the NAC for 10 minutes. Then, the NAC was washed by aCSF for 50 minutes. The perfusion and washing procedures were repeated for four times. (2) The no-calcium condition experiment includes three steps. First, high potassium aCSF was perfused into the NAC for 10 minutes. We used aCSF to wash the NAC for 50 minutes right after the perfusion and then used aCSF without calcium (145 mM NaCl, 2.7 mM KCl, 1.0 mM MgCl2, 2 mM EGTA, 0.45 mM monobasic phosphate, 1.55 mM dibasic phosphate, pH 7.0-7.4) to wash for another 30 minutes. Second, we perfused high potassium aCSF without calcium (95 mM NaCl, 50 mM KCl, 1.0 mM MgCl2, 2 mM EGTA, 0.45 mM monobasic phosphate, 1.55 mM dibasic phosphate, pH 7.0-7.4) into the NAC for 10 minutes. Then it was washed with aCSF without calcium for 50 minutes and washed with aCSF for another 30 minutes. Third, we perfused high potassium aCSF into the NAC for 10 minutes and washed NAC by aCSF for 50 minutes (Figure 3-1A). (3) Quinlorane (100 µM), pargyline (100 µM) and sulpiride (100 µM) experiments are on the basis of a three-time high potassium stimulation. We added these drugs into the second high potassium aCSF (Figure 3-1B). (4) In the pargyline/sulpiride experiment, the procedure is the same as the third experiment (drug experiment) except that we included the sulpiride into all of the aCSF solutions (normal aCSF and high potassium aCSF) (Figure 3-1B). (5) In the PEA without pargyline experiment, 20 µM PEA was perfused into NAC for 30 minutes was repeated twice more: 30-min 50 µM PEA stimulation followed by 30-min aCSF washing and 30-min 100 µM PEA stimulation followed by 30-min aCSF washing (Figure 3-1C). (6) In the PEA combined with pargyline experiment, we first perfused 100 µM pargyline into NAC for 1 hour. Then, NAC
was stimulated with 20 µM PEA combined with 100 µM pargyline for 30 minutes and washed off with only 100 µM pargyline for another 30 minutes. The stimulation and washing step was repeated two more times except we used 50 µM PEA and 100 µM PEA instead of 20 µM PEA the second and third time, respectively (Figure 3-1C).

**CE-LIF**

As described in Chapter 2.

**Data Analysis and Histology**

Each hour of data collection using the CE-LIF instrument results in about 300 electropherogram “files”. Each file contains over 11,000 data points. The detection of all peaks and the calculation of peak heights and peak areas in this vast data set are easily handled by “Cutter”, a Lab-View-constructed VI in which electropherograms are batch post-processed (Shackman, et al., 2004). The first twenty or more samples collected for each hour (prior to different stimulations or drug treatments) were used to calculate a baseline. If there was considerable individual variability in the baseline values of analytes across animals, subsequent data was expressed as a percent of this baseline and was calculated across animals as the mean ± SEM. We calculated the “relative ratio” to evaluate the change caused by different drug effects on the K+-induced GABA and taurine release. Basically, we calculated the sum of 100 points of the percent basal level. The 100 points definitely included the stimulated peak. The sum of the 100 points was subtracted by 10,000. This result presented the area of the stimulated peak (Figure 3-2). Finally, we divided the second and the third stimulated peak values by the first stimulated peak value. It gave us the relative ratios (see Figures 3-4, 5, 6, 7, 10, 11).

Additionally, for the experiments causing neurotransmitters decrease lower than basal line, we divided all peaks by the sum of N points of the percent basal level. The area under the curve (AUC) was calculated for each amino acid by summing the % baseline value of each amino acid
calculated over time. As an internal standard, we used a peak that is comprised of all the non-charged analytes that are labeled by OPA. This peak (which we will refer to as the OPA peak) has been shown to be very consistent from 1 to 6 hrs after probe implantation since the only factor which might alter its size is the injection efficiency of the flow gate. Thus, it is a good indicator of instrument variability and can be used as a control for the levels of the amino acids. In some cases, we present the OPA data as a reference peak (e.g., Figures 3-15, 3-16). In addition to the OPA peak, we also calculated the area under the curve for each time-response curve for every analyte of interest. To summarize these data, ∆AUC was calculated for each amino acid by subtracting the OPA AUC from the amino acid AUC. The AUC ratio for each amino acid was calculated by dividing amino acid ∆AUC by the OPA ∆AUC.

Relative ratio values and AUC ratio values are applied to JMP data table to perform statistical analysis. Statistic significance within and among the groups were determined using one-way ANOVA with post hoc Tukey-Kramer or Student-Newman-Keuls Multiple Comparison Test. Numerical data in the text and error bars in figures are expressed as the mean ± SEM (standard error of the mean). Statistical significance was considered to be p<0.05. At the end of each experiment, the rats were deeply anesthetized with pentobarbital sodium (120mg/kg, i.p.). The brains were removed and sectioned to verify the correct position of the probe. Only the data from rats with accurate probe placement were included in data analyses (Figure 3-3).

Results

Effect of Four-time High K⁺ Stimulation and No-Ca²⁺ Conditions on the Release of GABA, Glutamate and Taurine

In our study, we applied high potassium stimulations into NAC four times in each experiment. The basal concentrations of glutamate, GABA and taurine in the high potassium stimulation experiment are shown in Table 3-1. K⁺ stimulation increased the extracellular GABA
and taurine level in the NAC to 340±53% and 320±72%, respectively, compared to the basal value. There was no significant difference in either K⁺-induced GABA or K⁺-induced taurine release among the four stimuli during the high potassium stimulation experiment (Figure 3-4B, 3-4C and 3-4D). Therefore, high concentrations of K⁺ stimulation can increase the release of GABA and taurine at least four times and the process is reversible and repeatable. On the other hand, high K⁺ stimulation increased the extracellular glutamate concentration in NAC to 174±24%, which was much smaller than K⁺-induced GABA or K⁺-induced taurine release. In addition, K⁺-induced glutamate release significantly decreased after the second, third and fourth stimulation (F (3, 11) =6.03, p≤0.05) (Figure 3-4A, 3-4D). Furthermore, perfusion with Ca²⁺-free high K⁺ aCSF at the second stimulation resulted in a significant decrease in K⁺-induced glutamate (F (2, 11) =330.98, p<0.0001), GABA (F (2, 11) =36.5, p<0.0001) and taurine (F (2, 11) =193.5, p<0.0001) release, by about 50%, 50% and 40%, respectively. These reductions can be totally recovered by perfusion with the third high potassium stimulation again in the presence of calcium (Figure 3-5). The basal concentrations of glutamate, GABA and taurine under the no calcium condition experiment are also shown in table 3-1.

Effects of Pargyline, Sulpiride, Quinelorane and Pargyline Combined with Sulpiride on K⁺-induced Release of GABA, Glutamate and Taurine.

Direct perfusion of 100 µM pargyline, which is a monoamine oxidase B inhibitor and should increase extracellular levels of dopamine, into the NAC during the second potassium stimulation caused a significant decrease of the K⁺-induced GABA release (F (2, 19) =36.7, P<0.0001) and the K⁺-induced glutamate release (F(2,19)=66.17, P<0.0001). The average decrease of the K⁺-induced GABA and glutamate release were 54±6% and 56.7±6% respectively. The reductions were not reversible because it was not recovered by taking pargyline out of the aCSF for the third stimulation. The third high potassium stimulation still had 45±11%
of K⁺-induced GABA release reduction and 78.4±5% of K⁺-induced glutamate release reduction, which were significantly different from the first high potassium stimulation (p≤0.05) (Figure 3-6A, 3-6C, 3-8A and 3-8B). However, pargyline had no effect on the K⁺-induced taurine release (Figure 3-6B and 3-6C). The average of taurine release for the three K⁺ stimulations were 340±26%, 351±9% and 334±12% , respectively.

In order to determine that the reductions of the K⁺-induce GABA or glutamate release caused by pargyline were mediated by the D2 receptor, which are colocalized with GABA neurons in the NAC (Hemmati et al., 2001), we repeated the pargyline experiment but with sulpiride added to the aCSF through the whole experiment. Combining sulpiride, which is a selective D2/D3 receptor antagonist, with pargyline blocked the reduction of K⁺-induced GABA release caused by pargyline (Figure 3-7A and 3-7C). There was no significant effect of sulpiride and pargyline on the K⁺-induced taurine release (Figure 3-7B and 3-7C). However, combining sulpiride with pargyline did not block the reduction of K⁺-induce glutamate release caused by pargyline. There was still an average of 52% reduction on K⁺-induce glutamate release caused by pargyline in the presence of sulpiride (Figure 3-9A and 3-9B).

As a control experiment, we directly infused 100 µM sulpiride into the NAC during the second high potassium stimulation to test the hypothesis that endogenous dopamine may already be inhibiting GABA release via D2 receptors. The results showed that there was no significant difference between the normal high potassium stimulation and sulpiride-K⁺ stimulation on glutamate, GABA or taurine release (Figure 3-10A-D). In order to further confirm that pargyline caused the reduction of K⁺-induce GABA release via D₂ receptor-mediated mechanism, 100 µM quinelorane, a selective D₂/D₃ receptor agonist, was directly perfused into NAC during the second high potassium stimulation period. There was a significant decrease of K⁺-induce GABA
release by 27±2.6% (F(2,14)=11.17, P<0.05), but no effect on the K+-induce glutamate and taurine release (Figure 3-11A-D). The basal concentrations of glutamate, GABA and taurine in each drug perfusion experiment are shown in Table 3-1. Unfortunately, we didn’t observe any increase in DA regardless of high potassium stimulation or pargyline and sulpiride perfusion (data not shown).

**Effects of PEA on DA, GABA and Glutamate Release in the Presence or Absence of 100 µM Pargyline**

We directly perfused different concentrations of PEA, which can increase extracellular concentration of DA by reversing DA transporters, into the NAC. Without pargyline present, the levels of DA in NAC were increased by 7.7 ± 5.2% by 20 µM PEA, 19.3 ± 9.3% by 50 µM PEA and 47.2 ± 13.0% by 100 µM PEA (Figure 3-12, 3-13B-D). However, with continuous exposure to 100 µM pargyline, DA concentrations were increased by 38 ± 8.0% by 20 µM PEA, 82 ± 6.0% by 50 µM PEA, and 126 ± 19.0% by 100 µM PEA (Figure 3-13A, 3-13C, 3-13D). Therefore, DA release induced by PEA combined with pargyline is significantly higher than that stimulated by only PEA (F(1,6)=16.1, P<0.05 for 20 µM PEA; F(1,6)=15.9, P<0.05 for 50 µM PEA; F(1,6)=18.2, P<0.05 for 100 µM PEA). In addition, the escalation of DA release in NAC had a very good correlation with the dose of PEA. Our study demonstrated that it is possible to monitor DA in vivo using CE-LIFD but only under certain conditions.

Interestingly, basal GABA release was decreased by 10 ± 4% by 20 µM PEA, 16± 7% by 50 µM PEA and the reduction became significant to 20 ± 7% after the addition of 100 µM PEA (F(1,6)=21.55, P<0.05). The PEA suppression of basal GABA levels was reversed by removing PEA from the aCSF (Figure 3-14A and 3-14B). Additionally, in the presence of pargyline, PEA caused a much bigger decrease in the GABA release by 74.8 ± 13% (F(1,6)=94.1, P<0.001) and this reduction was not reversible (Figure 3-15A and B). On the other hand, PEA also induced
significant increase of glutamate release by 57.6±17.8% in the presence of pargyline (F(1,5)=7.9, P<0.05) (Figure 3-16B and 3-16C). However, PEA by itself had no effect on glutamate release in NAC (Figure 3-16A and 3-16C). The basal concentration of GABA and glutamate are listed in table 3-1.

**Discussion**

The current study confirmed and extended the previous findings that there is dopaminergic inhibition of potassium-induced GABA release via D2 receptors. A dopaminergic inhibitory regulation of potassium-induced glutamate release was also observed. However, this inhibition did not appear to be regulated by D2 receptors. In addition, PEA-induced DA release decreased spontaneous GABA release but increased spontaneous glutamate release. Although we were able to measure some drug-induced increases in DA levels in NAC, our ability to detect potassium-induced DA release was limited by our detection sensitivity.

**Relationship between DA and Glutamate Release in NAC**

In the present study, our data showed that the high K⁺-induced glutamate release is much smaller than either K⁺-induced GABA or K⁺-induced taurine release. There are two possible explanations. The first is that glutamate is released from a variety of sources through two different mechanisms, vesicular and non-vesicular mechanisms. The non-vesicular mechanism includes glial release and cystine/glutamate exchange (Danbolt, 2001). A previous study demonstrates that about 50~70% of the basal extracellular glutamate is released by cystine/glutamate exchange in the NAC (Baker et al., 2002). It suggests that most basal release of glutamate in NAC does not depend on depolarization of neurons. Thus, a higher non-neuronal basal value might minimize the apparent increase caused by neuronal stimulation. Another explanation is based on the different resting membrane potential of GABA and glutamate in NAC. The resting potential of glutamate is around -90 mV in NAC which is more negative than
that of GABA neurons in NAC, about -75mV (Muramatsu et al., 1998; Shi and Rayport, 1994). Therefore, glutamate neurons are less sensitive than GABA neurons in NAC upon to the same concentration of high $K^+$ stimulation. In either case, the release of glutamate through either vesicular or non-vesicular mechanism is calcium dependent, which is supported by our results that perfusion with Ca$^{2+}$-free high $K^+$ aCSF at the second stimulation resulted in a significant decrease in $K^+$-induced glutamate by 50%.

Our data showed that pargyline perfusion led to a significant reduction in the $K^+$-induced glutamate release, and this reduction was irreversible. PEA enhanced the basal release of glutamate in NAC. The increase was significant in the presence of pargyline. These results confirmed previous findings of DA regulation of glutamatergic neurotransmission in rat neostriatum (Godukhin et al., 1984). Application of DA, apomorphine or beta-phenylethylamine enhances the basal release of glutamate in striatum. While apomorphine and beta-phenylethylamine inhibit the release of glutamate induced by electrical stimulation of the frontal cortex and that by $K^+$-depolarization. These inhibitions are completely abolished by haloperidol, a D2 receptor antagonist. However, the current study showed that combining sulpiride with pargyline did not block the reduction on the $K^+$-induced glutamate release caused by pargyline. Our following results further confirm the finding by showing that quinelorane has no effect on $K^+$-induced glutamate release. The possible explanation would be the dopaminergic inhibitory regulation of the glutamate release in NAC through $D_1$ or 5-HT$_2$ receptors, but not $D_2$ receptors. Electrophysiological evidence has emerged supporting D1 mediated reduction in excitatory input to the nucleus accumbens (Pennartz 1992).

**Relationship between DA and GABA Release in NAC**

Previous studies report that $K^+$ stimulation can increase the release of GABA detected in NAC using microdialysis measurement (Smith and Sharp 1994a; Pin and Bockaert, 1989). The
K+ stimulated GABA release is decreased about 50% following perfusion with no Ca2+ medium (Bernath and Zigmond, 1988; Smith and Sharp, 1994a). The current data supported and extended the previous results by showing that high concentrations of K+ stimulation can increase the release of GABA and the process is reversible and repeatable. Perfusion with Ca2+-free high K+ aCSF at the second stimulation resulted in a significant reduction in K+-induced GABA release, about 50%.

Additionally, we found that pargyline selectively decreased K+-induced GABA levels by 55%, and the effect is not reversible. Combining sulpiride with pargyline blocked the reduction of K+-induced GABA levels by pargyline. At same time, sulpiride has no effect on K+-induced GABA release by itself, which may be due to a low concentration of endogenous agonist DA in NAC. Moreover, subsequent results showed that quinelorane significantly decrease K+-induced GABA release in NAC and this effect is reversible. On the other hand, this previous study suggests D2 receptor inhibits the release of GABA in the nucleus accumbens core (AcbC) not in the nucleus accumbens shell (AcbSh) (Hemmati et al., 2001). Although our study did not distinguish AcbC and AcbSh, over 90% of our probe placement was identified in AcbC. A number of microanatomy studies have revealed that NAC includes three main subregions: shell, core and rostral pole (Zahm and Brog, 1992; Wright and Groenewegen, 1995). Each subregion has different morphology and function. Although AcbSh appears to be more important than AcbC for drug reward, more recent works show that AcbC plays a much more important role on the choice between delayed, certain and immediate, uncertain rewards than AcbSh (Cardinal et al., 2006; Pothuizen et al., 2005). Additionally, it has been demonstrated that extracellular DA levels were significantly higher in AcbC compared with shell (McKittrick and Abercrombie,
2007; Pierce and Kalivas, 1995; King et al., 1997; Heidbreder and Feldon, 1998). Therefore, it emerges that the roles of AcbC in drug reward are similarly important.

