TEMPORAL RESOLUTION AND DETERMINATION OF THE MECHANISM OF ETHANOL-INDUCED TAURINE EFFLUX

By

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The mesocorticolimbic dopamine pathway is closely associated with the drug reward system. Drugs of abuse enhance neural transmission of this pathway and are responsible for the reinforcing properties of ethanol. The key neurotransmitter involved in this pathway and with addictive behaviors is dopamine. However, when ethanol is given intraperitoneal an increase in the efflux of taurine is observed.

Taurine is a sulfonated amino acid found in millimolar concentrations in the brain. Although taurine has many functions it is known as a major osmolyte in neuronal tissue; however it can act as an agonist at $GABA_A$ receptors and antagonist at NMDA receptors, similar to ethanol. In addition, taurine can be released in several different ways such as a typical neurotransmitter or through an osmotic channel or, in extreme conditions, the reversal of the taurine transporter. Since taurine is increased in response to

ethanol in a known addiction pathway, it is necessary to understand the mechanism of this release to better ascertain the function of ethanol-induced taurine efflux.

Capillary electrophoresis with laser-induced fluorescence was used to examine ethanol-induced taurine efflux. A dose-dependent release of taurine in response to increasing cumulative dosages of ethanol was observed. The efflux of taurine was specific and larger in the nucleus accumbens compared to the striatum, while other amino acids were not affected. In addition, rats trained to self-administer ethanol displayed an increase in taurine efflux in the nucleus accumbens. To further examine the mechanism of efflux, the sodium from the artificial cerebrospinal fluid (ACSF) was reduced to induce the osmotic release of taurine. Osmotic inhibitors were tested, and 4-Acetamido-4'-isothiocyanato-2,2'-stilbenedisulfonic acid disodium salt hydrate (SITS) was successful at blocking osmotic-induced taurine efflux. Several conditions to the ACSF were applied to block the ethanol-induced efflux of taurine by reverse dialysis. SITS and calcium-free ACSF with EGTA were successful at reducing ethanol-induced taurine efflux. The results suggested that the mechanism of ethanol-induced taurine efflux was osmotically mediated. However, local perfusion of ethanol did not induce any efflux. Instead of the local osmotic effect of ethanol, ethanol-induced taurine efflux could be due to neuronal firing in other areas of the brain that causes the osmotic release of taurine.

CHAPTER 1
LITERATURE REVIEW

Alcoholism

Nearly 14 million Americans abuse alcohol or are alcoholic (Narconon, 2004). Approximately 43% of the U.S. adult population (76 million people) have a relative who was an alcoholic or have either grown up with or married a problem drinker. The financial and human cost of alcohol abuse is enormous. Alcohol contributes to 100,000 deaths annually, making it the third leading cause of preventable mortality in the United States (Narconon, 2004). For these reasons alcohol could be considered one of the most deadly drugs in America. In order to understand and stop this alcoholism an emphasis must be placed upon examination of the escalating usage from casual to dependency and abuse, the process of addiction.

Addiction

Addiction is the process of spiraling dysregulation of the brain reward systems that progressively increases to a point of total loss of control of drug consummatory behaviors (Koob and Le Moal, 2001). The addiction cycle can be further defined as having three distinct components: preoccupation-anticipation, binge-intoxication, and withdrawal-negative affect (Koob and Le Moal, 1997). Superimposed upon this cycle of components are sources of reinforcements. For example, the positive reinforcement of a drug, and the pleasurable effects that stimulate drug taking when the drug is presented, is associated with the binge-intoxication cycle of addiction. In contrast, negative reinforcement occurs when drug consumption is driven by the potential alleviation of the

aversive effects of the drug. The preoccupation-anticipatory component of addiction can be associated with conditional positive and negative reinforcement, which are the neutral stimuli associated with drug taking (positive) or the withdrawal of the drug (negative).

The question then becomes what leads to this spiraling state. Several hypotheses have been put forth to explain the process from drug taking to drug abuse to drug addiction. Psychomotor behavioral sensitization is one hypothesis which states that an increase in drug taking causes an increase in the sensitization process, where a shift in the incentive salience "drug wanting" state becomes heightened upon increasing exposure to the drug (Robinson and Berridge, 1993). The opponent-process theory also attempts to explain the addiction process (Solomon and Corbit, 1974). This theory states that two processes, a positive hedonic response and a negative hedonic response, balance each other, and that drug exposure can cause a shift in the balance resulting in drug seeking behaviors. Recently, the allostasis hypothesis has been set forth to encompass several other theories and to further explain the process of addiction. In this model the brain is always under the process of achieving stability through change, " allostasis." Specifically, allostasis is the process of maintaining apparent reward function stability by changing brain reward mechanisms. Chronic deviations of the reward thresholds upon drug exposure cause a dysregulation of the reward and stress systems, which produce changes in the motivational systems. These changes further drug use and the allostatic state. Furthermore, the changes of the reward circuits in addition to the activation of brain and hormonal stress responses can be long-term. These long-term changes provide an explanation for persistent vulnerability to relapse. It is hypothesized that the origin of this dysregulation comes from the compromised function of the dopamine and opioid systems

in the extended amyglada, which are composed of the shell of the nucleus accumbens (NAc), bed nucleus of the stria terminalis, and the central nucleus of the amyglada (Koob and Le Moal, 2001).

Although the cortico-striatal-thalamic loop is known to be involved in compulsive behaviors, the mesocorticolimbic dopamine system has long been thought to be the primary area involved in the drug reward system (Le Moal and Simon, 1991). The major components of the reward circuit are the ventral tegmental area and the basal forebrain, which includes the NAc, amyglada, frontal and limbic cortices (Di Chiara, 2002; Jentsch and Taylor, 1999; Nevo and Hamon, 1995; Volkow and Fowler, 2000). The NAc and amyglada are interconnected and are modulated by dopamine (both areas are included in the ventral tegmental area and extended amyglada) and are key areas for the reinforcement and withdrawal (Koob and Le Moal, 2001). In addition, other components which have inputs or outputs involved in this system utilize other neurotransmitters such as GABA and glutamate (Figure 1-1) (Koob, 1992). Drugs that are addictive, such as alcohol, cocaine, opiates, and nicotine, modify the neurotransmitters in these areas (Carboni et al., 2000; Di Chiara and Imperato, 1988). Modification of the neural substrates in these areas of the brain can lead to dysregulation of the reward set point and eventually to an allostatic state.

The Neuronal Effects of Ethanol

Ethanol has been shown to modulate many neurotransmitter systems in the central nervous system (Deitrich et al., 1989; Nevo and Hamon, 1995). One of the major neurotransmitter systems ethanol affects is the mesolimbic dopaminergic system. As stated previously, several drugs of addiction modify dopamine in the extended amyglada

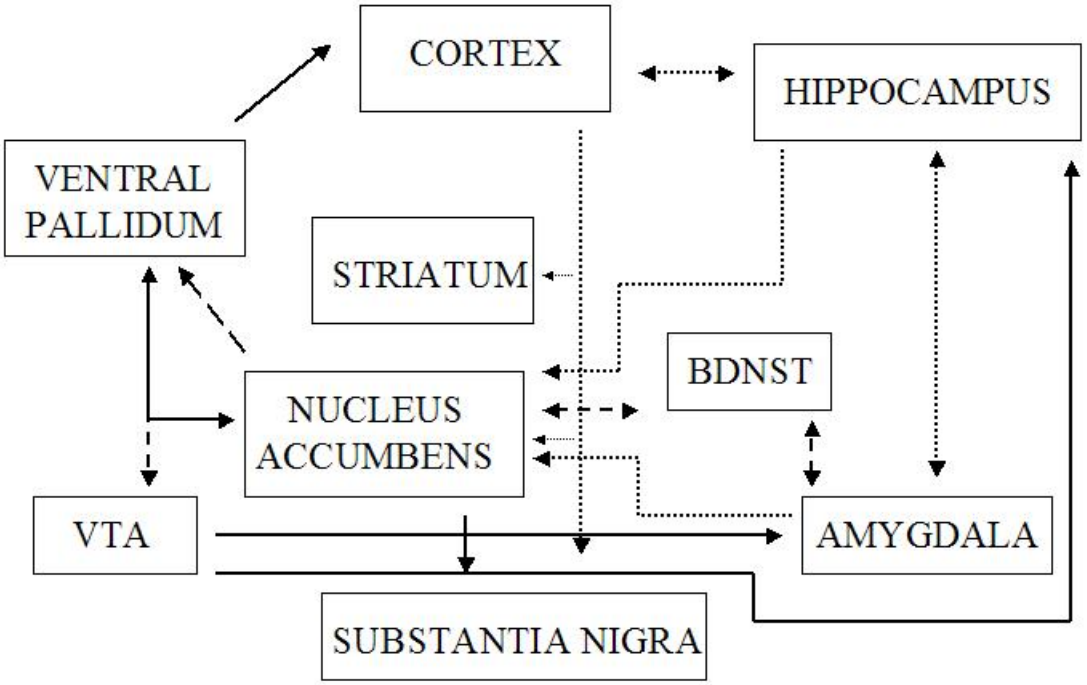

Figure 1-1 Schematic diagram of brain sites and neurotransmitters in the reward circuit. Solid lines represent the dopaminergic afferents; the glutamatergic afferents are represented by dotted lines, and dashed lines represent GABA afferents. VTA, ventral tegmental area; BDNST, bed nucleus of the stria terminalis (Koob and LeMoal, 2001; Koob, 1992).

(Carboni et al., 2000; Di Chiara and Imperato, 1988), which is believed to be one of the factors that leads to allostatic dysregulation. The interactions between ethanol and the dopaminergic system are well established (Brodie and Appel, 1998; Brodie et al., 1999; Brodie et al., 1990; Fitzgerald and Nestler, 1995; Mereu et al., 1984). For example, ethanol has been shown to directly affect the D_2 dopamine receptor either by altering protein kinase C (PKC) signal transduction or increasing receptor binding (Gordon et al., 2001; Souza-Formigoni et al., 1999). In addition, alteration of dopaminergic function by

animals lacking dopamine receptors or the perfusion of agonists/antagonists has been shown to alter ethanol self-administration and drug seeking (El-Ghundi et al., 1998; Hodge et al., 1997; Kaczmarek and Kiefer, 2000; Phillips et al., 1998; Rassnick et al., 1992b; Samson and Chappell, 1999). Several microdialysis studies have shown that acute ethanol can increase the release of dopamine in the NAc (Budygin et al., 2001; Engleman et al., 2000; Imperato and Di Chiara, 1986; Mocsary Z, 1996; Weiss et al., 1993; Wozniak et al., 1991; Yim et al., 1997; Yim and Gonzales, 2000).