In the present study, beta-phenylethylamine administration produced a dose-dependent reduction of GABA basal release and a dose-dependent increase of DA basal release in NAC. Combing pargyline with beta-phenylethylamine made both changes became significant. In this case, CE-LIF showed particular advantages in monitoring catecholamine and amino acids simultaneously which, for the first time, directly gave evidence that increase of DA release leads to reduction of GABA basal release in NAC.

**Relationship between DA and Taurine Release in NAC**

Our data showed that taurine was increased by the high concentration of potassium stimulation and this increase is significantly reduced by 40% through perfusion with Ca$^{2+}$-free high K$^+$ aCSF at the second stimulation. These data support the view of taurine as an inhibitory neurotransmitter (Davison and Kaczmarek, 1971). It has been suggested that taurine can open chloride channels and increase intracellular chloride which hyperpolarizes neuronal cells (Hausser et al., 1992; Billard, 1990; Taber et al., 1986). Several studies have shown that high potassium stimulation can increase the release of taurine in different brain regions (Hada et al., 1996; Bockelmann et al., 1998). However, because there is not a specific taurine receptor found on postsynaptic membranes, taurine is not recognized as a neurotransmitter. On the other hand, taurine also has been considered as a neuromodulator (Oja and Kontro, 1990). Much evidence suggests that taurine has a neuromodulatory function particularly on GABA receptors (Bureau and Olsen, 1991; Medina and De Robertis, 1984). Furthermore, taurine is involved in osmoregulatory modulation in the brain since many in vitro and in vivo studies showed that cell swelling induced by different conditions increase the basal level of extracellular taurine (Kimelberg et al., 1990; Schousboe et al., 1990).
Overall, the present study gave the novel evidence that there were different patterns of DA inhibitory regulation between GABA and glutamate release in NAC. PEA-induced DA release increased basal release of glutamate but decreased spontaneous GABA release. The dopaminergic inhibition of K+-induced GABA release appeared to be mediated via D2 receptors while DA inhibitory regulation on K+-induced glutamate release did not fully follow D2 receptor pharmacology but could occur instead via D1 or 5-HT2 receptor.
Figure 3-1. Different experiment procedures. A) Four-time high potassium stimulation experiment and no calcium condition experiment. B) Agent plus high potassium stimulation. Agent included 100 µM quinelorane, 100 µM pargyline, or 100 µM sulpiride. C) PEA experiment with the presence or absence of pargyline. For the PEA with pargyline experiment, pargyline was perfused starting one hour before the experiment and then throughout the whole experiment period.
Figure 3-2. Calculation of the relative ratio. The result is given as percent of baseline. The value of each stimulation is calculated by summing the 100 points of percent basal level (from time point A to B, C to D, and E to F) subtract 10,000. Grey rectangles between time point A and B, C and D, and E and F represent 10,000 (100X100). The peak areas filled with solid line represent the value of each stimulation. Relative ratio is calculated by dividing the second and the third (and if tested, the fourth) stimulated peak value by the first stimulated peak value.
Figure 3-3. Coronal sections showing microdialysis probe placement within the nucleus accumbens. Lines indicate the active dialysis regions. Numbers below the figure represent the position of the slice relative to Bregma. The figure was adapted from Paxinos and Watson (2005).
Figure 3-4. Effect of four-time high potassium stimulation on glutamate, GABA and taurine release in nucleus accumbens. A) Effect of four-time high potassium stimulation on glutamate release (N=3). B) Effect of four-time high potassium stimulation on GABA release (N=3). C) Effect of four-time high potassium stimulation on taurine release (N=3). The results are given as percent of the basal level. The value of SEM is shown every ten data points. D) The relative ratios for glutamate, GABA and taurine in the four-time high potassium stimulation experiments. The relative ratios of glutamate release for each time stimulation are 1 ±0, 0.78 ±0.008, 0.77±0.013, 0.57±0.14, F(3,11)=6.03 (p<0.05). There is significant difference among the K⁺-induced glutamate release. The relative ratios of GABA release for each time stimulation are 1 ±0, 0.83 ±0.16, 0.85±0.20, 0.85±0.065, F(3,11)=0.54 (p>0.67). The relative ratios of taurine release for each time stimulation are 1 ±0, 0.93 ±0.02, 0.97±0.13, 0.90±0.14, F(3,11)=0.32(p>0.81).
Figure 3-5. Effect of calcium-free perfusion on K⁺-induced glutamate, GABA and taurine release in nucleus accumbens. A) Effect of calcium-free perfusion on K⁺-induced glutamate release (N=4). B) Effect of calcium-free perfusion on K⁺-induced GABA release (N=4). C) Effect of calcium-free perfusion on K⁺-induced taurine release (N=4). The results are given as percent of the basal level. The value of SEM is shown every ten data points. D) The relative ratios for glutamate, GABA and taurine in the calcium-free perfusion experiments. The relative ratios for GABA for each stimulus are 1 ±0, 0.50±0.056, and 0.95±0.07. There is a significant difference among all three stimuli F (2, 11) =36.5, p<0.0001. The relative ratios for glutamate for each stimulus are 1 ±0, 0.52±0.010, and 0.81±0.02. There is a significant difference among all three stimuli F (2, 11) =330.98, p<0.0001. The relative ratios for taurine after each stimulus are 1 ±0, 0.61±0.02, and 0.89±0.02 (F (2, 11) =193.5, p<0.0001), which also has significant difference.
Figure 3-6. Effect of pargyline on the \(K^+\)-induced GABA and taurine release. A) Effect of pargyline on the \(K^+\)-induced GABA release (N=7). B) Effect of pargyline on the \(K^+\)-induced taurine release (N=7). The results are given as percent of the basal level. The value of SEM is shown every ten data points. C) The relative ratios for GABA and taurine in the pargyline experiments. The relative ratios for GABA for each stimulus are 1 ±0, 0.44±0.054, and 0.71±0.14. There is a significant difference among all three stimuli \(F (2,19) =36, P<0.0001\). The relative ratios for taurine after each stimulus are 1 ±0, 0.93±0.09, and 0.93±0.07, \(F (2,19) =0.45, P>0.64\).
Figure 3-7. Effect of pargyline/sulpiride on the K$^+$-induced GABA and taurine release. A) Effect of pargyline/sulpiride combination on the K$^+$-induced GABA release (N=7). B) Effect of pargyline/sulpiride combination on the K$^+$-induced taurine release (N=7). The results are given as percent of the basal level. The value of SEM is shown every ten data points. C) The relative ratios for GABA and taurine during the pargyline/sulpiride experiment. The relative ratios for GABA for each stimulus are 1 ±0, 0.79±0.095, and 0.85±0.21. F (2, 19) =0.91, P>0.42. The relative ratios for taurine after each stimulation are 1 ±0, 1.1±0.1, and 1.35±0.18, F (2, 19) =2.68, P>0.10.
Figure 3-8. Effect of pargyline on the K⁺-induced glutamate release in NAC. A) Effect of pargyline on the K⁺-induced glutamate release (N=7). The results are given as percent of the basal level. The value of SEM is shown every ten data points B) The AUC ratio for glutamate during pargyline experiment. The AUC ratios for glutamate for each stimulus are 0.21 ±0.05, -0.36±0.042, and -0.57±0.05. F(2,19)=66.17, P<0.0001.
Figure 3-9. Effect of pargyline/sulpiride on the $K^+$-induced glutamate release in NAC. A) Effect of pargyline/sulpiride on the $K^+$-induced glutamate release (N=7). The results are given as percent of the basal level. The value of SEM is shown every ten data points. B) The AUC ratio for glutamate during pargyline/sulpiride experiment. The AUC ratios for glutamate for each stimulus are $0.011 \pm 0.026$, $-0.27 \pm 0.016$, and $-0.53 \pm 0.046$. F(2,19) = 54.2, P<0.05.
Figure 3-10. Effect of sulpiride on the K⁺-induced glutamate, GABA and taurine release. The results are given as percent of basal level. The value of SEM is shown every ten data points. A) Effect of sulpiride on the K⁺-induced Glutamate release (N=3). B) Effect of sulpiride on the K⁺-induced GABA release (N=3). C) Effect of sulpiride on the K⁺-induced taurine release (N=3). D) The relative ratio for GABA and taurine in the sulpiride experiment. The relative ratio of glutamate for each stimulus are 1 ±0, 0.74 ±0.13, 0.79±0.098, F (2, 7) =2.7, (p>0.14). The relative ratio of GABA for each stimulus are 1 ±0, 0.88 ±0.14, 0.93±0.004, F (2, 7) =1.03, (p>0.42). The relative ratio of taurine after each stimulation are 1 ±0, 1.07±0.052, 1.03±0.041, F (2, 7) =0.49 (p>0.64).
Figure 3-11. Effect of quinelorane on the K$^+$-induced glutamate, GABA and taurine release. A) Effect of quinelorane on the K$^+$-induced Glutamate release (N=5). B) Effect of quinelorane on the K$^+$-induced GABA release (N=5). C) Effect of quinelorane on the K$^+$-induced taurine release (N=5). The results are given as percent of basal level. The value of SEM is shown every ten data points. D) The relative ratio for GABA and taurine in the sulpiride experiment. The relative ratio of glutamate for each stimulus are 1 ±0, 0.94 ±0.04, 0.91±0.06, F (2, 14) =0.95, (P>0.42). The relative ratio of GABA for each stimulus are 1 ±0, 0.72 ±0.016, 1.10±0.057, F(2,14)=11.17, (P<0.05). The relative ratio of taurine after each stimulation are 1 ±0, 1.10±0.040, 1.20±0.109, F (2, 14) =2.68 (P>0.108).
Figure 3-12. Effect of PEA combined with pargyline stimulation on the DA release in NAC. The DA peak is right in the middle. Following the stimulation of different concentration of PEA combined with 100 µM pargyline, there is a significant increase of DA peak. The different doses of PEA are indicated by different shape of line.
Figure 3-13. The effects of PEA on the level of DA and PEA in NAC in the presence or absence of 100 µM pargyline. A) The level of DA and PEA in NAC by stimulation of only PEA. B) The level of DA and PEA in NAC by stimulation of PEA and pargyline. The results are given as percent of the basal level in figure A and B. The value of SEM is shown every ten data points. C) The AUC ratio of PEA in NAC induced by PEA perfusion in the presence (1.52±0.25 for 20 µM PEA; 4.12±0.34 for 50 µM PEA; 5.75±0.65 for 100 µM PEA) or absence of pargyline (1.37±0.17 for 20 µM PEA; 3.77±0.66 for 50 µM PEA; 5.51±0.75 for 100 µM PEA). D) The AUC ratio of DA in NAC induced by PEA perfusion in the presence (0.38±0.08 for 20 µM PEA; 0.82±0.18 for 50 µM PEA; 1.26±0.19 for 100 µM PEA) or absence of pargyline (0.08±0.052 for 20 µM PEA; 0.19±0.0.09 for 50 µM PEA; 0.47±0.13 for 100 µM PEA). It indicated that there is no significant difference on the level of PEA induced by PEA with pargyline comparing to only PEA. PEA-stimulated DA release in the NAC is better detected when PG is present. PEA is 2-phenylethylamine hydrochloride. PG is pargyline.
Figure 3-14. Effects of PEA on the extracellular concentration of GABA and DA in NAC. A) Effect of PEA on GABA and DA basal release (N=4). The results are given as percent of the basal level. The value of SEM is shown every ten data points. B) The AUC ratio for GABA during PEA experiment. The AUC ratios for GABA for each stimulus are -0.09 ±0.04 for 20 µM PEA, -0.16±0.07 for 50 µM PEA, and -0.20±0.068 for 100 µM PEA. Only 100 µM PEA cause significant decrease of GABA level (F(3,11)=21.55, P<0.05).
Figure 3-15. Effect of PEA combined with pargyline on GABA and ornithine (control) basal release in NAC. A) Effect of PEA combing with pargyline on GABA basal release (N=3). The results are given as percent of the basal level. The value of SEM is shown every ten data points. B) The AUC ratio for GABA during pargyline/PEA experiment. The AUC ratios for GABA and ornithine are -0.53±0.05 and 0.22±0.08, F(1,5)=94.1, P<0.001.
Figure 3-16. Effects of PEA on the level of glutamate and aspartate (control) in NAC in presence or absence of pargyline. A) Effects of PEA combining with pargyline on the level of glutamate (N=3). B) Effects of PEA on the level of glutamate. The results are given as percent of the basal level (N=4). The value of SEM is shown every ten data points. C) The AUC ratios for glutamate are 0.58±0.18 for PEA/pargyline experiment and 0.17±0.16 for only PEA experiment. The AUC ratios for aspartate are 0.006±0.5 for PEA/pargyline experiment and 0.02±0.05 for only PEA experiment. Only PEA/pargyline caused significant increase of glutamate basal level comparing to aspartate.
Table 3-1. Basal concentrations for GABA, taurine and glutamate in NAC.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>GABA (µM)</th>
<th>Taurine (µM)</th>
<th>Glutamate (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High potassium stimulation</td>
<td>0.2±0.013</td>
<td>4.4±0.27</td>
<td>10.2±0.31</td>
</tr>
<tr>
<td>No calcium condition</td>
<td>0.32±0.008</td>
<td>3.9±0.29</td>
<td>11.4±0.30</td>
</tr>
<tr>
<td>Quinlorane</td>
<td>0.25±0.017</td>
<td>4.4±0.16</td>
<td>8.9±0.27</td>
</tr>
<tr>
<td>Pargyline</td>
<td>0.2±0.009</td>
<td>3.4±0.21</td>
<td>9.86±0.54</td>
</tr>
<tr>
<td>Sulpiride</td>
<td>0.19±0.015</td>
<td>3.1±0.26</td>
<td>10.3±0.19</td>
</tr>
<tr>
<td>Pargyline and sulpiride</td>
<td>0.36±0.021</td>
<td>4.1±0.16</td>
<td>10.7±0.76</td>
</tr>
<tr>
<td>PEA</td>
<td>0.24±0.042</td>
<td>4.2±0.17</td>
<td>11.2±0.23</td>
</tr>
<tr>
<td>PEA and pargyline</td>
<td>0.34±0.009</td>
<td>2.9±0.48</td>
<td>11.2±0.32</td>
</tr>
<tr>
<td>AMPH</td>
<td>0.28±0.005</td>
<td>3.7±0.52</td>
<td>9.67±0.64</td>
</tr>
</tbody>
</table>
CHAPTER 4
PERSISTENT INCREASE IN BREAKPOINTS FOR SELF ADMINISTRATION OF ETHANOL-CONTAINING GELATIN IN RATS FOLLOWING INTERMITTENT HIGH DOSE ETHANOL EXPOSURE

Introduction

Recently an ethanol-containing gelatin (10% ethanol, 10% Polycose®, 0.25% gelatin, wt/vol) has been used to induce reliable and robust self-administration without food or water restriction (Rowland et al., 2005). The ethanol-containing gelatin produces pharmacologically relevant concentrations of ethanol in the brain that are comparable to previously published reports after similar amounts of voluntary ethanol drinking (Peris et al., 2006). Even so, it is not clear whether the reinforcing properties of this gel are due to the ethanol or to the Polycose-gelatin vehicle. In the present study, the feasibility of using this procedure for establishing operant self-administration of ethanol was determined. We chose fixed ratio (FR) schedules to establish baseline responding for ethanol reinforcement and progressive ratio (PR) schedules to explore subsequent maintenance of intake as a function of motivation. At the same time, we also measured the effects of a number of conditions previously shown to increase ethanol self-administration under operant conditions, including periods of ethanol deprivation and intermittent high dose ethanol exposures.