As mentioned previously, several other neurotransmitters are also involved in the brain reward system and accordingly several other neurotransmitter systems are also altered by ethanol. Serotonin release is increased in response to ethanol in the NAc (Yoshimoto et al., 1992) and antagonism of serotonin receptors prevents ethanol-induced dopamine release (Wozniak et al., 1990). The GABA receptor function is enhanced by ethanol (Allan and Harris, 1987; Ma et al., 2001; Proctor et al., 2003) and antagonism of the receptor has been shown to decrease ethanol self-administration (Hyytia and Koob, 1995; Rassnick et al., 1992a). Similarly, ethanol has been shown to inhibit NMDA receptor function by acting as an antagonist (Hoffman et al., 1989; Lovinger et al., 1989; Peoples and Stewart, 2000; Steffensen et al., 2000). In addition, microdialysis studies have also shown that ethanol can increase the efflux of glutamate in the NAc (Moghaddam and Bolinao, 1994; Yan et al., 1998) and hippocampus (Moghaddam and Bolinao, 1994). Ethanol self-administration is diminished due to glutamate receptor antagonism in the NAc (Rassnick et al., 1992b).

Recently, another neuro amino acid has been shown to be affected by ethanol. Microdialysis studies have shown that taurine is released in response to an acute dose of

ethanol in the NAc (Dahchour et al., 2000; Dahchour et al., 1994; Dahchour et al., 1996; De Witte et al., 1994; Quertemont et al., 2000; Quertemont et al., 2002), hippocampus (Dahchour and De Witte, 2000a), frontal cortex (Dahchour and De Witte, 2000a), and striatum (Smith et al., 2004). From these studies ethanol-induced taurine efflux is 70% larger in the NAc than other areas of the brain in naive animals. In addition, several lines of alcohol-preferring rats have been shown to vary the release of taurine in response to an acute ethanol injection (Dahchour et al., 2000; Quertemont et al., 2000; Quertemont et al., 2002).

Taurine itself has been shown to modulate the effects of ethanol such as reducing sleep time (Boggan et al., 1978; Ferko, 1987; Ferko and Bobyock, 1988; Mattucci-Schiavone and Ferko, 1985; McBroom et al., 1986), aversion place preference (Aragon and Amit, 1993), open field locomotor activity (Aragon et al., 1992), ethanol-conditioned stimulus preference and (Quertemont et al., 1998b). In conclusion, ethanol affects several different neurotransmitters in areas involved in the brain reward system.

Systems that are dysregulated are targets for medications used to help decrease addiction. For example, naltrexone, an opiate antagonist, can reduce the relapse rates of alcoholics (Volpicelli et al., 1992) and has been shown to modulate ethanol-induced dopamine increase in the NAc (Gonzales and Weiss, 1998). Another medication that can reduce the relapse rates of alcoholics is acamprosate (Geerlings et al., 1997). Although the exact mechanism is unknown for acamprosate, several studies have indicated that acamprosate affects the NMDA receptor (al Qatari et al., 2001; Allgaier et al., 2000; Berton et al., 1998; Dahchour et al., 1998; Lovinger, 1996; Popp and Lovinger, 2000; Rammes et al., 2001), GABA receptor (Berton et al., 1998; Boismare et al., 1984; Daoust

et al., 1992), and dopamine release in the NAc (Olive et al., 2002). Structurally, acamprosate is a taurine derivative and similar derivatives of taurine have been shown to reduce ethanol self-administration (Boismare et al., 1984; Olive et al., 2002), as well as modulate ethanol-induced dopamine release in the mesolimbic system (Olive et al., 2002). Ethanol modulates many of the neural systems in the brain reward system and taurine is one of these neural substrates altered by ethanol. In addition, taurine can modify several ethanol-induced behaviors such as ethanol-induced sleep time (Ferko, 1987), conditioned taste aversion (Aragon and Amit, 1993), and open-field locomotor activity (Aragon et al., 1992). In addition, taurine is very similar in structure to acamprosate that is used to treat alcoholism. Due to these properties it is hypothesized that taurine could be involved in the addiction process.

The Biochemistry of Taurine

Taurine (known chemically as 2-aminoethanesulfonic acid) is the most abundant neuroactive amino acid in the brain extracellular fluid (Huxtable, 1989; Jacobson and Hamberger, 1984). Several microdialysis studies have measured taurine in the low-to mid micromolar concentrations throughout different brain regions (Semba et al., 1995; Solis et al., 1986). In addition, taurine can be found in several CNS cell types such as neuronal cell bodies (Della Corte et al., 1987; Jacobson and Hamberger, 1984; Korpi and Oja, 1983; Lehmann et al., 1985; Palkovits et al., 1986), glial cells (Decavel and Hatton, 1995; Dutton et al., 1991), cerebellar granule and stellate cell types (Holopainen et al., 1989; Kontro and Oja, 1989; Okamoto et al., 1983a), as well as in synaptosomes and synaptic vesicles (Bonhaus et al., 1984; Lombardini, 1977; Meiners et al., 1980). From these studies it is concluded that taurine can be found in all cells in the CNS (Huxtable, 1989).

Taurine Synthesis

Most animals can synthesize taurine and there are three synthesis pathways: the cysteine sulfinic acid (CSA), the cysteic acid, and the cysteamine routes. The most important route for taurine biosynthesis and the most widely accepted for physiological significance in the CNS is the CSA pathway (Griffith, 1983; Huxtable, 1989; Tappaz et al., 1992; Wright, 1988). In this pathway cysteine dioxygenase (CSD) synthesizes CSA from cysteine and CSA decarboxylase produces hypotaurine. The final product of TAU is regulated by hypotaurine dehydrogenase and CSD is the rate-limiting step in this pathway (de la Rosa and Stipanuk, 1985). CSD is subject to endpoint feedback inhibition in the liver as dietary taurine supplementation has been shown to cause a significant decline in hepatic CSD activity (Eppler and Dawson, 2001). Although hepatic biosynthetic enzymes produce taurine, body levels are maintained by dietary intake (Huxtable, 1989). Several studies have indicated that most of the brain taurine content is derived from the diet (Huxtable, 1992; Huxtable et al., 1979). The lack of a correlation of taurine concentration and enzyme activities indicates that the bulk of taurine is obtained by transport, while a marginal amount is synthesized in the neurons (Huxtable, 1989).

Taurine Transport

Taurine is a very hydrophilic compound but does not readily diffuse across membranes (Della Corte et al., 2002). Taurine transporters are found on many cells (Clarke et al., 1983; Liu et al., 1992) and function to maintain the intracellular concentration greater than the extracellular concentration. In some studies the ratio of intracellular:extracellular is 400:1 (Pasantes-Morales et al., 1986).The taurine transporter is very specific for amino monobasic acids, and taurine can be competitively inhibited by other beta amino acids such as beta alanine (Jessen, 1994; Ramanathan et al., 1997),

hypotaurine (Sebring and Huxtable, 1986; Tamai et al., 1995), GABA (Saransaari and Oja, 2000d; Smith et al., 1992) and guanidinoethyl sulphonate (GES) (Huxtable et al., 1979; Moran et al., 1994). Several studies have shown that taurine transport occurs through two saturable systems, one with high affinity and one low affinity (Hanretta and Lombardini, 1987; Meiners et al., 1980; Saransaari and Oja, 1994b; Tamai et al., 1995). The taurine transporter is a sodium dependent transporter (Jessen, 1994; Tamai et al., 1995) with a stoichiometry of two sodium ions per one taurine molecule (Meiners et al., 1980; Ramamoorthy et al., 1994; Ramanathan et al., 1997) and is unaffected by the removal of K^+ or Ca^{2+} (Huxtable, 1989). The taurine transporter has been found on both glial and neuronal cells (Pow et al., 2002) and taurine transport is an active transport system and is reduced or abolished in the presence of metabolic inhibitors, Na^+/K^+ ATPase inhibitors, or lowering the temperature to 4°C (Huxtable, 1989). The properties of the uptake kinetics indicate that taurine is removed from the synaptic cleft more by diffusion rather than re-uptake (Huxtable, 1989). Several studies have indicated that the taurine transporter can be reversed and taurine can be transported out of the cell (Chen et al., 1996; Ohkuma et al., 1996; Saransaari and Oja, 1999). These cases rely on a depleted sodium state within the cell that would only occur in the most extreme cases of ischemia (Saransaari and Oja, 1997; Saransaari and Oja, 1999; Saransaari and Oja, 2000c).

Several biological roles have been proposed for taurine such as neurotransmission(Chepkova et al., 2002; Kurachi and Aihara, 1985b; Liljequist, 1993), antioxidation, osmoregulation (Schaffer et al., 2000; Trachtman et al., 1992), Ca^{2+} modulation (Huxtable, 1992; Huxtable and Sebring, 1987), and membrane stabilization(Wright et al., 1986). It is only fitting that one of the most abundant amino

acids in the CNS also plays a role in most cellular processes. While the multitude of roles of taurine in the CNS is intriguing, for this discussion taurine's involvement in neurotransmission and neuromodulation as well as osmoregulation will be discussed in greater detail.

Taurine as a Neuromodulator

Taurine has been suggested to be an inhibitory neurotransmitter through electrophysiological experiments (Huxtable, 1992; Okamoto et al., 1983a). Furthermore, studies have indicated that taurine can exert an overall inhibitory tone on several different types of neurons (Engelmann et al., 2001; Kurachi et al., 1983b; Ye et al., 1997). Several of taurine's effects on different neurotransmitter receptors are summarized below. Interestingly, the actions of taurine on the GABA, NMDA receptors, and on the dopaminergic transmission are very similar to the actions of ethanol on these systems (Narahashi et al., 2001).