The alcohol deprivation effect (ADE) is defined as a temporary increase in alcohol intake when ethanol is reinstated following a period of alcohol deprivation (Sinclair and Senter, 1967) and is considered to be a model of developing alcoholism. It has been observed after a deprivation period as short as 12 hours or as long as 75 days. Usually, after initial stabilization of baseline ethanol self-administration, rats are deprived of ethanol access for 7-10 days which causes a temporary increase in alcohol intake (Heyser et al., 1997; Spanagel and Holter, 1999) and preference (Spanagel and Holter, 1999) under operant conditions. Multiple periods of
deprivation-access cycles, similar to what might occur in the development of human alcoholics, increase the magnitude and prolong the expression of the ADE (Koros et al., 1999; Rodd-Henricks et al., 2001). Additionally, repeated alcohol deprivation–access cycles increases the reinforcing properties of ethanol as indicated by significantly increasing breakpoints found when using PR operant schedules (Rodd et al., 2003).

Another method to increase ethanol self-administration utilizes intermittent exposure to high ethanol concentrations which results in a marked and long-lasting increase in subsequent ethanol intake by almost 3-fold (Rimondini et al., 2002, 2003). Intermittent ethanol exposure has been reported to produce a larger increase in operant self-administration of ethanol relative to continuous ethanol vapor (O’Dell et al., 2004). The increase in ethanol self-administration after intermittent ethanol pre-exposure should be distinguished from that occurring during withdrawal from ethanol which generally only occurs during the short period after ethanol withdrawal (O’Dell et al., 2004).

By manipulating the conditions of ethanol exposure such that ethanol consumption is increased, presumably by a change in the set-point for ethanol craving (Koob, 2003), we can determine how these relationships are altered in the development of the addiction process. The present experiments examined the feasibility of using the novel ethanol-containing gelatin procedure for establishing operant self-administration of ethanol; including higher effort FR schedules or PR schedules. PR schedules were used to assess motivation for ethanol reinforcement. Since the response requirement for each subsequent reinforcer increases until the animal stops responding, this breakpoint is typically taken as an index of the reinforcing strength of that drug. Lastly, the effects of periods of ethanol deprivation and intermittent high dose ethanol exposures on motivation for ethanol responding were also examined.
Materials and Methods

Animals and Housing

Nine female Sprague-Dawley rats were used. Rats were ~3 months of age and weighed 325±10 g at the beginning of the study. Body weight was recorded once a week during testing. All rats were singly housed in a vivarium with a reverse 12:12 light dark cycle (lights off @ 0900) and an ambient temperature maintained at 23±2 °C. Purina 5001 Rodent Chow was available ad libitum at all times except during daily 30-min operant sessions. 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.

Behavioral Apparatus

As described in Chapter 2.

Procedure

Gelatin administration in home cage

Rats received access to gelatin containing 10% ethanol in jars in their home cage in different stages. Rats received the gelatin in their home cage for 24 hrs for the first two days of exposure, then for 6 hrs for two days, then 3 hrs for two days and finally for 1 hr for about 2 weeks as described earlier (Peris et al., 2007)

Gelatin administration in the operant conditioning chamber

Magazine training. After reliably consuming the 10% ethanol-containing gelatin for a 1 hr period, rats were acclimated to the operant conditioning chamber via a 60 min session where gelatin was automatically delivered at fixed intervals. During this session, a visual cue light was illuminated above the left lever at all times. Another visual cue, the house light, was illuminated to signal gelatin delivery and was terminated upon a 9.5s infusion. The first three rats went through the magazine training. The other six rats were tested without magazine training and
successfully acquired the task; therefore magazine training is clearly not a requirement for this behavior.

**Fixed ratio schedule.** Next, rats were moved to daily 30 minute operant self administration sessions that occurred between 10 AM and 2 PM. Rats were trained to press once for a 0.28 g delivery of 10% ethanol-containing gelatin (FR1). In these sessions, there were three cue lights on over the left lever and a 1s illumination of the house light signaled gelatin delivery. Sessions occurred once a day for 7 days a week. After a period on an FR1 schedule, rats were moved to an FR5 schedule, where five presses on the left lever resulted in one bolus delivery of gelatin. Presses on the right lever had no programmed consequence. Both schedules lasted for 20 days. To attain FR 30 responding, reinforcement schedules were increased across several days (see Table 4-1). Any spillage that occurred was removed from the lickometer and weighed to the nearest .01g and subtracted from the daily total. All operant sessions were run approximately the same time each day during the dark cycle.

**Progressive ratio schedule.** After 10 days of FR30 training, rats were moved to daily 30 minute PR sessions where the lever press requirement increased with each successive reinforcement (Figure 4-1). Two PR5 and seven PR10 schedules were used where the first reinforcer required 5 and 10 responses and the contingency subsequently increased by the order of 5 and 10, respectively. The last completed ratio level was used as the breakpoint. In keeping with methodology used in the FR schedules, sessions were 30 min in length regardless of the rats’ performance. Each round of PR5 or PR10 schedule was implemented for 17 days total; the first 7 days responding was reinforced with 10% ethanol-containing gelatin and the next 10 days responding was reinforced with nonethanol-containing gelatin (see Table 4-1). The environment
of the chamber during these sessions was similar to the FR sessions with the exception that the house light was not illuminated during stimulus delivery.

**Binge injections.** Rats were divided into two groups based on their performance under the first two PR10 schedules such that they had equal consumption and breakpoints. The ethanol (EtOH) group (N = 5) received “binge” injections consisting of 3 g/kg i.p. ethanol (20% wt/vol) and the Saline group (N = 3) received equivolume saline injections. All injections occurred at least one hour after the daily operant session on Days 4 and 6 of the third, fourth, fifth and sixth 10-day ethanol deprivation period when rats were responding for plain gel reinforcement (Figure 4-1). Each binge injection was divided into three equal volumes given at 5-min intervals. The duration of the loss of righting reflex was measured as the time after the third injection at which the animals lost righting to the time they were able to right themselves three times in one minute. After the last PR10 period of ethanol reinforcement (i.e., after the fourth set of binge exposures and subsequent testing), rats were transferred to an FR5 schedule for 30 days and then back to a PR10 schedule for 5 days (Figure 4-1). At no point during this latter period did rats receive additional ethanol injections.

**Blood Ethanol Determinations**

As described in Chapter 2.

**Statistics**

Breakpoints and amount of ethanol consumption were the main dependent variables for the PR schedules. The data were analyzed using one way and two way ANOVA with JMP-stat software (SAS Institute Inc.) and p-values less than 0.05 were considered statistically significant. Newman–Keuls post hoc analyses were used to follow up any significant interactions.
Results

The feasibility of using ethanol-containing gelatin for establishing operant self-administration of ethanol was determined (Figure 4-2). Rats were trained to press once for a 0.28 g delivery of ethanol-containing gelatin (FR1). Since there was very little spillage, the average ethanol consumption during these 30-min sessions was 1.3 ± 0.05 g/kg (Figure 4-2A and B). Blood ethanol concentrations were determined immediately after the 30-min session on Day 11 by sampling tail blood from a subset of these rats (Figure 4-3). There was a significant correlation between ethanol dose and blood ethanol concentration. Rats were then moved to an FR5 schedule for another 20 days and responding increased accordingly with average ethanol consumption during the last 10 days of responding at 1.2 ± 0.05 g/kg (Figure 4-2C and D). To attain FR 30 responding, reinforcement schedules were incrementally increased every several days. Over 10 days of FR30 responding, rats exhibited high response levels and consumed an average of 0.7 ± 0.05 g/kg ethanol per session (Figure 4-2E and F).

When rats were moved to daily PR5 sessions, where the response requirement increased by 5 with each successive reinforcement, ethanol consumption fell to 0.6 ± 0.03 g/kg (Figure 4-4). The PR5 schedule was implemented for 7 days with ethanol-containing gelatin followed by 10 days responding for gelatin vehicle, repeated twice (Figure 4-1). There was no difference in breakpoints for ethanol gel (34.5 ± 1.3) versus plain gel (36.2 ± 1.8). Similar results were found for a PR10 schedule with breakpoints of 51.8 ± 3.1 for ethanol gel and 52.2 ± 3.8 for plain gel with ethanol consumption decreased to 0.5 ± 0.02 g/kg (Figure 4-4). There was not an ADE when rats returned to ethanol reinforcement after the 10-day plain gel reinforcement periods for either PR schedule (Figure 4-5). There was a significant decrease in ethanol consumption as the response requirements became more difficult (Figure 4-6).
When the number of responses on the non-reinforced lever were tabulated, there was initially very little responding on this lever (2.7±0.5 per FR1 session) and this decreased further over days (FR5 = 2.2±0.3; FR30 = 0.7±0.1; PR5 = 0.7±0.1; PR10 = 0.4±0.1). The average numbers of responses on the reinforced lever were: FR1 = 22.5±1.2; FR5 = 80.6±3.1; FR30 = 263.4±17.1; PR5 = 325.9±19.6; PR10 = 264.0±21.5.

We then examined whether “binge” exposure to ethanol would affect operant intake. The EtOH group (n=5) received 3 g/kg i.p. ethanol “binge” injections and the Saline group (n=3) received saline injections on Days 4 and 6 during each of the cycles of plain gel responding (see Figure 4-1). Breakpoint and ethanol consumption rose in the EtOH group (Figure 4-7) reaching a maximum after the fourth pair of binge injections (F (4,121) =3.9; P<0.01). There was no effect of saline injections on either breakpoint or ethanol consumption (Figure 4-7) or an effect of binge exposures on breakpoint or consumption of plain gel reinforcement (Figure 4-8).

When the pattern of reinforcements over the 30-minute operant session was examined, over 97% of left-lever presses occurred during the first 15 minutes of each session regardless of reinforcement type or whether the rats had received saline or binge ethanol injections (Figure 4-9). There was a significant increase in responding during the first 10 minutes of the third through sixth sessions for rats in the group receiving binge ethanol injections (Figure 4-9C). There was no difference in responding during any of the time periods for saline rats receiving ethanol gel (Figure 4-9A) or in any of the rats receiving plain gel reinforcements (Figure 4-9B and D). These data were supported by a significant Group X Time interaction (F(5,30) = 2.92; p < 0.05) for ethanol-reinforced responding but only a significant effect of Time (F(5,30) = 18.33; p < 0.001) with no effect or interactions involving Group for plain gel-reinforced responding. The Group X Time interaction that occurred during ethanol-reinforced sessions was due to significantly higher
responding during the first 5 and 10 min periods for EtOH rats during the later ethanol-
reinforced sessions (4E, 5E and 6E) compared to earlier sessions (2E and 3E) (F(4,133) = 11.8
and 11.5, respectively for 5 and 10 min). There was no difference during these time periods for
Saline rats receiving ethanol reinforcement or for any rats during plain gel-reinforced
responding.

There was no evidence for tolerance to the loss of righting induced by the eight binge
injections (Figure 4-10). Interestingly, there was no increase in the EtOH group compared to the
Saline group when they were switched back to an FR5 schedule even though the EtOH group
still exhibited increases in PR10 breakpoints and consumption when retested one month after the
last binge injection (Figure 4-11). These data were supported by a significant main effects of
Group during the PR10 sessions (F(1,6) = 6.02). There was also a significant interaction of
Group X Session for the FR5 responding (F(2,12) = 3.42) but this appeared to be due to changes
in the Saline group, which started out higher (we only matched groups for PR10 responding
before binge treatments), compared to the EtOH group which exhibited stable responding over
sessions.

**Discussion**

Our results have demonstrated that our ethanol-gel can support robust operant responding
and that resultant blood ethanol concentrations are significantly correlated with the ethanol dose
consumed during an FR1 30 min-session. This confirmed our previous finding that ethanol-
containing gelatin can produce pharmacologically relevant concentrations of ethanol in the rat
brain (Peris et al., 2006). Additionally, this reliable and pharmacologically relevant level of
ethanol self-administration was easily established without the need for any food or water
restriction based on palatability of the ethanol-gel. In studies using short time water deprivation
or the sucrose fading procedure, Wistar rats self administer about 0.625g/kg ethanol in a 30 min
FR1 operant session (Heyser et al., 1997, 2003); Long-Evans rats consume 0.45g/kg ethanol during a 30-min FR3 session (Arolfo et al., 2004); and, sP rats have a mean ethanol intake in the 0.6 to 1.1 g/kg range over a 30 min FR1 session (Vacca et al., 2002). Compared to these results, the “jello shot” procedure establishes robust operant responding in Sprague-Dawley rats from FR1 (1.3 ± 0.05 g/kg ethanol per 30min-session) even up to FR30 (0.7 ± 0.05 g/kg ethanol per 30min-session) operant schedules, without the need for any food or water restriction. Despite Sprague-Dawley rats being known for poor levels of ethanol self-administration under free access condition (Mormede et al., 2004; Vengeliene et al., 2005), using the “jello shot” procedure, they self administer much more ethanol in the same length operant session than the other outbred or even a selected line of rats. Thus, the “jello shot” procedure provides a reliable and efficient method for studying an animal model of self-administration of ethanol.

Using PR reinforcement schedules, we found that response breakpoints did not differ for 10% ethanol gel versus plain gel under either PR5 or PR10 schedules. This indicates that the motivational properties for both kinds of gel are similar. In other words, there is no indication that, at least initially, the rats are responding for the pharmacological effects of ethanol as opposed to the other reinforcing properties of the Polycose-gelatin vehicle. It will be of interest to test whether rats that are selectively bred for preference to ethanol will differ in their initial breakpoints for ethanol gel and plain gel. However, in Sprague-Dawley rats, this equal responding for ethanol-containing gelatin and plain gelatin provides a good baseline for the subsequent binge exposure experiments. Although the amount of ethanol consumed using the PR5 schedule was significantly lower than that consumed under the easier FR schedules, it was not significantly different than ethanol consumed under the FR30 schedule. Thus it is not clear whether motivation or satiation contributed to the cessation of responding at the breakpoint of
the PR5 schedule. On the other hand, consumption was decreased by about 50% on the PR10 schedule, indicating that motivation was the major contributing factor to the breakpoint, and not satiation. The fact that breakpoints were similar for ethanol gel and plain gel on the PR10 schedule also indicates that ethanol intoxication did not have response suppressive effects.

Although we didn’t compare responding on low FR schedules for ethanol gel and plain gel, when rats are given 60-min free access to ethanol gel or plain gel, they consume much more plain gel (data not shown) which indicates that the higher doses consumed under this free access (around 1 g/kg) are probably having response suppressive effects. Thus it is likely that under the low FR schedules of reinforcement, ethanol is also having response suppressive effects.

We did not observe an ADE when periods of ethanol reinforcement were interspersed with 10-day periods of plain gel reinforcement, either on the PR5 schedule or during any of the PR10 periods of testing. Since Sprague-Dawley rats are known to usually consume lower amounts of ethanol (Mormede et al., 2004; Vengeliene et al., 2005), it is not surprising that a report of an ADE has not previously been published with Sprague-Dawley rats (Martinetti et al., 2006). In most outbred strains of rats, when the ethanol intake is lower, the magnitude of the ADE is usually modest (Heyser et al., 1997, 2003; Rodd et al., 2004). However, in the present study, even after two and half months of daily 30 min-sessions of responding for amounts of ethanol similar to that seen in studies using other strains of rats, Sprague-Dawley rats still did not exhibit an ADE. It is possible that prior to binge exposures, ethanol gel had no more reinforcement value than plain gel. Since rats were not deprived of plain gel during the deprivation period, it is not surprising an ADE did not occur. However even after the binge exposures increased motivation for ethanol, there was still no evidence for an ADE. Thus, the absence of an ADE in our study supports the possibility that Sprague-Dawley rats are less sensitive to temporary increases in
ethanol consumption such as the ADE. Another possibility is that we did not detect an ADE in our animals because the PR sessions ended at 30 minutes. It may be possible that longer sessions would reveal a second bout of responding. Additionally, PR schedules have not yet been shown to be susceptible to the effects of an ADE, therefore, subsequent experiments using longer sessions might be more successful if lower effort FR schedules are employed.

On the other hand, binge ethanol injections (3 g/kg, i.p.) produced a long-lasting increase in ethanol consumption and in the breakpoints for ethanol responding under PR10 schedules. This occurred in the absence of tolerance to the sedative effects of this high dose and did not appear to have any temporal relationship to ethanol withdrawal. Although the N for this study ended up being small (based on 1 animal dying in the saline group), we feel that there was sufficient power based on the repeated design used, which indicated very stable responding in the saline group and very stable responding in both groups for plain gel reinforcement. The data used for the bar graphs in Figures 4-7 and 4-8 were derived from multiple observations (7 or 10 days of testing) which when graphed over days (data not shown) were very similar in appearance to Figure 4-4 and 4-5 in their stability from day to day. Even so, we have since replicated these findings using a larger N (Binge group = 10; Saline group = 7) that are currently undergoing microdialysis experiments, which show similar statistically significant increases in breakpoints for ethanol gel reinforcement and in ethanol consumption on a PR10 schedule but only after binge ethanol treatments. Thus, high dose exposure to ethanol appears to cause a long-lasting increase in motivation for ethanol self-administration even during periods when rats are not undergoing withdrawal from ethanol.

Analysis of responding for each 5-min period during each session indicated that rats had completed over 97% of responding during the first 15 minutes of each session with no
responding occurring during the last 10 minutes of each session. Thus, this pause in responding was not due to slowing of responding due to intoxication since it was very similar in rats receiving plain gel reinforcement. After binge exposure, the increase in responding was primarily during the first 10 minutes of a session. This also argues against the development of tolerance to a response slowing effect of intoxication since that would not be expected to occur so quickly in the session.

What is particularly interesting in the absence of an effect of binge exposures on ethanol consumption under relatively easy reinforcement schedules indicating that this treatment does not alter overall satiety levels of ethanol. It has been reported there is a long term increase in voluntary ethanol consumption in the rat after intermittent exposure to alcohol (Rimondini et al., 2002). However, our study is the first to report an effect of intermittent ethanol binge exposure on motivation instead of total ethanol consumption. The increase in motivation may be involved in the earliest stages of development of alcoholism.

Although this effect of binge ethanol on PR breakpoints is a novel finding for the alcohol field, there are a few studies showing the effects of binge exposure on sensitization to cocaine reinforcement. PR schedules have been widely used to assess cocaine’s reinforcing efficacy (Katz, 1990). Morgan and his colleagues have focused on manipulations that alter the strength of cocaine to function as a reinforcer using operant schedules. They reported that there is an escalation of breakpoints after cocaine binge exposure which is only sensitive on the PR schedule and not the FR schedule (Morgan et al., 2005). It has been also suggested that early binge exposure during the drug exposure period plays a much more important role in the development of sensitization for the reinforcing effect of cocaine than later high level of drug exposure (Morgan et al., 2006).
In terms of ethanol self administration, a temporal threshold has also been suggested to exist for induction of long-lasting changes in voluntary alcohol consumption (Rimondini et al., 2003). Therefore, we hypothesize that there are at least two distinct stages involved in the development of alcohol dependence. The first one, demonstrated here, illustrates that intermittent high dose ethanol exposures can increase the reinforcing strength of alcohol such that motivation to work for ethanol access is increased. Only during the subsequent stage(s), is satiation decreased such that ethanol intake increases even under free access or easy operant schedules. Under these conditions, chronic high dose ethanol exposure produces a deteriorating behavioral profile including tolerance, physical dependence and a withdrawal syndrome. The next step for the current study will be to identify if there is an ethanol dose or exposure pattern threshold between ethanol exposures that alters motivation versus that altering satiation.

In summary, we have successfully determined a reliable model of ethanol exposure that can alter motivation for ethanol self administration. This animal model should be useful for studying the early stages of development of alcoholism particularly in adolescents. Additionally, the neurochemical and molecular changes that underlie this alteration in ethanol responding will be of great interest.
Figure 4-1. A summary of the PR training regimen and the “binge” ethanol exposures.
Figure 4-3. Blood ethanol concentrations determined immediately after the 30-min session on Day 11 by sampling tail blood. There was a significant correlation between ethanol dose and blood ethanol concentration. (N=6)
Figure 4-4. Response breakpoints of rats during 7 days of a PR5 and PR10 schedule for 10% ethanol in gelatin, followed by 10 days of plain gelatin as the reinforce (PR5 Plain). Shown are means and SEMs for N = 9.
Figure 4-5. Ethanol consumption of rats during 4 different ethanol responding periods (PR5: EtOH-1 and EtOH-2 and PR10: EtOH-3 and EtOH-4), which were separated by 10-day ethanol deprivation periods during which rats responded for plain gel (data not shown). There was no increase in ethanol responding or consumption during the first few days after the ethanol deprivation periods during any of the ethanol access periods. Shown are means and SEMs for N = 9.
Figure 4-6. Mean ethanol consumption during the different ethanol reinforcement schedules. There was a significant lower ethanol consumption during FR30 and PR5 sessions compared to FR1 and FR5 sessions. Ethanol consumption was the lowest during PR10 sessions compared to all other schedules of reinforcement. N = 9 for FR schedules and 8 for PR schedules.
Figure 4-7. Binge ethanol injections increased both ethanol consumption and response breakpoints. A) Binge ethanol injections increased ethanol consumption. B) Binge ethanol injections increased response breakpoints. Binge ethanol injections (3 g/kg, i.p.) were given on Days 4 and 6 of each of the 10-day plain gel periods occurring between the second and sixth PR10 ethanol sessions. Therefore, by 6 PR10, each rat in the EtOH group had received 8 binge injections. Since there was no difference in responding on any of the days during the 7-day ethanol periods, data shown are means and SEMs for the average of all 7 days for N = 5 and 3 per group. * P < 0.05.
Figure 4-8. Binge ethanol injections had no effect on plain gel consumption or response breakpoints. A) Binge ethanol injections had no effect on plain gel consumption. B) Binge ethanol injections had no effect on response breakpoints. Binge ethanol injections (3 g/kg, i.p.) were given on Days 4 and 6 of each of these 10-day plain gel periods occurring between the second and sixth PR10 ethanol sessions as described in figure 6. Data shown are means and SEMs for the average of all 10 days for N = 5 and 3 per group. * P < 0.05.
Figure 4-9. The time course of left lever responses. A) The time course of left lever responses during each 5-minute period of the 30-min PR10 sessions for ethanol gel in saline rats. B) The time course of left lever responses during each 5-minute period of the 30-min PR10 sessions for plain gel in saline rats. C) The time course of left lever responses during each 5-minute period of the 30-min PR10 sessions for ethanol gel in binge rats. D) The time course of left lever responses during each 5-minute period of the 30-min PR10 sessions for plain gel in binge rats. The majority of lever presses (over 97%) occur during the first 15 minutes of each session regardless of reinforcement type or whether rats received binge or saline injections.
Figure 4-10. Loss of righting reflex (LORR) did not differ after any of the 4 pairs of binge injections. N = 5.
Figure 4-11. Rats received additional operant sessions after the last set of binge injections and PR10 testing. A) Binge injections do not affect FR5 schedule. This consisted of 30 daily sessions of FR5 before binge injections, then 4 days of FR10 after 4 sets of binge injections and another 3 days of FR5 one month later. B) Binge injections have persistent effects on PR schedules. At no time did rats receive additional binge injections. 1PR10 indicates the first set of PR10 sessions, 6PR10 indicates the data from the PR10 sessions occurring one week after the last set of binge injections while 7PR10 indicates the PR10 sessions occurring over a month after the last set of binge injections. * P < 0.05.
Table 4-1. A summary of the operant FR self administration training regimen.

<table>
<thead>
<tr>
<th>FR</th>
<th>Number of days on schedule</th>
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<tbody>
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<td>30</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td>76</td>
</tr>
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CHAPTER 5

HIGH TEMPORAL RESOLUTION OF AMINO ACID LEVELS IN RAT NUCLEUS ACCUMBENS DURING OPERANT ETHANOL SELF-ADMINISTRATION: INVOLVEMENT OF ELEVATED GLYCINE IN ANTICIPATION

Introduction

One of the most valid preclinical models of alcoholism is that of self-administration, particularly operant procedures (the use of consequences to modify the occurrence and form of behavior) which allow quantification of the reinforcing effects of drugs (Poling and Bryceland, 1979). These procedures allow the study of different contingencies for alcohol reward, with good temporal resolution of self-administration behavior in a highly controlled environment, and low day-to-day variability within both subject and condition (Sanchis-Segura et al., 2006).

Many studies suggest that the mesolimbic dopaminergic system plays a very important role in the ethanol self-administration process. It has been reported that operant self-administration of ethanol is associated with increases in extracellular levels of DA in the NAC (Weiss et al., 1993; Doyon et al., 2003). Conversely, drugs such as naltrexone, which attenuate the rewarding properties of ethanol, may attenuate ethanol-induced stimulation of DA activity in NAC (Gonzales et al., 1998). However, the precise role of DA in the rewarding properties of ethanol is far from clear. A recent study pointed out that a transient increase of DA in NAC may be related to the stimulus properties of ethanol presentation rather than its pharmacological or reinforcing actions (Doyon et al., 2004).

Alternative neurochemical mechanisms for regulation of operant ethanol self-administration have not been thoroughly explored. The few studies measuring amino acid changes following forced ethanol administration report increased basal levels of extracellular glutamate as measured by microdialysis (Dahchour et al., 2003). Taurine levels also are increased in a dose-dependent manner by ethanol (Dahchour et al., 2000) and this escalation was enhanced
after repeated ethanol treatment (Dahchour et al., 1994, 1996; Dahchour and De Witte, 2000; Smith et al., 2004). This finding suggests that taurine may be a possible neurobiological molecule involved in ethanol reinforcement. This hypothesis is supported by findings that acamprosate (a calcium homolog of taurine) and homotaurine (a taurine precursor) both modulate the mesolimbic DA system and decrease ethanol reinforcement processes (Olive et al., 2002; Cowen et al., 2005). These studies used free drinking or forced administration of ethanol, and no study has reported changes in taurine during operant ethanol self administration. One of the objectives of the present study is to investigate taurine changes during operant self-administration of ethanol.

Ethanol in water is not extremely palatable, and many protocols in rodents require some form of deprivation and/or sucrose fading to establish high levels of intake. However, these are quite unlike the way that humans start to use ethanol. To better emulate this, we developed an ethanol-containing gelatin (10% ethanol, 10% Polycose®, 0.25% gelatin, wt/vol) that induces reliable and robust self-administration without food or water restriction (Rowland et al., 2005). Voluntary consumption of this “jello shot” results in pharmacologically relevant concentrations of ethanol in brain (Peris et al., 2006) comparable to those previously reported after similar amounts of voluntary ethanol drinking (Nurmi et al., 1999).

Changes in neurotransmitter release are thought to occur over a short time period during most behavioral events. Thus, in vivo detection of neurotransmitters under these conditions should have a high temporal resolution. To date, fast-scan cyclic voltammetry (FSCV) and microdialysis sampling with high-performance liquid chromatography (HPLC) have been used most often. FSCV provides good chemical selectivity as well as subsecond temporal resolution (Robinson et al., 2003). However, it is not able to determine the basal concentrations or to detect
DA changes for periods longer than 90 seconds (Heien et al., 2005). In contrast to FSCV, microdialysis sampling with HPLC can estimate basal concentrations and detect both DA and amino acid changes over longer times, but it requires longer sampling times, usually 5 to 20 minutes per sample. Each sample represents an average concentration during this period and so precludes detection of more rapid changes of extracellular chemicals associated with neurotransmission. A newer method, capillary electrophoresis (CE), possesses a high mass sensitivity (Zhou et al., 1995; Robert et al., 1998; Shou et al., 2006) and so requires only a very low volume of sample (≈20nl). This decreases the sampling time to 1sec. Coupled with a separation time of about 14 sec, this brings the sampling frequency within the range to allow monitoring of rapid changes that may correlate with behavioral episodes. CE also may be used to detect both catecholamines and amino acids simultaneously in dialysates of extracellular fluid (Bowser et al., 2001).

In the present study, we used CE coupled with laser induced fluorescence detection (CE-LIFD), to provide for the first time, almost real time measurement of amino acid changes in microdialysate during operant responding. This approach allows exploration of neural mechanisms underlying ethanol self-administration as well as those involved in positive reinforcement and anticipation of reward.

Methods

Animals and Housing

Male and female Sprague-Dawley rats were used in this study. Male rats used in Experiments 1 and 2 were approximately 6 months of age and weighed 485±11 g at the beginning of the study. Female rats used in Experiments 2 and 3 were approximately 6 weeks of age and weighed 200±8 g at the beginning of the study. Body weight was recorded weekly. All
rats were individually housed in a vivarium with a 12:12 light dark cycle (lights off at 0900) and an ambient temperature maintained at 23±2 °C. Rat use was approved by the IACUC and was consistent with the NIH Guide for the Care and Use of Laboratory Animals. Purina 5001 Rodent Chow and tap water was available ad libitum at all times except during daily operant sessions.

**Behavioral Apparatus**

As described in Chapter 2.

**Reinforcers**

Two types of gel reinforcement were used. The ethanol gel (1.03 kcal/g) was made with 10% ethanol (wt/vol), 10% Polycose® (wt/vol) (Abbott Laboratories, Abbott Park, IL) and 0.25% gelatin (wt/vol) (Knox brand, Kraft Foods, Northfield, IL) in water. Polycose is an oligosaccharide mixture (~4 kcal/g) that is sweet to rats. The second reinforcer was control (plain) gel (0.45 kcal/g) not containing alcohol, and was made from gelatin and Polycose at the above concentrations. The standard amount of gel delivered per reinforcement was 0.3 mL over 10 sec.

**Experimental Procedures**

**Experiment 1**

**Acclimation to control gel in home cage**

Fourteen male rats were given free access to a 50 mL glass jar of control gel, containing polycose but no ethanol, hung from a metal stirrup in their home cage. The gel was available for 24 hrs for the first two days of exposure, then for 6 hrs for two days, then 3 hrs for two days and finally for 1 hr per day for approximately 2 weeks as described earlier (Peris et al., 2006).

**Gelatin administration in the operant chamber**

**Shaping.** When 1 h free access consumption of the control gel stabilized (Peris et al., 2006), rats were placed in the operant chamber for a 10 min shaping session. During shaping, gel
reinforcements were delivered as the rats made successive approximations towards pressing the lever for a total of 3-5 reinforcements. This shaping session was immediately followed by a 30 min session in which gel delivery was contingent an FR1 schedule (one lever press yielded a reinforcement). A visual cue light was illuminated above the left lever at all times. Another visual cue, the house light, was illuminated to signal gelatin delivery and was terminated at the end of the 10 s gel delivery.

**Fixed ratio (FR) schedule.** After 3 days of shaping followed by FR1 sessions, rats were tested daily in 30 minute FR1 sessions for 30 days with only control gel reinforcement. They were then divided into two groups based on their performance under the FR1 schedule. The ethanol group (n=9) then only received reinforcements of 10% ethanol-containing gelatin and the control group (n=5) continued responding for the non-alcoholic control gel. At the same time, the reinforcement requirement was increased to FR5, i.e., five presses on the left lever were required for each reinforcement. During this training, a time out was introduced after each reinforcement which was gradually incremented (by 5 s per day) from 5 s to 120 s. Session length was also gradually increased to 50 min to allow for the same total number of reinforcements for each rat to remain stable. There was little spillage of the gel and this mostly occurred during initial training sessions. Any spillage that occurred was removed from the lickometer and weighed to the nearest 0.01g and subtracted from the daily total. All operant sessions were run during the light period at approximately the same time each day.

**Experiment 2.**

Several procedures were used to investigate the possibility that the effects observed in experiment 1 might be due to the operant task itself. We thus examined non-operant, free access to various non-alcoholic commodities in a standard home cage (40 cm X 40 cm X 30 cm high).
**Gel administration in home cage**

Ten male rats and 6 female rats received free access to the control gelatin in the home cage. The procedure was otherwise the same as Exp 1.

**Free access to moist chow or sugar fat whip in home cage**

Six male rats received free access to moist chow (equal weights chow and water) for 1 hr after overnight food deprivation on at least three occasions before microdialysis surgery. Another 6 male rats received free access to sugar fat whip (shortening: sugar =2:1) for 24 hrs (chow was also available) for the first two days of exposure, then for 6 hrs for two days, then 3 hrs for two days and finally for 1 hr for about 3~7 days before microdialysis surgery.