Taurine and GABA

Structurally taurine is very similar to GABA and not surprisingly taurine can modulate GABA receptor function. For example, taurine appears to act at the $GABA_A$ receptor by more than one mechanism: stimulating Cl^- fluxes to hyperpolarize membranes (del Olmo et al., 2000; Oja et al., 1990; Okamoto et al., 1983b; Ye et al., 1997), being antagonized by bicuculline (Haas and Hosli, 1973; Horikoshi et al., 1988), increasing Cl^- uptake (Luu et al., 1987), and competitively inhibiting muscimol binding and enhancing benzodiazepine binding (Bureau and Olsen, 1991; Malminen and Kontro, 1986). In addition, taurine has shown partial agonist qualities by enhancing flunitrazepam binding yet decreasing maximal activation in the presence of GABA (Quinn and Miller, 1992b). Other studies have demonstrated the partial agonist qualities of taurine (Jiang et

al., 2004; Liljequist, 1993). Furthermore, taurine has been shown to displace ligand binding to $GABA_B$ receptors (Kontro and Oja, 1990), and activation on presynaptic $GABA_B$ autoreceptors is suggested by taurine's ability to block GABA release (Kamisaki et al., 1993). Taurine release due to K^+ stimulation is potentiated by ligands for both $GABA_A$ and $GABA_B$ receptors (Saransaari and Oja, 2000d). From these studies it appears that taurine exerts its inhibitory tone through its actions on the GABA receptor complex.

Taurine and Glutamate

Taurine can inhibit NMDA, kainate and quisqualate receptors (Kurachi and Aihara, 1985a; Kurachi and Aihara, 1985b; Kurachi et al., 1983a; Lehmann et al., 1984). In addition, the agonists to these receptors have been shown to induce taurine release *in vivo* (Del Arco and Mora, 1999; Del Arco et al., 2000; Lehmann et al., 1985; Menendez et al., 1989a). However, taurine could be released due to cell swelling in association with neurotoxicity that occurs due to application of the these glutamate receptor agonists. However, several studies have shown that taurine release due to NMDA is not osmotic dependent (Shibanoki et al., 1993) but rather dependent on Ca^{2+} mobilization (Menendez et al., 1993; Rogers et al., 1990) or nitric oxide cascade (Scheller et al., 2000). From these studies it can be concluded that there is a mutual interaction with the excitatory amino acid function.

Taurine and Dopamine

Taurine has also been suggested to modulate dopaminergic transmission in the striatum (Kontro, 1987) and has been shown to have mutual interactions with neurotransmitters in this area (Colivicchi et al., 1998; Exposito et al., 1999; Kontro and Oja, 1988a; Segovia et al., 1997). Further studies have shown that taurine infused into the

striatum can increase dopamine levels (Ruotsalainen and Ahtee, 1996); however taurine infused into the substania nigra actually decreases dopamine levels in the striatum, indicating the importance of site of administration of taurine (Ruotsalainen et al., 1996). In addition, taurine can activate the glycine receptors on dopaminergic neurons (Hausser et al., 1992; Jiang et al., 2004), further displaying various ways taurine can alter a neural system.

Taurine as an Osmolyte

The best-established function for taurine is that of osmoregulation. Taurine is a good osmolyte based upon its abundance within the cell and its ability to be retained by the cell (poor diffusion capabilities due to its lipophobic nature) and the largest shift in osmolar equivalents within a cell in response to osmotic stress is contributed by taurine (Huxtable, 1989). When cells are presented with hypotonic media, an initial cell swelling occurs followed by a regulatory volume decrease (RVD) (Kimelberg and Frangakis, 1985; Olsen et al., 1986) in which organic osmolytes are released (Pasantes-Morales and Schousboe, 1989). Several cell types such as astrocytes (Pasantes-Morales et al., 1993a; Pasantes-Morales et al., 1990b; Pasantes-Morales and Schousboe, 1989; Sanchez-Olea et al., 1991), erythrocytes(Kirk et al., 1992), C6 glioma(Jackson and Strange, 1993), and Ehrlich ascites tumor cells(Hoffmann, 2000; Lambert and Hoffmann, 1994) release taurine due to hypoosmotic stress. Taurine is involved in osmoregulation in the brain as several studies have shown taurine to be released due to neuronal and glial cell swelling (Kimelberg et al., 1990; Schousboe et al., 1990a; Schousboe and Pasantes-Morales, 1992) and *in vivo* microdialysis studies have shown that taurine release is increased dramatically when hypotonic solutions are perfused into the brain (Estevez et al., 1999; Lehmann, 1989; Solis et al., 1988b). The efflux pathway seems to be very similar in the

different cell types tested. Swelling-induced taurine loss occurs through a passive (Sanchez-Olea et al., 1991), Na^+ independent (Kirk et al., 1992) transport system that does not seem to be saturable (Roy and Malo, 1992). In addition, this transport pathway appears to have a very distinct pharmacological profile (Jackson and Strange, 1993; Sanchez-Olea et al., 1996).

Taurine is one of the most abundant amino acids in the CNS and can modulate many systems. In order to determine the function of taurine due to a physiological condition, it is necessary to examine the mechanism by which taurine is released. For example, if taurine is co-released with several neurotransmitters due to neuronal depolarization, then a closer examination of taurine's neuromodulatory characteristics is warranted. Conversely, if the mechanism of taurine release is due to cell swelling, then the osmoregulation characteristic should be examined. The mechanism by which taurine is released gives insight to the function of taurine.