**Experiment 3.**

Ten female rats received free access to control gel in the home cage. The procedure was the same as Exp 1. After 3 days shaping followed by FR1 sessions, rats received daily 30 minute FR1 sessions for 30 days. At this point, the female rats were divided into two groups based on their performance under the FR1 schedule. The first group (n=5) were moved to 50-min FR5 session with no time out after reinforcement, and the second group (n=5) were moved to 50-min FR5 session with a gradually increased time out (10 sec/ day) after each delivery reaching a maximum 5-min time out.

**Surgery and Microdialysis Experiments**

As described in Chapter 2.

**CE-LIF**

As described in Chapter 2.

**Data Analysis and Histology**

About 300 electropherogram files, each containing over 11,000 data points, are collected each hour using the CE-LIF instrument. The detection of all peaks and the calculation of peak
heights and peak areas in these files were performed by “Cutter”, a Lab-View-constructed VI in which electropherograms are batch post-processed. The first twenty or more samples collected for each hour (prior to different stimulations or drug treatments) were used to calculate a baseline. Since there was considerable individual variability in the baseline values of analytes across animals, subsequent data were expressed as a percent of this baseline and calculated across animals as the mean ± SEM. The area under the curve (AUC) was calculated for each amino acid by summing the % baseline value of each amino acid calculated over time.

As an internal standard, we used a peak that is comprised of all the non-charged analytes that are labeled by OPA. This peak (which we will refer to as the OPA peak) has been shown to be very consistent from 1 to 6 hrs after probe implantation (see Figures 5-4, 5, 6,9,10) since the only factor which might alter its size is the injection efficiency of the flow gate. Thus, it is a good indicator of instrument variability and can be used as a control for the levels of the amino acids. In some cases, we present the OPA data as a reference peak (e.g., Figures 5-4, 5, 6,9,10) and in some cases, in order to reduce sample to sample variability caused by the instrument, we present the data as a ratio of the analyte of interest to the OPA peak (e.g., Figure 5-3).

In addition to the OPA peak, we also calculated the area under the curve for each time-response curve for every analyte of interest. To summarize these data, Δ AUC was calculated for each amino acid by subtracting the OPA AUC from the amino acid AUC. The AUC ratio for each amino acid was calculated by dividing amino acid Δ AUC by the OPA Δ AUC.

Data were analyzed using ANOVA or paired T-test. A significance level of p < 0.05 was used or all statistical analyses. At the end of each experiment, the rats were deeply anesthetized with pentobarbital sodium (120 mg/kg, i.p.). The brains were removed and sectioned to verify
the correct position of the probe. Only the data from rats with accurate probe placement were included in data analyses (Figure 5-1).

**Results**

**Experiment 1.**

**Gelatin consumption**

The number of ethanol gel and plain gel reinforcements obtained before surgery, after surgery, and on the dialysis day are shown in Figure 5-2. Rats took more reinforcements with plain gel than ethanol gel mainly because rats responded for plain gel throughout the session while those rats consuming ethanol gel stopped after 10-15 min (Figure 5-2B). These observations were supported by a significant time×treatment×gel delivery interaction (F(10,100) =21.71, P<0.0001).

**Effect of operant ethanol self-administration on taurine release in NAC**

There was a significant increase in taurine levels in NAC during 50 min of operant responding for ethanol gelatin compared with no change in the plain gel group (Figure 5-3A and 5-3C). This observation was supported by a significant ethanol main effect (F(1,9)=18.2, P<0.05). For the rats consuming higher than 0.4 g/kg ethanol (N=5), the average increase in taurine was 8.4±2.5%. In addition, the taurine increase was well correlated with ethanol dose (R²=0.81) with a maximum increase of 16.7% at intake of 1.12g/kg ethanol (Figure 5-3B).

**Effect of gelatin consumption on the glycine and serine levels in NAC during operant self administration**

There were large, consistent increases in glycine and small but consistent increases in serine in both the ethanol and plain gel groups during operant responding (Figure 5-4). This observation was supported by significant time X neurotransmitter interactions (F(1841, 12929)=1621, P<0.001 for ethanol gel operant; F(1841,12929)=1718, P<0.001 for plain gel
operant). Glycine increases correlated significantly with the amount of gelatin consumption in both groups (Figure 5-4D. There were no changes in either GABA or glutamate (data not shown).

**Experiment 2**

In order to determine whether the gel-induced increase in glycine is due to gelatin exposure or operant responding for gelatin, we also measured the change of amino acid levels during free access to plain gel in the home cage. The levels of glycine and serine increased in NAC during free access self administration (F(1841,12929)=439, P<0.001 for plain gel free access) (Figure 5-4C). The glycine increases during free access were also significantly correlated with gelatin consumption (Figure 5-4D); however, the slope of this relationship in the free access condition was much less steep than in the operant condition. In addition, there were small but significant increases of glycine and serine level about 10 min before the start of the operant session (Figure 5-5A and 5-5B) but no change in glycine or serine levels before access to plain gel (Figure 5-5C). These results was supported by significant time X neurotransmitter interactions (F(1276,8980)=70.08, P<0.001 for ethanol gel operant; F(1276,8980)=56.60, P<0.001 for plain gel operant; F(1276,8980)=1.95, NS for plain gel free access).

**Effect of gelatin consumption on the glycine and serine levels in hippocampus**

To determine if the increase in glycine is specific to the NAC, we repeated the plain gel free access experiment with the probe location in hippocampus. We did not find any increase of glycine or serine in hippocampus during free access plain gelatin consumption (Figure 5-6A and 5-7). The observation was supported by no time X neurotransmitter interaction.

**Effect of moist chow and sugar fat whip on glycine and serine levels in NAC**

In order to determine whether glycine was increased by other food commodities, we investigated the levels of glycine and serine during intake of moist chow or sugar fat whip. We
found no increase in the extracellular concentration of glycine in NAC during either wet chow (Figure 5-6B and 5-7A) or sugar fat whip (Figure 5-6C and 5-7A) intake. The observation was supported by no time X neurotransmitter (OPA and glycine) interactions. Slight increases in levels of serine were observed under both conditions (Figure 5-5B, 5-5C and 5-6B). One-way ANOVAs revealed a significant effect of gel intake on glycine release (F(2, 15)=9.09, P<0.005). A significant effect of brain region on glycine (F(1,10)=37.41, P<0.0001) and serine release (F(1, 10)=6.94, P<0.05) has been supported by the result of T test. There was no effect of moist chow and sugar fat whip on taurine release in NAC (data not shown).

At this point, we chose to use female rats in all subsequent operant experiments, since they have been found to have more consistent and slightly higher self-administration data (Eriksson 1968). Therefore, we needed to ascertain whether there is a sex difference in the changes of glycine and serine levels during plain gel consumption. Thus, we compared glycine and serine levels in NAC during free access presentation of plain gelatin in female and male rats. Both genders ate similar amounts of gel (21.9±6.5 g/kg for female; 16.5±2.5g/kg for male) and had similar increases in glycine (31.2±15.2% for females; 20.6±3.6% for males) and serine (6.6±2.5% for females; 4.8±1.4% for males).

**Experiment 3**

**Effect of time out periods during operant self-administration of plain gel**

Based on our findings that glycine increases more under operant conditions than during free access (Figure 5-4D) and that glycine starts to increase during the 10 min before the session (Figure 5-5A and 5-5B), we hypothesized that the increase of glycine in NAC may be involved in anticipation of reward. Therefore, we trained female rats to respond for plain gelatin under an FR5 schedule either with 0 or 5 min time-outs after each delivery. As before, responding for plain gel was not disrupted by either surgery or microdialysis sampling. Deliveries in the no
time-out group were significantly higher than the 5 min time out group both before and after surgery (Fig 5-8).

The levels of glycine were increased less in the no time out group (Figure 5-9A) compared to the 5min time out group (Figure 5-9B). Glycine increases were significantly correlated with gelatin consumption in both groups but with a much steeper slope in the 5-min time out group (Figure 5-10).

In addition, substantial increases of glycine and serine levels were also observed in both groups about 30 minutes before the session started (Figure 5-10). The observation was supported by two ANOVA statistic results that there was a significant neurotransmitter effect over all (F(5, 29)=6.25, P<0.05).

Discussion

The Effect of Operant Ethanol Self-administration on Taurine Levels in NAC

The present findings are the first to show that extracellular taurine levels increase in NAC during operant ethanol self-administration and that the magnitude of the increase is correlated with the amount of ethanol consumed. Several previous studies have shown that taurine release increases in NAC following IP ethanol injection in a dose-dependent manner (Dahchour et al., 1994, 1996; Dahchour and De Witte, 2000; Smith et al., 2004). Our results support and extend these observations by measuring taurine release during operant ethanol self-administration which, in Sprague-Dawley rats, results in fairly low brain ethanol levels. We found statistically reliable increases in taurine in rats that consumed more than 0.5g/kg ethanol. It has been reported that this low dose of ethanol (0.5g/kg, given IP) significantly increases taurine levels in NAC by 20% for about 10 mins using CE-LIFD (Smith et al., 2004). Our rats consumed 0.47±0.018 g/kg ethanol and the taurine level increased by 8.4±2.5%, which is only about half the increase reported after IP ethanol at the same dose. There is a similar two-fold difference in the brain
ethanol levels found after self-administration of 0.44±0.06g/kg ethanol compared to intra-gastric gavage of 0.5g/kg ethanol (Peris et al., 2006). In addition, the rats in the present study were self-administering ethanol under an FR5 reinforcement schedule with 2-min time out after each delivery schedule. This schedule ensures that ethanol intake is spread over ~20 min (Figure 5-2). Thus, it is not surprising that any alterations in taurine levels caused by ethanol concentrations rising in the brain would be more subtle under these conditions and would require a very sensitive measure of small changes in amino acid levels. Our studies provide the first evidence that the level of taurine increases in NAC during operant ethanol self-administration, probably due to the high temporal resolution of the CE-LIF technique.

Taurine is a non-essential sulfur-containing amino acid in the brain, present both in neurons and glial cells (Huxtable et al., 1989, 1992). It has many biological roles including neurotransmission and osmoregulation (Huxtable et al., 1992; Wright et al., 1986). The neural mechanism of the increase of taurine release following ethanol administration is not fully understood. Several previous studies suggested ethanol-induced taurine efflux may be due to neuronal depolarization (Smith et al., 2004), while other studies have indicated that ethanol-induced taurine efflux may be primarily due to osmoregulation (Quertemont et al., 2003).

In contrast to taurine, we found no effect on glutamate or GABA during operant ethanol self-administration. A previous study suggested that an increased level of extracellular glutamate is a key neurochemical feature associated with ethanol withdrawal (Melendez et al., 2005). In the current study, Sprague-Dawley rats had an average daily ethanol consumption of 0.67± 0.01g/kg, which is not high enough to develop withdrawal symptoms in the hours following ethanol exposure. Only one previous study described an increase in NAC extracellular GABA levels during alcohol consumption (Szumlinski et al., 2007). The discrepancy in findings with this
early study may be related to alcohol dose (0.67 g/kg vs 1.5–1.6 g/kg) and/or the species used (Sprague–Dawley rat vs C57BL/6J mouse).

The Relationship between the Increase of Glycine and Anticipation

A large and constant increase in glycine levels in NAC was observed during both plain gel and ethanol gel operant responding periods. Gelatin is high in the non-essential amino acids glycine and proline, however it does not appear that this is the cause of the increase in glycine seen in the NAC after gel consumption. First, the increase of glycine was greatest under operant conditions even though much more gel was consumed under free access conditions. This increase did not occur in hippocampus indicating that it is not due to an overall increase in brain glycine levels that might reflect high glycine content of gelatin. Normally, people consume approximately 0.023 g/kg of glycine per day. This dietary glycine does not normally influence brain glycine level (Lehninger, 1982). In contrast to humans, our rats consumed much smaller amount of glycine (0.016 g/kg in FR5; 0.0044 g/kg in FR5-2 min time out; 0.0028 g/kg in FR5-5 min time out; 0.0107 g/kg in male rats during free access and 0.014 g/kg in female rats during free access). On the other hand, some previous studies have shown that peripherally administered glycine may pass the blood-brain barrier and functionally elevate brain glycine levels when administered in sufficient quantity (Peterson, 1994; Toth et al., 1986; Toth and Lajtha, 1986). However, the doses of glycine used in these previous papers are at least 50~200 times higher than the amount of glycine that our rats consumed. Therefore, it suggested that the amount of glycine consumed by our rats is not high enough to elevate the level of glycine in the brain.

Interestingly, there was also a small increase of glycine about ten minutes before the start of the operant session for both ethanol and plain gel groups but not prior to free access sessions. In the operant experiments, rats were trained for 1 month to wait in the operant chambers for exactly 2 hr before the session started. Thus, environmental and temporal cues were present for
these rats concerning the impending gel reinforcement. Since the free access experiments were performed in cages that were identical to the home cage, these cues were not present. Finally, the longer the rat had to wait between barpressing episodes during a session, the greater the subsequent increase in glycine in the NAC, again, regardless of the fact that fewer reinforcements were given under these conditions. Together, these finding indicate that an increase in glycine in NAC may be more involved with anticipation of reward rather than reward itself.

There were no changes in glycine levels after presentation of rat chow or other palatable food. One previous study suggests that glycine levels decrease 30% in NAC during normal food consumption (Saul'skaya et al., 2001). Our data showed that there was no change in glycine level during normal chow intake. The discrepancy in the findings with the earlier study may relate to the different food administration before microdialysis. Our rats were food deprived for the 18 hrs prior to microdialysis to ensure that they consumed the food upon presentation. In the earlier study, rats received regular food administration which may suggest that food deprivation blocked or compensated for the food intake- induced reduction of glycine level in NAC. In addition, the level of glycine also did not change in the NAC during consumption of sugar fat whip intake, which would have been expected if glycine increased purely as a result of consumption of highly palatable food. In other studies, we have found that rats consume more sugar fatty whip than gelatin, further suggesting that the increase of glycine in NAC was not caused by intake of any highly palatable food.

Recent work demonstrates that glycine and strychnine alter extracellular DA levels in the NAC, probably via GlyR stimulation and blockade, respectively (Molander et al., 2005). The taurine-induced DA increase in the NAC is completely blocked by perfusion of the competitive
GlyR antagonist strychnine (Ericson et al., 2006). Therefore, it suggested that accumbal glycine receptors may be an access point for brain reward system. However, the present findings are the first to report that the glycine system plays a role in anticipation of reward. On the other hand, DA, serotonin and acetylcholine systems have been suggested to be involved in anticipation (Katner and Weiss, 1999; Doyon et al., 2003; Melendez et al., 2002).