The Mechanism of Taurine Release

Taurine is released by two physiologically significant mechanisms, which are neuronal depolarization and osmoregulation. These two mechanisms of release are discussed in the following sections. A third mechanism of release does exist, as mentioned previously, the reversal of the taurine transporter in extreme conditions. Characteristics of this release, involves the release of taurine with Na^+ out of the cell in exchange for either a taurine molecule, or an α,ω-amino acid such as, GABA, β-alanine or hypotaurine (Huxtable, 1989). The reversal of the active transport system is different from osmoregulation because the exchange of other amino acids to transport taurine out of the cell is not required and release due to neuronal depolarization does not involve a

transporter but does require Ca^{2+}. In efforts to declare taurine a neurotransmitter, one of the criteria is that release be due to depolarization either by high potassium or electrical stimulation, or by opening the fast-acting sodium channels (veratrine or veratridine). Upon depolarization, voltage-gated Ca^{2+} channels are activated and the influx of Ca^{2+} initiates interactions between secretion-related proteins, which leads to vesicle docking, and membrane fusion. Therefore, Ca^{2+} is important in the synaptic release due to depolarization. In the quest to prove that taurine is a neurotransmitter most of the literature has been devoted to the mechanisms of taurine release due to neuronal depolarization.

Taurine Release Due to Neurotransmission

High potassium has been shown to induce taurine release in cerebellar neuronal cell cultures (Dutton et al., 1991; Philibert et al., 1989b; Rogers et al., 1991), cereberal cortex slices (Kontro and Oja, 1987a; Kontro and Oja, 1987d; Oja and Kontro, 1989),striatal and hippocampal slices (Kontro and Oja, 1988a; Saransaari and Oja, 1999), primary astrocytes (Holopainen et al., 1989; Philibert et al., 1988b; Philibert et al., 1989a), and *in vivo* (Colivicchi et al., 1998; del Rio et al., 1987; Heron et al., 1993; Jacobson and Hamberger, 1984; Semba et al., 1995; Singewald et al., 1993). There are a few interesting characteristics of this release. The release of taurine due to high potassium has been shown to be lesser in magnitude when compared to other neuro amino acids such as GABA and glutamate (Dutton et al., 1991; Saransaari and Oja, 1992). In addition, lower levels of potassium (3-6mM) that did not induce the release of GABA or glycine did evoke the release of taurine (del Rio et al., 1987). Furthermore, it has been shown that potassium-stimulated taurine release is greater in developing or immature animals than in adults (Kontro and Oja, 1989; Kontro and Oja, 1988b; Oja and Kontro, 1989; Saransaari

and Oja, 1994a). Another interesting characteristic taurine exhibits due to high potassium stimulation are delay phenomena. High potassium stimulation evokes neurotransmitter release very rapidly, however the efflux of taurine occurs several minutes after the potassium stimulation (Dutton et al., 1991; Kontro and Oja, 1987b; Philibert et al., 1988a; Saransaari and Oja, 1994b). In addition to high potassium, taurine release can be evoked by electrical stimulation (Colivicchi et al., 1998; Paleckova et al., 1992; Saransaari and Oja, 1992) and veratridine (Heron et al., 1993; Holopainen et al., 1989; Jacobson and Hamberger, 1984; Kontro and Oja, 1987b; Philibert et al., 1989b). There are several unique characteristics associated with potassium-stimulated taurine release, veratridine and electrical stimulation evoked taurine release that would seem to indicate that taurine is released due to neuronal depolarization. To further complicate matters, tetrodotoxin, which blocks the fast-acting sodium channels and prevents neuronal depolarization, has been shown to block taurine release due to high potassium (Philibert et al., 1989b), veratridine (Jacobson and Hamberger, 1984; Philibert et al., 1989b) and electrical stimulation (Paleckova et al., 1992) or partially block (Singewald et al., 1993) or have no effect on the stimulated release of taurine (Philibert et al., 1989b; Semba et al., 1995). Due to the unique characteristics of taurine release due to depolarizing agents (e.g. lag phenomena, efflux lower in magnitude compared to other neurotransmitters, semi-sensitive to tetrodotoxin), it has been suggested that there could be two mechanisms of taurine release, a neuronal and a non-neuronal component (Huxtable, 1989). The role of Ca^{2+} in depolarizing evoked taurine release does not clarify this issue.

Neurotransmitter release is dependent on Ca^{2+} for synaptic release. Several studies have shown that potassium-stimulated evoked taurine release was diminished by

the omission of Ca^{2+} and was "not strictly" Ca^{2+}-dependent (Kontro and Oja, 1989; Korpi and Oja, 1983; Oja and Kontro, 1989). However, in most of the studies exploring the properties of taurine release due to depolarization have shown the release to be Ca^{2+}-independent (Dutton et al., 1991; Rogers et al., 1991; Semba et al., 1995). Interestingly, in one study the removal of Ca^{2+} actually stimulated the basal and potassium stimulated release of taurine (Dutton et al., 1991). Perhaps, the internal stores may have been adequate to cause synaptic release and therefore the omission of Ca^{2+} was not enough to examine Ca^{2+}-dependency. Several studies have shown a complete block of potassium and veratridine evoked taurine release by the omission of Ca^{2+} with the addition of EGTA (Philibert et al., 1988a). Interestingly, it appears that the amount of potassium used for depolarization determines Ca^{2+}-dependency (Philibert et al., 1989b). The lower concentrations of potassium (below 40mM) evoked taurine release that was Ca^{2+}-dependent but at higher concentrations of potassium (above 40mM) at evoked release was Ca^{2+}-independent. In addition, Ca^{2+} channel blockers abolished the stimulated release of taurine (Kontro and Oja, 1987d; Philibert et al., 1989a; Philibert et al., 1989b). These studies indicate the dependency on both the intracellular and extracellular concentrations of Ca^{2+}. In spite of the previous studies, the role of Ca^{2+} in the neuronal release of taurine is still unclear.