Our study, for the first time, showed the real time measurement of changes of amino acid levels during operant responding by using CE-LIFD coupled with on line microdialysis. Our data supports previous findings that ethanol increases the extracellular concentration of taurine in NAC, even with the very low brain ethanol concentrations achieved during operant ethanol self-administration. In addition, our novel findings concerning alterations of glycine in NAC in response to different reinforcement schedules strongly suggests this neurotransmission as being involved in anticipation of reward.
Figure 5-1. Coronal sections showing microdialysis probe placement for all animals. A) Coronal sections showing microdialysis probe placement within the nucleus accumbens. B) Coronal sections showing microdialysis probe placement within the hippocampus. Lines indicate the active dialysis regions. Numbers below the figure represent the position of the slice relative to Bregma. The figure was adapted from Paxinos and Watson (2005).
Figure 5-2. Gelatin consumption for FR5 schedule with a 2 min time out. A) Comparison of the number of deliveries before surgery, after surgery and on the day of the microdialysis experiment. Responding for either ethanol or plain gel was not disrupted by either surgery or microdialysis. Deliveries of plain gel were significant higher than ethanol gel at all times. B) The number of ethanol and plain gel deliveries per 5 minute period during the 50-min FR5 session during microdialysis. There were fewer deliveries in the ethanol group, but only during the last 30 minutes of the session ($F(5,50) = 16.04, P<0.0001$). Each data point is the average of the number of deliveries per 5 min period with $N = 7$ for the ethanol group and $N= 5$ for the plain gel group.
Figure 5-3. Taurine levels during the 50-min FR5 session with 2 min time out. A) During the 15 min following the first reinforcement, there was a significant increase in taurine levels (N=7). The data are shown as % baseline of taurine divided by the % baseline of the OPA peak (which stayed at 100% throughout the session – see Figure 4) and lined up by the first reinforcement for each rat (indicated by the arrow). The basal level of taurine was 2.9±0.02 µM for ethanol gel group and 2.64±0.09 µM for plain gel group 1.5 hrs after implantation of probe. B) For the rats consuming ethanol higher than 0.4 g/kg (N=5), there was a significant increase in taurine compared to no change in the plain gel group (F(1,9)=18.2, P<0.05). The data are shown as the AUC ratio. C) There was a significant correlation between the taurine increase and amount of ethanol consumed for each individual rat (R2=0.81, P<0.05, N=7).
Figure 5-4. The levels of glycine and serine during and 1 hour following operant responding or free access to gel reinforcement. The levels of the OPA-labeled neutral peak are included in each panel for comparison. A) Levels of glycine and serine during and after operant ethanol gel self-administration (N=7). The basal levels of glycine and serine were 0.57± 0.07 µM and 10.68±0.15 µM, respectively. B) Levels of glycine and serine during and after operant plain gel self-administration (N=5). The basal levels of glycine and serine were 0.71± 0.1 µM and 13.51±0.23 µM, respectively. C) Levels of glycine and serine during and after free access plain gel presentation and 1hr following the session (N=10). The basal levels of glycine and serine were 0.67± 0.04 µM and 11.15±0.25 µM, respectively. The data in Panel A-C are shown as % baseline. D) Glycine increases were significantly correlated with gelatin consumption in all groups. (R2=0.81, P<0.05 in operant conditioning plain gel group, R2=0.84, P<0.05 in operant conditioning EtOH group, R2=0.88, P<0.05 in free access plain gel group). The data are shown as the AUC ratio.
Figure 5-5. The levels of glycine and serine before, during and after operant responding or free access. The results are showed as % baseline using the data as the baseline right after implantation of probe. A) The operant ethanol gel group. B) The operant plain gel group. C) The free access plain gel group. There are small increases of glycine and serine levels about ten minutes before the session started for both operant plain gel and ethanol groups compared to very little change in the free access plain gel group.
Figure 5-6. The levels of glycine and serine in the hippocampus during free access plain gelatin presentation, and in the NAC during moist chow and sugar fat whip intake. A) Levels of glycine and serine in the hippocampus during free access plain gelatin presentation. The basal levels of glycine and serine 1.5hrs after implantation of probe were 0.62±0.07 µM and 12.04±0.21 µM, respectively. B) Levels of glycine and serine in the NAC during moist chow intake. The basal levels of glycine and serine 1.5hrs after implantation of probe were 0.70±0.10 µM and 11.48±0.13 µM, respectively. C) Levels of glycine and serine in the NAC during sugar fat whip intake. The basal levels of glycine and serine 1.5hrs after implantation of probe were 0.72±0.13 µM and 10.48±0.29 µM, respectively. All data are shown as % baseline.
Figure 5-7. The levels of glycine and serine in the hippocampus and NAC during free access plain gelatin intake, in the NAC during moist chow and sugar fat whip intake. A) Levels of glycine in the hippocampus and NAC during free access plain gelatin presentation; in the NAC during moist chow and sugar fat whip intake. The level of glycine only increased when the probe in the NAC and during gelatin consumption (F(3,20)=30.4, P<0.05). The data are shown as the AUC of glycine divided by the amount of gelatin consumed and body weight. B) Levels of serine in the hippocampus and NAC during free access plain gelatin presentation; in the NAC during moist chow and sugar fat whip intake. The increases of serine level are same for all experiments except when the probe in the hippocampus and during gelatin consumption (F(3,20)=14.2, P<0.05). The data were shown as the AUC of serine divided by the amount of gelatin consumed and body weight.
Figure 5-8. Gelatin consumption for no time out and 5 min time out groups. A) Comparison of delivery number before surgery, after surgery and on the microdialysis day. Rat responding for plain gel were not disrupted by either surgery or microdialysis. Deliveries in the no time out group were significant higher than in the 5 min time out group both before and after surgery. B) Number of deliveries per 5 min period during the 50-min FR5 session during microdialysis for no time out and 5 min time out groups. (N=5 for no time out, N=5 for 5 min time out).
Figure 5-9. The levels of glycine and serine during, and 1 hour following, operant responding under an FR5 schedule with 0 min or 5 min time out after each delivery. A) Levels of glycine and serine during operant responding under FR5 schedule with no time out (N=5). The basal levels of glycine and serine 1.5 hrs after implantation of probe were 0.64± 0.17 µM and 11.31±0.17 µM, respectively. B) Levels of glycine and serine during operant responding under FR5 with 5 min time out (N=5). The data in Panel A and B are shown as % baseline. The basal levels of glycine and serine 1.5 hrs after implantation of probe were 0.52± 0.06 µM and 10.59±0.26 µM, respectively. C) Glycine increases were significantly correlated with gelatin consumption in all groups but much larger increases were seen in the 5 min time out group even though they ate much less gel (R²=0.72, P<0.05 in 5 min time out group, R²=0.97, P<0.05 in no time out group). The data are shown as the AUC ratio.
Figure 5-10. The levels of glycine and serine using the baseline right after implantation of probe. A) The no time out group. B) The 5 min time out group. There are slight increases of glycine and serine levels about forty minutes before the session start. C) Area under curve (% baseline) of OPA, glycine and serine during waiting period. Comparing to OPA, there were significant increase of glycine and serine which was support by a main effect of analytes but no effect or interaction involving delay time as indicated by *.
CHAPTER 6
NEURONAL MECHANISM OF INCREASED MOTIVATION FOR OPERANT ETHANOL SELF-ADMINISTRATION CAUSED BY BINGE EXPOSURE

Introduction

Alcoholism is a severe disease that affects millions of American each year. As a common pattern of excessive alcohol use, binge drink affects the highest percentage of drinking people, especially young people. Binge drinking is defined as a pattern of drinking that elevates blood alcohol concentrations (BAC) to 0.8% or higher, normally after five or more drinks per session for man and four or more for woman. In 2006, about 10.8 million adolescent aged 12 to 20 (28.3 percent) reported using alcohol. Approximately 7.2 million (19.0 percent) were binge drinkers and 2.4 million (6.2 percent) were heavy drinkers in the United State (SAMHSA, 2006). Recently, clinical alcohol studies indicate that the more binge exposure people have in adolescence and young adulthood, the higher chance they develop to be alcoholics (Enoch, 2006). Therefore, binge drinking has been considered as one of the most important beginning steps for the development of alcoholism. One of the major goals of basic science alcoholism research is to identify alcohol-induced neuroadaptation contributing to the development of alcoholism which may provide pharmacological treatment principles for alcohol dependence or abuse.

In order to investigate the possible neuronal mechanism underlying binge exposure, development of an animal model of alcoholism is a key step. Rimondini (et al., 2002) developed a rat model of alcoholism that uses intermittent high dose ethanol exposure resulting in long-lasting increases in voluntary ethanol consumption in rats. In addition, several recent works reported a new mice model named scheduled high alcohol consumption (SHAC) (Cronise et al., 2005; Finn et al., 2005). In this animal model, C57BL/6 mice consume about 1.5–2 g/kg alcohol during a 30-min period over multiple alcohol presentations. Such high ethanol consumption
results in BACs above 100 mg%, which produces signs of intoxication. However, C57BL/6 mice represent only a very small group of drinkers because they have very high basal ethanol consumption compared to other rodents. Our lab recently developed a rat model of increasing motivation for operant ethanol self-administration caused by multiple binge exposures, which attempts to parallel early aspects of alcoholic conditions. This model termed Increased Motivation of Alcohol Consumption (IMAC) provides a good tool to investigate the neurobiological consequences of repeated binge alcohol exposures in rodents.

Ethanol administration affects many neurotransmitter systems in the NAC which is an important brain area mediating reward/reinforcement. In particular, alcohol administration increases DA levels in NAC. About thirty years ago, the first reports of ethanol induced-DA release in the NAC appeared in the literature (Imperato and Di Chiara, 1986). Following that, a large number of laboratories replicated and extended the findings through all kinds of ethanol administration including 60% intraperitoneal administration (Imperato and Di Chiara, 1986; Carboni et al., 1989; Yoshimoto et al., 1991, 1992; Diana et al., 1992; Acquas et al., 1993; Tanda and Di Chiara, 1998; Olive et al., 2000a; Gonzales et al., 2002; Lominac et al., 2006), 16% local perfusion of ethanol in NAC (Wozniak et al., 1991; Yoshimoto et al., 1991, 1996; Benjamin et al., 1993; Yan et al., 2003; Ericson et al., 2003), 10% nonoperant oral self-administration (Nurmi et al., 1998; Engelman et al., 2000; De Montis et al., 2004), 8% operant oral self-administration (Weiss et al., 1993, 1996; Melendez et al., 2002; Doyon et al., 2003) as well as intravenous administration (Bradberry, 2002). Therefore, DA release in NAC is considered to be the neurobiological substrate of ethanol reward (Tupala and Tiihonen, 2004).

In addition, ethanol inhibits NMDA (Lovinger and Zieglsanger0, 1996; Lovinger et al., 1989, Wirkner et al., 2000), kainate (Dildy-Mayfield and Harris, 1995) and metabotropic
glutamate receptors. Depending on the dose of ethanol administration, the change of glutamate release in NAC is biphasic. Acute low dose ethanol administration has been reported to increase glutamate release but high dose ethanol administration decreases it (Moghaddam and Bolinao, 1994; Dahchour et al., 1994, 1996; Dahchour and De Witte, 2000; Selim and Bradberry, 1996; Piepponen et al., 2002; Szumlinski et al., 2005; Lominac et al., 2006). Moreover, with repeated ethanol injections, a sensitized glutamate release has also been observed in the NAC (Szumlinski et al., 2005, 2007).

The glycine system has also recently emerged to be important for investigation of neural mechanism of ethanol. Most previous studies focused on the relationship between the glycine receptor (GlyR) or transporter (GlyT1) and ethanol. It is suggested that glycine and strychnine alter extracellular DA levels in the NAC, probably via GlyR stimulation and blockade, respectively (Molander et. al., 2005). Strychnine-sensitive GlyR in the NAC regulate both basal and ethanol-induced mesolimbic DA activity (Molander and Söderpalm, 2005a, 2005b). In addition, the taurine-induced DA increase, observed after ethanol administration, is completely blocked by perfusion of the competitive GlyR antagonist strychnine in the NAC (Ericson et. al., 2006). Therefore, GlyRs in the NAC might constitute targets for EtOH in its mesolimbic DA-activating effect. Recent works proved that org 25935, a GlyT1 inhibitor, decreased EtOH intake and EtOH preference in EtOH high-preferring rats (Molander et. al., 2007). These data indicate that developing selective GlyR agonists, antagonists, or GlyT1 inhibitors could represent a new pharmacological treatment principle for alcohol dependence or abuse (Molander et. al., 2005). Similarly, administration of glycine and strychnine locally into the NAC also alters EtOH consumption in high EtOH-preferring male Wistar rats (Molander et. al., 2005). However, few studies have investigated the release of glycine in NAC during alcohol consumption. Although
our previous study suggests that increases of glycine are involved in anticipation of reward, the
increase of glycine does not differ between operant responding for ethanol gel and plain gel (Li
et al., 2008). It may be due to the fact that Sprague-Dawley rats with only daily access to ethanol
have equal anticipation for both plain gel and ethanol gel. Therefore, in order to characterize the
relationship among glycine, anticipation and ethanol, our current experiment investigated the
change of glycine level using the IMAC model with increased the motivation for ethanol
consumption.

On the other hand, many studies suggest that taurine may be a possible neurobiological
molecule involved in ethanol consumption. Previous studies showed that acamprosate (a calcium
homolog of taurine) and homotaurine (a taurine precursor) modulate the mesolimbic
dopaminergic system and may thereby decrease ethanol reinforcement processes (Olive et. al.,
2002; Cowen et al., 2005). In addition, an elevation of taurine level was observed to significantly
correlate with the acute ethanol dose, and this escalation was enhanced after repeated ethanol
treatment (Dahchour et. al., 1994, 1996; Dahchour and De Witte, 2000; Smith et. al., 2004; Li et
al, 2008). Thus, we are also interested in how taurine changes in our IMAC model.

Evidence from pharmacology, electrophysiology and neurochemistry studies suggest that
DA and amino acid neurotransmitter systems play a very important role in the pathophysiology
and treatment of alcoholism (De Witte, 2004; Johnson, 2005; Tupala and Tiihonen, 2004;
Williams, 2005). A recent study demonstrates that there are accumbens neurochemical
adaptations produced by binge-like alcohol consumption in B6 mice. Particularly, a sensitized
glutamate release is observed in NAC (Szumlinski et al., 2007). Although there is no effect on
DA release in NAC in this study, some previous studies reported a sensitization of alcohol
induced DA release after repeated, intermittent alcohol injections (Szumlinski et al., 2005; Zhao
et al., 2006). The present study employed the IMAC model to determine whether a history of repeated high dose ethanol exposures influences the neurotransmitter response to subsequent operant ethanol self-administration or to an IP alcohol binge injection. We hypothesize that there are sensitized releases of glutamate and DA in NAC in binge treated animals. In addition, according to our previous finding that the accumbal release of glycine may be involved with anticipation (Li et al., 2008), we also expect that the release of glycine will be much higher in the binge treated group than the control group.

Materials and Methods

Animals and Housing

Seventeen female Sprague-Dawley rats were used. Rats were ~2.5 months of age and weighed 204±2 g at the beginning of the study. Body weight was recorded once a week during testing. All rats were singly housed in a vivarium with a normal 12:12 light dark cycle (lights off @ 1800) and an ambient temperature maintained at 23±2 °C. Purina 5001 Rodent Chow was available ad libitum at all times except during daily 30-min operant sessions. 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.

Behavioral Apparatus

As described in Chapter 2.

Surgery and Microdialysis Experiments

As described in Chapter 2.

CE-LIF

As described in Chapter 2.
**Dopamine Spiking**

Preliminary experiments indicated that the limit of detection of our capillary electrophoresis instrument is about 50 nM for DA. We developed a DA spiking method in which 50 nM standards were added into dialysate right before mixing with the OPA derivatization solution. In this way, the basal levels of DA were brought into the range of the limit of detection and increases in DA could now be quantified. In vivo experiments proved that DA spiking was stable for at least 6 hours which allowed most experiments to be completed. In addition, cocaine administration (20 mg/kg, ip.) increased DA release in NAC by 3 fold which is similar to previous studies (Carboni et al., 1989; Pettit and Justice, 1989; Kuczenski et al., 1991) (Figure 6-1).

**Data Analysis and Histology**

About 300 electropherogram files, each containing over 11,000 data points, are collected each hour using the CE-LIF instrument. The detection of all peaks and the calculation of peak heights and peak areas in these files were performed by “Cutter”, a Lab-View-constructed VI in which electropherograms are batch post-processed. The first twenty or more samples collected for each hour (prior to different stimulations or drug treatments) were used to calculate a baseline. Since there was considerable individual variability in the baseline values of analytes across animals, subsequent data were expressed as a percent of this baseline and calculated across animals as the mean ± SEM. The area under the curve (AUC) was calculated for each amino acid by summing the % baseline value of each amino acid calculated over time.

As an internal standard, we used a peak that is comprised of all the non-charged analytes that are labeled by OPA. This peak (which we will refer to as the OPA peak) has been shown to be very consistent from 1 to 6 hrs after probe implantation since the only factor which might alter its size is the injection efficiency of the flow gate. Thus, it is a good indicator of instrument
variability and can be used as a control for the levels of the amino acids. In order to reduce sample to sample variability caused by the instrument, we present the data as a ratio of the analyte of interest to the OPA peak (e.g., Figures 6-6,7,9).

In addition to the OPA peak, we also calculated the area under the curve for each time-response curve for every analyte of interest. To summarize these data, ΔAUC was calculated for each amino acid by subtracting the OPA AUC from the amino acid AUC. The AUC ratio for each amino acid was calculated by dividing amino acid ΔAUC by the OPA AUC (e.g., Figure 6-8).

Data were analyzed using ANOVA or paired T-test. A significance level of p < 0.05 was used for all statistical analyses. At the end of each experiment, the rats were deeply anesthetized with pentobarbital sodium (120 mg/kg, i.p.). The brains were removed and sectioned to verify the correct position of the probe. Only the data from rats with accurate probe placement were included in data analyses (Figure 6-2).

**Results**

Before the binge injections, the binge and control rats consumed similar amounts of ethanol (0.46±0.019g/kg) during 30-min operant self-administration sessions using 10% ethanol-containing gel. The binge group (n=10) received 3 g/kg i.p. ethanol “binge” injections and the control group (n=7) received saline injections on Days 4 and 6 during each of the cycles of plain gel responding (see Figure 4-1). Breakpoint and ethanol consumption rose in the binge group reaching a maximum increase of 40% by the fourth pair of binge injections (F (4,346) =3.7; P<0.05). There was no effect of saline injections on either breakpoint or ethanol consumption (Figure 6-3) or an effect of binge exposures on breakpoint or consumption of plain gel reinforcement (Figure 6-4).
Intake did not vary across the five alcohol drinking days before microdialysis, nor did the microdialysis procedures influence significantly alcohol intake in binge and control rats (Figure 6-5A and B). However, there were significant effect of microdialysis surgery on both breakpoint and consumption of ethanol gelatin responding (F (1,118) = 5.7, P<0.05) (Figure 6-5C) but not plain gel responding (Figure 6-5D).

As in the operant drinking studies, no significant group differences were found in baseline concentrations of all neurotransmitters except glutamate. On-line detection of the change of DA and amino acids showed that that there was greater release of taurine, DA and glutamate in the NAC in the binge rats than the control rats during 10% ethanol-containing gelatin operant responding (Figure 6-6). An overall significant time X group X operant responding interaction (F (1028, 7503) =38.7, P<0.001 for taurine; F (1028, 7503) =26.5, P<0.001 for glutamate; F (1028, 7503) =9.18, P<0.005 for DA; F (1028, 7503) =90.95, P<0.001 for glycine) was detected. During the 30 min operant ethanol gel responding, taurine level increased by 15% in both binge rats and control rats. It was supported by a significant effect of time (F (99, 1485) = 1.34, P<0.05) regardless group with no interaction. During the following 30 min, taurine level was still slightly higher in binge rats than control rats. There was a significant effect of group (F (1, 15) = 24.34, P<0.05) with no interaction or the effect of time.

During the 30 min operant ethanol gel responding, glutamate level gradually increased by 10% in binge rats compared with no change in control rats. It was supported by a significant group X time interaction (F (99, 1485) = 1.40, P<0.05) which was due to a significant effect of time in the binge rats (F (99, 891) = 1.48, P<0.05) but not in the control rats (F (99, 594) = 1.19, NS). During the following 30 min, glutamate level still slightly increased in binge rats. There was a significant group X time interaction (F (99, 1485) = 1.36, P<0.05) which was due to a
significant effect of time in the binge rats (F (99, 891) = 1.61, P<0.05) but not in the control rats (F (99, 594) = 0.91, NS).

During the 30 min operant ethanol gel responding, DA level increased by 15% in binge rats compared with no change in control rats. It was supported by a significant group X time interaction (F (99, 1485) = 1.84, P<0.05) which was due to a significant effect of time in the binge rats (F (99, 891) = 2.34, P<0.0001) but not in the control rats (F (99, 594) = 1.04, NS). During the following 30 min, DA level came back to basal in the binge rats. There was no interaction or effect of time or group.

In addition, a greater release of glycine was observed in the binge rats during operant responding for 10% ethanol-containing gelatin than plain gel responding (Figures 6-6D and 6-7D). During the 30 min operant ethanol gel responding, there was a significant group X time interaction (F (99, 1485) = 1.65, P<0.05) which was due to a significant effect of time in the binge rats (F (99, 891) = 4.99, P<0.0001) but not in the binge rats (F (99, 594) = 1.02, NS). During the following 30 min, there was a significant group X time interaction (F (99, 1455) = 1.31, P<0.05) which was due to a significant effect of time in the control rats (F (97, 582) = 1.97, P<0.0001) but not in the binge rats (F (97, 873) = 0.85, NS).

As also illustrated in figure 6-3, binge and control rat differed in alcohol consumption during microdialysis which is an indicator that binge rats had more motivation than the control rats. In order to prove that the changes of amino acids and DA in binge rats is due to intermittent ethanol binge exposures not the greater amount of ethanol consumed in the binge group, we analyzed the data from all rats with 2 or 3 reinforcements. In this way, the binge group and control groups had similar breakpoints and ethanol consumption (binge group: N=6, BP=25±2.24, ethanol consumption=0.41±0.03; control group: N=6, BP=23.3±2.11, ethanol
consumption=0.39±0.02). We also observed greater release of DA, glutamate, taurine and glycine in binge rats than control rats.

Each individual rat has different responding pattern during operant session and consumed different amount of ethanol. Therefore, we correlated neurotransmitter release and ethanol consumption in figure 6-8. There was much greater release of DA, glutamate, taurine and glycine per amount of ethanol consumed in binge group which is indicated by much greater slope in the binge group than the control group. In addition, neurotransmitter release was well correlated with the amount of ethanol intake for each group.

In order to determine that sensitized release of DA and amino acids was an effect related to ethanol exposure, each rat received an operant plain gel responding session one day after ethanol gel responding. However, only part of the rats responded for plain gel (N=6 for the binge group; N=6 for the control group). The data indicated that there was no effect on the DA, taurine and glutamate release in either group during plain gel responding (Figure 6-7). The observations were supported by statistical results of two-way ANOVA as follows. During the 30 min of operant plain gel responding and 30 min after, there was no interaction or effect of time or group on the taurine, DA and glutamate release. The increase of glycine levels did not differ between the binge and control rats (Figure 6-7D). Statistic results showed that during the 30 min of operant plain gel responding, there was a significant time effect (F (99, 1485) = 1.66, P<0.05) regardless of group with no interaction or effect of group. During the following 30 min after operant plain gel responding, there was no interaction or effect of time or group on glycine release.

In order to determine if the differences in the DA and amino acid responses to operant self-administered ethanol reflected alterations in pharmacological responsiveness to alcohol, the
binge and control rats received an IP injection of a fixed ethanol dose (3g/kg) one hour after ethanol/plain gel responding. Intermittent ethanol binge exposure history influenced extracellular NAC levels of DA, glutamate and taurine after a 3 g/kg alcohol injection (Figure. 6-9). As illustrated in figure 6-9A, injection elicited a large taurine increase that did not differ between binge and control rats. However, the increase of taurine lasted for at least 2 hours after injection in binge group compared only lasted for 25-30 mins in the control group. The observations were supported by the results of two-way ANOVA as follows. An overall significant time X group interaction (F (944, 8054) = 273.24, P<0.0001) was detected. During the first 20 min sampling, there was a significant effect of time regardless of group (F (80, 1200) = 20.35, P<0.0001) with no interaction or effect of group. However, during 20 to 45 min after binge injection, there was a significant group X time interaction (F (99,1485) = 2.35, P<0.05) which was due to a significant effect of time in the control rats (F (99,594) = 9.89, P<0.0001) but not in the binge rats (F (99,891) = 0.97, NS). During 45 to 70 min after injection, there was again a significant group X time interaction (F (99,1485) = 1.32, P<0.05) but now it was due to a significant effect of time in both the control rats (F (99,594) = 2.43, P<0.0001) and the binge rats (F (99,891) = 2.10, P <0.0001). During the last 48 mins, there was a main effect of groups with not effect on interaction of time.

In addition, a group difference in NAC glutamate produced by 3.0 g/kg ethanol injection was observed (Figure 6-9B). Ethanol slightly decreased glutamate levels in the control rats, but elicited a large and latent rise in extracellular glutamate in binge rats. It was supported by an overall significant time X group interaction (F (944, 8054) = 491.86, P<0.0001). During the first 20 min of sampling, a significant effect of time (F (80, 1200) = 1.28, P<0.05) was detected. It was due to a significant effect of time both in the binge rats (F (80, 720) = 2.02, P<0.0001) and
in the control rats (F (80,480) = 1.31, P<0.05). However, during 20 to 45 min after binge injection, there was a significant group X time interaction (F (99, 1485) = 2.34, P<0.05) which was due to a significant effect of time in the binge rats (F (99, 891) = 3.29, P<0.0001) but not in the binge rats (F (99, 594) = 0.74, NS). During the last 73 mins, there was a main effect of groups with not effect on interaction of time.

Similar to glutamate, there was a group difference in NAC DA produced by a 3.0 g/kg ethanol injection (Figure 6-9C). Acute ethanol injection did not affect DA levels in the control rats, but increased the extracellular concentration of DA by 20% in binge rats. The observations were supported by the results of two-way ANOVA as follows. An overall significant time X group interaction (F (944, 8054) =858.82, P<0.0001) was detected. During the first 20 min sampling, there was no main effect of groups or no interaction or effect of group. However, during 20 to 118 min after binge injection, there was a main effect of groups with not effect on interaction of time.

Following the 3.0 g/kg ethanol injection, a slight increase of glycine release was observed in the binge group but not the control group in the first 20 min. During 20 to 45 min after binge injection, there was a big decrease of glycine release by 40% in the control rats while only 20% in the binge rats (Figure 6-9D). The observations were supported by an overall significant time X group interaction (F (944, 8054) =472, P<0.0001) was detected. In the first 20min sampling, there was no interaction or effect of group but a significant effect of time (F (80, 1200) = 1.28, P<0.05) which was due to a significant effect of time in the binge rats (F (80, 720) = 1.58, P<0.001) but not in the control rats (F (80,480) = 0.91, NS). During 20 to 45 min after binge injection, there was a significant group X time interaction (F (99,1485) = 1.99, P<0.05) which was due to a significant effect of time both in the control rats (F (99,594) = 12.02, P<0.0001) and
in the binge rats (F (99,891) = 9.83, P<0.05). During the last 73 mins, there was a main effect of groups with not effect on interaction of time.

**Discussion**

The present study demonstrated that there was greater release of taurine, DA and glutamate in the NAC in the binge rats during 10% ethanol-containing gelatin operant responding than the control rats. Similar results were observed after 3 g/kg ethanol i.p injection, but the sensitized release was much larger than during operant responding period. In addition, a sensitized release of glycine was observed in the binge rats during 10% ethanol-containing gelatin operant responding but not plain gel operant responding. These data further confirmed our previous finding that the release of glycine in NAC is involved with the anticipation.

A time course has been suggested to exist for induction of long-lasting changes in voluntary alcohol consumption (Rimondini et al., 2003) which is further demonstrated in our previous findings. We hypothesize that there are at least two distinct stages involved in the development of alcohol dependence. The first one illustrates that intermittent high dose ethanol exposures can increase the reinforcing strength of alcohol such that motivation to work for ethanol access is increased. Only during the subsequent stage(s) is satiation increased, such that ethanol intake increases even under free access or easy operant schedules. Under these conditions, chronic high dose ethanol exposure produces a deteriorating behavioral profile including tolerance, physical dependence and a withdrawal syndrome. The traditional rodent model of ethanol self-administration shows a marked increase in the amount of alcohol it takes to satiate voluntary alcohol consumption after multiple binge exposures (Rimondini et al., 2002, O’Dell et al., 2004) which limits investigation of alcohol-induced neural adaptation to the latter stage of development of alcoholism. The IMAC model was employed in the current study to examine early aspects of neuronal adaptation produced by intermittent binge exposures. This
animal model has demonstrated that intermittent binge ethanol exposure persistently increases motivation for ethanol self-administration not satiation. In this case, it should be especially useful for studying the early stages of development of alcoholism particularly in adolescents.

Traditionally, accumbal DA release is considered to be the neurobiological substrate of the development of alcoholism (Tupala and Tiihonen, 2004). However, during 20 years of investigation which consistently demonstrate that alcohol administration increase DA levels in NAC, only 8% studies have examined NAC DA during operant self-administration of ethanol (cf. “Introduction”). Weiss (et al., 1993) reported the first investigation of DA release in NAC during operant ethanol self-administration. It suggests there is a significant, dose-dependent rise in accumbal DA release with maximal effects at approximately 15 min after peak intake. In addition, it also proves that elevation of extracellular levels of DA is from the pharmacological effects of ethanol not from the effects of operant responding by comparing operant self-administration of ethanol vs saccharin. Consistent with our observations, several studies confirm and extent the results that accumbal DA is increased in the first 5 min of ethanol but not water (Weiss et al., 1996; Gonzales and Weiss, 1998; Melendez et al., 2002; Doyon et al., 2003).

Conflicted results exist from studies investigating the sensitization of ethanol-induced NAC DA release. Some studies show a sensitization of DA release following intermittent ethanol exposure (Szumlinski et al., 2005; Zhao et al., 2006). However, other studies have failed to detect sensitized DA release in NAC (De Montis et al., 2004; Zapata et al., 2006). For an example, a recent study shows that repeated binge-like drinking of pharmacologically relevant alcohol doses does not sensitize NAC DA responses to either ingested or injected alcohol despite altering alcohol-induced changes in NAC serotonin, GABA, and glutamate (Szumlinski et al., 2007). In contrast to this recent study, the present data demonstrated a sensitized DA release in
NAC in IMAC during operant ethanol response and challenge ethanol injections. The
discrepancy in our findings with this earlier study may relate to differences in the route of
alcohol binge exposure (IP injection vs oral self-administration), alcohol dose (3 g/kg vs 1.5–1.6
g/kg), species (Sprague–Dawly rat vs C57BL/6J mouse), or the duration of deprivation period (7
days vs 2–3 days). Additionally, an alcohol deprivation effect was not observed in either the
Szumlinski’s study or the current study.

For several decades, the glutamatergic system has been proposed to play a very important
role in the development of neuroadaptation in alcohol withdrawal (Littleton, 1998). The
adaptation process mainly involves a straightforward homologous upregulation in the number
and/or function of receptors, especially NMDA receptors (Samson and Harris, 1992) based on
the inhibitory actions of ethanol on these receptors. On the other hand, it has been shown that
there are alterations of glutamate transmission following either the acute or chronic behavioral
effects of alcohol (Siggins et al., 2003; Volkow et al., 2003). Systemic alcohol administration
elevates glutamate levels in the NAC (Dahchour et al., 2000; Lominac et al., 2006). Repeated
ethanol injections cause a sensitized release of glutamate in NAC (Szumlinski et al., 2005).
Although consistent with existing reports that repeated or chronic alcohol exposure can sensitize
 glutamate levels within NAC of both self-administered and experimenter-administered alcohol
(Szumlinski et al., 2007), the sensitized glutamate release in current study is much less than in
this earlier study. This may be because C57BL/6J mice have much higher motivation to
consume alcohol compared to Sprague–Dawly rats. Furthermore, we also repeated previous
findings that there was an upregulation of the basal level of accumbal glutamate (Melendez et al.,
2005).
Taurine is a non-essential sulfur-containing amino acid in the brain, present both in neurons and glial cells (Huxtable et al., 1989, 1992). It is involved in many biological roles mainly including neurotransmission and osmoregulation (Huxtable et al., 1992; Wright et al., 1986). It has been suggested that taurine may provide an alternative neuronal mechanism underlying ethanol function. Previous studies reported a low dose (0.5g/kg) ethanol-induced increase of NAC taurine during both operant self-administered (Li et al., 2008) and experimenter-administered alcohol (Smith et al., 2004) by using CE-LIFD. This present study, for the first time, describes a sensitized taurine release during ethanol self administration in rats that had received intermittent ethanol binge exposures. The neuronal mechanisms mediating the sensitized taurine release in the IMAC group are not fully understood. Additionally the impact of this sensitized taurine on other neurotransmitters is unknown. Early studies indicate that local perfusion of taurine can upregulate the level of DA (Ericson et al., 2006) but decrease basal release of glutamate and glycine in NAC (Olive et al., 2000b). In addition, it has been demonstrated that taurine can block accumbal glutamate release during the initial ethanol withdrawal period (Dahchour et al., 1996). Therefore, the sensitized taurine release may inhibit glycine release which is due to increased motivation for ethanol, or inhibit sensitized glutamate release in NAC or initiate sensitized NAC DA release contributing to decreased alcohol craving behavior and increased alcohol consumption behavior.