Taurine Release Due to Osmoregulation

Osmoregulation is another process that can produce the release of taurine from cells. Hypoosmotic conditions cause cells to rapidly swell which is then followed by the extrusion of osmolytes leading to cell volume recovery, this process is called regulatory volume decrease (RVD) (Pasantes-Morales, 1996; Pasantes-Morales et al., 1993b; Vitarella et al., 1994). Taurine has been well characterized as a preferential osmolyte

released due to RVD in many cell types (Bothwell et al., 2002; Deleuze et al., 1998; Hussy et al., 2000; Miyata et al., 1997; Pow et al., 2002) and *in vivo* (Estevez et al., 1999; Solis et al., 1988a). The property of this efflux mechanism indicates a diffusion process as there is essentially no contribution of energy-dependent carrier (Pasantes-Morales and Schousboe, 1988; Schousboe et al., 1991), does not saturate over a broad range (Roy and Malo, 1992) and is dependent on the taurine concentration gradient (Pasantes-Morales and Schousboe, 1997). Furthermore, the taurine transporter is not involved as it is Na^+ independent (Deleuze et al., 1998; Schousboe et al., 1991), uptake blockers have no effect (Bres et al., 2000) and is temperature insensitive (Bres et al., 2000). The diffusion nature of this efflux pathway would indicate that a channel is involved.

Patch-clamp studies have characterized a swelling-activated, outwardly-rectifying anion channel and suggests that the channel mediates both taurine and chloride movement (Jackson and Strange, 1993). The described volume-sensitive organic anion channel (VSOAC) is blocked by several chloride channel inhibitors (i.e. sodium borate, 4,4'-Diisothiocyanatostilbene-2,2'-disulfonic acid disodium salt hydrate (DIDS), SITS, 5-nitro-(3-phenylpropylamino)benzoic acid (NPPB), niflumic acid) in several cell types (Bothwell et al., 2002; Bres et al., 2000; Deleuze et al., 1998; Jackson and Strange, 1993; Okada, 1997; Rosso et al., 2003; Sakai, 1999; Sanchez-Olea et al., 1996) and *in vivo* (Estevez et al., 1999; Solis et al., 1988b) which indicates the ubiquitous nature of this channel. Although the pharmacological profile and the physical behavior of VSOAC are found in several cell types, the molecular identity has yet to be ascertained. Several studies have postulated that the constituents involved are part of the chloride channel family (CCl) (Clapham, 1998; Duan et al., 1999; Jentsch et al., 1999; Strange, 1998;

Strange et al., 1996). The best candidate of the CCl family appears to be CCl-3 which when expressed results in a volume-activated chloride channel with similar VSOAC properties (Duan et al., 1997; Strange, 1998). However, in mice in which the CCl-3 expression was disrupted, VSOAC properties were very similar compared to native VSOACs (Yamamoto-Mizuma et al., 2004). As a compensatory mechanism, the deletion of CCl-3 increased the expression of several proteins associated with VSOAC subunit composition. Similarly, VSOACs with different pharmacological properties were discovered in supraoptic glial cells which suggests that changes in subunit composition or associated regulatory proteins could give rise to VSOACs with different properties (Bres et al., 2000). The regulation and composition of VSOAC remains unsettled and correspondingly the exact signaling mechanism of the taurine efflux pathway due to hypoosmolarity has not been fully established.

Hypoosmolarity induces cell swelling along with a rapid increase in cytosolic Ca^{2+} from both the influx of Ca^{2+} and from intracellular stores (Huang et al., 2001; Morales-Mulia et al., 1998; Pasantes-Morales and Morales-Mulia, 2000). In addition, hypoosmotic induced taurine efflux is blocked by the addition of a Ca^{2+} chelator in rat pituicytes (Rosso et al., 2003). However, VSOAC has been shown to be Ca^{2+} independent in several cell types (Deleuze et al., 1998; Moran et al., 1997; Nilius et al., 1997; O'Connor and Kimelberg, 1993; Okada, 1997). Nevertheless, ionomycin, a Ca^{2+} ionophore, can potentiate the hypoosmotic-induced taurine release (Cardin et al., 2003; Rosso et al., 2003). This effect is also seen with other ionophores (Philibert et al., 1989a; Philibert et al., 1989b). In addition to Ca^{2+}, recent evidence indicates the importance of protein kinases in the signaling cascade. For example, tyrosine kinase inhibitors have been shown

to abolish taurine (Cardin et al., 2003; Deleuze et al., 2000; Huang et al., 2001; Hubert et al., 2000; Tilly et al., 1993) and blockers of tyrosine phosphatases, prolonging the tyrosine phosphorylation reactions, potentiates taurine release in several cell types (Cardin et al., 2003; Morales-Mulia et al., 2001; Tilly et al., 1993). The problem with identifying the necessary steps involved in taurine efflux signaling is that hypoosmotic shock or cell swelling induces the activation of many cellular processes (i.e. ERK1/ERK2, p38, CaM/CaMK) yet these have no effect on the hypoosmotic efflux of taurine in several cell types (Cardin et al., 2003; Franco et al., 2001; Morales-Mulia et al., 2001; Tilly et al., 1996). Due to the complex nature of cell swelling, further experiments are warranted to fully understand the exact mechanism involved in hypoosmotic taurine efflux.