On the other hand, several recent studies pointed out that the glycine system is important for investigation of neural mechanism of ethanol. Most previous studies focus on relationship between the glycine receptor or transporter and ethanol action (cf “Introduction”), with very few alcohol studies investigating transmission of glycine in mesolimbic brain regions. It has been shown that glycine perfusion decreases extracellular concentrations of DA in NAC (Molander
and Söderpalm, 2005a). Our recent study gives novel evidence that NAC glycine may be involved in anticipation during longer waiting periods between responding, since larger glycine increases are observed (Li et al., 2008). The current finding confirmed our previous results by demonstrating a much larger increase of glycine in the IMAC group than in the control group during operant ethanol responding but not plain gel responding. This is due to the IMAC rats increased motivation for ethanol consumption compared to normal rats (Li et al., 2008).

Overall, the exploration of the imbalance of excitatory and inhibitory neurotransmission caused by intermittent ethanol binge exposures is the first step for understanding the neuronal adaptation underlying alcoholism. The characterization of the sensitized release of DA, glutamate, taurine and glycine and the investigation of the interactions amongst these neurotransmitter changes are the next logical steps in our understanding of alcoholism.
Figure 6-1  Cocaine administration (20 mg/kg, ip) induced release of DA in NAC (N=6). A) Cocaine induced DA release in NAC. The data are shown as the % baseline. B) Cocaine induced DA release in NAC. The data are shown as AUC ratio. OPA (AUC ratio) =-0.072±0.018; DA (AUC ratio) =0.22±0.05. [DA]=(0.22+0.072) * 50 nM=14.6nM. Compared with 4~5 nM basal concentration of DA in NAC, there were around 3~4 times increase of DA after cocaine administration.
Figure 6-2  Coronal sections showing microdialysis probe placement within the nucleus accumbens for all animals. Lines indicate the active dialysis regions. Numbers below the figure represent the position of the slice relative to Bregma. The figure was adapted from Paxinos and Watson (2005).
Figure 6-3. Binge ethanol injections increased both ethanol consumption and response breakpoints. A) Binge ethanol injections increased ethanol consumption. B) Binge ethanol injections increased response breakpoints. Binge ethanol injections (3 g/kg, i.p.) were given on Days 4 and 6 of each of the 10-day plain gel periods occurring between the second and sixth PR10 ethanol sessions. Therefore, by 6 PR10, each rat in the EtOH group had received 8 binge injections. Since there was no difference in responding on any of the days during the 7-day ethanol periods, data shown are means and SEMs for the average of all 7 days for N = 10 and 7 per group. * P < 0.05.
Figure 6-4. Binge ethanol injections had no effect on plain gel consumption or response breakpoints. A) Binge ethanol injections had no effect on plain gel consumption. B) Binge ethanol injections had no effect on plain response breakpoints. Binge ethanol injections (3 g/kg, i.p.) were given on Days 4 and 6 of each of these 10-day plain gel periods occurring between the second and sixth PR10 ethanol sessions as described in figure 6. Data shown are means and SEMs for the average of all 10 days for N = 10 and 7 per group. * P < 0.05.
Figure 6-5. Comparison of ethanol consumption and gelatin consumption before surgery, during habituation and on the experimental day. A) Comparison of ethanol consumption between the control group and the binge group during habituation and on the experimental day. B) Comparison of gelatin consumption between the control group and the binge group during habituation and on the experimental day. C) Comparison of ethanol consumption between the control group and the binge group before surgery and during habituation. D) Comparison of gelatin consumption between the control group and the binge group during habituation and before surgery. Hab-D1~5 represented the ethanol/gelatin consumption during habituation days. Exp represented the ethanol/gelatin consumption on the microdialysis day. Pre-SG included five days ethanol/gelatin consumption before microdialysis surgery. Hab included five days ethanol/gelatin consumption during habituation days.
Figure 6-6. There was greater release of taurine, DA, glycine and glutamate in the NAC in the binge rats during 10% ethanol-containing gelatin operant responding. A) Taurine increased by 10~15% in both groups. However, the increase of taurine in the binge group lasted for at least 30 mins longer than that in the control group. B) Glutamate increased by 10% in the binge group with no change in the control group. C) DA increased by 20% in the binge group with no change in the control group. The data in panel A-C are shown as % baseline and lined up by the first reinforcement. D) Glycine increased in both groups. However, the increase of glycine in the binge group is significantly higher than in the control group during the first 20 mins. (N=10 for binge group, N=7 for control group).
Figure 6-7. There were no change in DA, taurine and glutamate release in NAC in either groups during operant plain gelatin responding. A) The level of taurine in NAC during operant plain gelatin self-administration. B) The level of glutamate in NAC during operant plain gelatin self-administration. C) The level of DA in NAC during operant plain gelatin self-administration. D) The level of glycine in NAC during operant plain gelatin self-administration. The data in panel A-C are shown as % baseline and lined up by the first reinforcement. (N=5 for binge group, N=6 for control group)
A. Taurine

\[ y = 0.0524x - 0.0107 \]
\[ R^2 = 0.8894 \]

B. Glutamate

\[ y = 0.0139x - 0.0325 \]
\[ R^2 = 0.0215 \]

C. Dopamine

\[ y = 0.0569x - 0.0277 \]
\[ R^2 = 0.345 \]

D. Glycine

\[ y = 1.2425x - 0.2702 \]
\[ R^2 = 0.8391 \]

Figure 6-8. The correlation between ethanol consumption and neurotransmitter release. A) Sensitized taurine release in NAC was well correlated with the amount of ethanol consumed in both groups. The slope in the binge group is significant higher than that in the control group. B) Sensitized glutamate release in NAC was well correlated with ethanol consumption only in the binge group not the control group. C) Sensitized DA release in NAC was well correlated with ethanol consumption only in the binge group not the control group. D) The releases of glycine in NAC were well correlated with the amount of ethanol consumed in both groups. The slope in the binge group is significant higher than that in the control group.
Figure 6-9. Effect of acute binge exposure (3g/kg ethanol i.p.) on the level of taurine, glutamate and DA in NAC for binge and control groups. A) Taurine significantly increased by 40% in both groups. However, the increase of taurine lasted for at least 1.5 hours longer than that in the control group after binge injection. B) Glutamate significantly increased by 30% in the binge group compared with slight decreases of glutamate in the control group. C) DA increased by 20% in the binge group compared with no change in the control group. The data in panel A-C are shown as % baseline. D) Glycine significantly decreased by 40% in the control group compared with only slight decrease of glycine in the binge group.
Table 6-1. Basal concentrations of DA, glutamate, glycine and taurine in NAC (2 hours after probe insertion).

<table>
<thead>
<tr>
<th></th>
<th>DA (nM)</th>
<th>Glutamate (µM)</th>
<th>Glycine (µM)</th>
<th>Taurine (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (N=7)</td>
<td>5 ±1.4</td>
<td>1.9±0.7</td>
<td>0.71±0.04</td>
<td>3.1±0.13</td>
</tr>
<tr>
<td>Binge (N=10)</td>
<td>3±2.8</td>
<td>3.6±0.5</td>
<td>0.76±0.15</td>
<td>2.9±0.24</td>
</tr>
</tbody>
</table>

The basal concentration of DA = the detected basal concentration DA – spiking DA concentration.
CHAPTER 7
GENERAL DISCUSSION AND FUTURE STUDIES

Alcoholism is the most severe stage of a group of alcohol drinking problems including alcohol abuse and alcohol dependence. In United States, one in 13 people displays alcohol related problems or alcoholism. Binge drinking has been proposed as one of the important initiation steps of the development of alcoholism. In the current study, we investigated the possible neuronal mechanism of increased motivation of ethanol drinking caused by intermittent ethanol binge exposure. The resulting data may provide new principles for the pharmacological treatment for alcohol dependence or abuse.

In general, ethanol has been demonstrated to alter the functions of neurotransmitters in several brain regions including the mesocorticicolimbic DA system and its connections to the nucleus accumbens and amygdala (Koob et al., 1992). The mesolimbic DA pathway is the most predominant addiction pathway studied, particularly the increase of DA release and alteration of DA receptors in NAC (Imperato and Di Chiara, 1986; Yoshimoto et al., 1992a). However, as suggested in previous studies, amino acids like taurine, glutamate and GABA are also altered in the NAC due to ethanol exposure (Szumlinski et al., 2005, 2007; Smith et al., 2004). Therefore, investigation of amino acids and catecholamine at the same time may give alternative mechanisms of the development of alcoholism.

CE-LIF has been recently adapted to measure the changes of amino acid in vivo with high temporal resolution, which provides a method to correlate rapid change of neurotransmission with behavioral episodes. However, previous CE-LIF techniques can not monitor catecholamine and amino acids in the same range. Therefore, the ability of monitoring catecholamine and amino acids simultaneously became a key step of this whole project. In Chapter 3, we tried to bring the extracellular level of DA in NAC within the normal concentration range of amino acids by using
various agents. Although some of our pharmacological manipulations were successful, since we would like to investigate the changes of neurotransmission during ethanol behavior episodes, the perfusion of these agents into rat brain was not an optimal choice to modify the detection ability of DA since they may also modify behavior. Therefore, we developed the DA spiking method which brings the basal level of DA into the normal concentration range of amino acids. Both in vitro and in vivo experiments indicated that the DA spiking method is valid at least for increases of DA. This method was then used in chapter 6. In addition, interactions between GABA and DA, and between glutamate and DA were characterized in NAC in chapter 3. As a result, we found that there were different patterns of DA inhibitory regulation between GABA and glutamate release in NAC. The dopaminergic inhibition of K+-induced GABA release appeared to be mediated via D2 receptors while DA inhibitory regulation on K+-induced glutamate release did not fully follow D2 receptor pharmacology but could occur instead via D1 or 5-HT2 receptor. These results are in agreement with previous studies (Pennartz et al., 1992; Hemmati et al., 2001) which may provide insight to the role of the release of glutamate in chapter 6. Further studies on K+-induced glutamate release with manipulation of D1 and 5-HT receptors are necessary to test the hypothesis.

As shown in chapter 4, the development of rat model of alcoholism is the next aim of this project. Our results demonstrated that we successfully developed a good rat model which showed long-lasting increases of ethanol consumption similar to previous studies (Rimondini et al., 2002, 2003; O’Dell et al., 2004). However, we gave the first evidence of increased ethanol consumption based on altered motivation not satiation since the increase of ethanol consumption is only sensitive in PR10 schedule not FR5. However, we did not observe alcohol deprivation effect (ADE) before the binge exposures or even after increased motivation for ethanol. Thus, the
absence of an ADE in our study supports the possibility that Sprague-Dawley rats are less sensitive to temporary increases in ethanol consumption such as the ADE. Another possibility is that we did not detect an ADE in our animals because the PR sessions ended at 30 minutes. It may be possible that longer sessions would reveal a second bout of responding that is sensitive to an ADE. Additionally, PR schedules have not yet been shown to be susceptible to the effects of an ADE, therefore, subsequent experiments using longer sessions might be more successful if lower effort FR schedules are employed.

The results in Chapter 5 provide the first investigation of real time monitoring of the changes of amino acids in NAC during self administration experiments. In chapter 5, we observed a significant increase of taurine in NAC during operant ethanol self-administration and that the magnitude of the taurine increase is well correlated with the amount of ethanol consumed. Several previous studies have shown that taurine release increases in NAC following IP ethanol injection in a dose-dependent manner (Dahchour et al., 1994, 1996; Dahchour and De Witte, 2000; Smith et al., 2004). Our results support and extend these observations by measuring taurine release during operant ethanol self-administration which, in Sprague-Dawley rats, results in fairly low brain ethanol levels. We found statistically reliable increases in taurine in rats that consumed more than 0.5g/kg ethanol.

Additionally, several results from Chapter 5 led to the formation of the hypothesis that an increase of glycine in NAC may be more involved with anticipation of reward rather than reward. First, a large and constant increase in glycine levels in NAC was observed during both plain gel and ethanol gel operant responding periods. However, the increase of glycine was greatest under operant conditions even though much more gel was consumed under free access conditions. This increase did not occur in hippocampus indicating that it is not due to an overall
increase in brain glycine levels that might reflect high glycine content of gelatin. Following that, there was also a small increase of glycine about ten minutes before the start of the operant session for both ethanol and plain gel groups but not prior to free access sessions. Also, the longer the rat had to wait between barpressing episodes during a session, the greater the subsequent increase in glycine in the NAC, again, regardless of the fact that fewer reinforcements were given under these conditions. This hypothesis was further confirmed in chapter 6 that a sensitized release of glycine was observed in the binge rat during 10% ethanol-containing gelatin operant responding but not plain gel operant responding because binge rats showed much more motivation for ethanol than control rats.

The first experiment performed in chapter 6 was to repeat the binge rat model in chapter 4. After four sets of ethanol binge injection, we observed a similar increase in motivation of ethanol consumption by 40% in binge rats but not in control rats. The microdialysis results showed that there was an imbalance of excitatory and inhibitory neurotransmission caused by intermittent ethanol binge exposures, including greater release of taurine, DA and glutamate in the NAC in the binge rats than the control rats during 10% ethanol-containing gelatin operant responding. Similar results were observed when all rats received a binge exposure of ethanol, such that rats that had previous binge ethanol also had sensitized responses to a subsequent binge ethanol injection compared to control rats. It confirmed that the changes on accumbal neurotransmitter release during operant responding for ethanol gel can reflect the alteration of alcohol pharmacological responsiveness. In addition, there were much greater glycine release in binge group than control group during operant ethanol gel responding while glycine release was very similar in both groups during plain gel responding. In line with our previous results (chapter 5) that glycine release is related to the amount of anticipation, the results indicated that binge rats
had more motivation for ethanol consumption than control rats. In contrast, they had similar anticipation for plain gel consumption.

On the other hand, in chapter 5 and 6, our results showed there are slight increases of serine during the gelatin, moist chow and sugar fatty whip consumption. Since our instrument cannot separate L-serine and D-serine. It is hard to explain the possible mechanism underlying the increase of serine. L-serine is a nonessential amino acid, it occurs naturally in an L-isomer form, synthesized from glycine or theonine. Therefore, in the case of a big increase of glycine experiments during gelatin consumption either operant condition or free access, the increased serine may be L-serine which is due to the metabolism of glycine. However, this explanation can not fit to the experiments of moist chow or sugar fatty whip consumption in which we didn’t observe significant increase of glycine. Therefore, the increase of serine may be at least partly due to the release of D-serine. In the recent couple of decades, D-serine has been widely studied as a neurotransmitter. It is synthesized from L-serine via one enzymatic step catalyzed by the enzyme racemase (Schell, 2004). D-serine is predominantly concentrated in the brain (Hashimoto et al., 1993), and its contents in the spinal cord, peripheral tissue or the blood are very low. Unlike other classical neurotransmitters, such as DA and glutamate, the level of D-serine is not increased but rather decreased by depolarization following high potassium perfusion (Hashimoto et al., 1995). Moreover, interruption of nerve impulse flow or removal of extracellular calcium ions fails to reduce the D-serine level. D-serine has three general physiological functions in the brain. The first and the best studied is regulation of the NMDA receptor. Like glycine, D-serine stimulates the glycine site of the NMDA type receptor consisting of NR1 and NR2 subunits and enhances the action of glutamate (Danysz and Parsons, 1998). In addition, D-serine may be implicated in the glia-neuron interaction that is required for
the control of the NMDA receptor in the brain (Mothet et al., 2005). Finally, previous study suggests that D-serine may be involved in neural circuit formation (Kim et al., 2005).

Overall, our studies demonstrated that the imbalance of excitatory and inhibitory neurotransmission caused by intermittent ethanol binge exposures which identified alcohol-induced neuroadaptation contributing to the early stages of the development of alcoholism, particularly in adolescents. It may provide pharmacological treatment principles for alcohol dependence or abuse.
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BIOGRAPHICAL SKETCH

Zhimin, Li was born on Nov 24, 1979 in Wuhan City, Hubei, China. There, she spent the remainder of her childhood. She graduated from Wugang Third High School in 1997 and started studies at the Hubei College of Traditional Chinese Medicine. In 2001, she received her BS in pharmacy. After graduation, as a junior pharmacist, she worked in the Research Center of Hubei College of Traditional Chinese Medicine. Zhimin Li joined the graduate program in the Department of Pharmacodynamics, College of Pharmacy, University of Florida, Gainesville, Florida, in July 2004.