Microdialysis and CE-LIF

Microdialysis is a common method employed to measure neurotransmitter/neuromodulator levels by the passive diffusion of these substances through a semi-permeable membrane (Ungerstedt 1981,Robinson 1991). The limitation of this technique occurs in the detection of amino acids in the dialysate. High pressure liquid chromatography (HPLC), with fluorescence or electrochemical detection, has been the common choice to measure analytes in microdialysis. This method of detection is limited by the sensitivity of detection, the inability to measure several analytes in one analysis, and most importantly the larger volume of samples required. The larger volumes required by this detection method usually translate to collection times from 15-30 minutes even with the perfusion rate at 2-3μL/minute (Adell 1998). Due to the longer

sample time required, a considerable lack of information could be lost when considering the rapid dynamics of the neural system.

Recently, capillary electrophoresis with laser-induced fluorescence (CE-LIF) has been developed and is a considerable advancement in the method of detection of amino acids due to its ability to detect a multitude of neural substances in seconds (Bowser and Kennedy, 2001; Boyd et al., 2000; Lada et al., 1998a; Lada and Kennedy, 1995; Lada and Kennedy, 1996; Lada and Kennedy, 1997; Lada et al., 1997; Tucci et al., 1997). Most recently, this method has been used on-line in an anesthetized animal to measure several amino acids in 20-second intervals and the changes in the levels of these amino acids have been correlated with neuronal activity (Bowser and Kennedy, 2001). CE-LIF, through increased sensitivity, small sample volumes and the detection of several amino acids at once create a greater temporal resolution in microdialysis experiments.

However, a disadvantage of this system is the expense of the laser and the complexity of the optics and operation involved. This system can be altered to utilize commercially available optics and a less expensive laser. The important alteration involves changing the fluorescent tag from OPA to an OPA analogue, NDA. Primary amines react with NDA and CN to produce 1-cyano-2-subsituted-benz[*f*]isoindoles (CBI) which have superior quantum fluorescence efficiencies than OPA-thiol reactions and can be detected in the visible spectrum (420-450nm) (Kawasaki et al., 1989; Matuszewski et al., 1987; Roach and Harmony, 1987). Several microdialysis studies using CE-LIF with NDA/CN derivatization reaction have reported detection of neuro-amino acids *in vitro* (Bert et al., 1996) and *in vivo* (Bert et al., 2002; Chen et al., 2001; Parrot et al., 2003; Robert et al., 1995; Robert et al., 1996; Zhou et al., 1995) with a temporal resolution from

10 seconds to 3 minutes, with changes in the basal levels of these amino acids being correlated to neuronal activity (Bert et al., 2002; Parrot et al., 2003). However, these experiments required the collection of dialysate for later analysis. Zhou et al. (1995), have successfully monitored glutamate and aspartate with a temporal resolution of less than 2 minutes in anaesthetized rat using on-line CE-LIF, which avoids the troublesome tasks of collection, storage, and the handling of small volumes for analysis.

Summary

Alcohol addiction is a severe disease that affects millions of people each year. Addiction is a very complex process that involves the regulation of numerous cellular functions. Generally, the mesolimbic pathway is believed to be involved such that ethanol alters dopamine transmission being altered. However, acute ethanol has been shown to increase the concentration of taurine in the CNS. Taurine has been shown to have several functions in the CNS, such as osmoregulation, neurotransmission, and neuromodulation. In addition, taurine has been shown to be able modify several of ethanol's effects on mice and rats. Interestingly, a drug widely used to treat alcoholics, acamprosate, is a structural analogue of taurine. Since taurine can modify the neurotransmission in areas associated with addiction, and an analogue of taurine is used to treat alcohol addiction, this implies that taurine release due to ethanol in the NAc and striatum could be an overlooked step in the addiction process. The exact function of taurine after acute dosage of ethanol is unknown. However, examination into the mechanism of taurine release could give insight into the purpose of ethanol-induced taurine efflux. Therefore the studies in this dissertation were designed to characterize ethanol-induced taurine efflux in the NAc and striatum.

Objectives

Ethanol increases the efflux of taurine but the mechanism and the purpose of this efflux is unknown. Due to taurine's ubiquitous nature, it is necessary to use an intact neural system. *In vivo* microdialysis was employed to gain insight into the ethanol-induced efflux. In addition, utilizing CE-LIF, which decreased the temporal resolution from 15 minutes to 11 seconds, enhanced my ability to characterize ethanol-induced taurine efflux.

The first aim of my research was to study the dose response relationship and regional selectivity of ethanol-induced taurine efflux (CHAPTER 3). Previous cell culture data indicate that ethanol can induce the efflux of taurine and aspartate and this efflux is due to osmoregulation (Kimelberg et al. 1993). Ethanol-induced taurine efflux could be due to an osmotic imbalance, in which taurine efflux is increased in response to cell swelling. If this were the case it would be expected, as seen in the previously mentioned study, that taurine would not display a dose response to ethanol, as low dosages of ethanol would not perturb the cellular environment. The preferential release of taurine would also contradict taurine being released due to osmoregulation as it would be expected that other osmolytes (i.e. aspartate) would be released in addition to taurine. In contrast, if ethanol-induced taurine efflux was due to neuronal depolarization then the areas of the brain that are stimulated the most by ethanol would be expected to have a larger increase in taurine efflux (i.e. NAc). In these studies, a cumulative dose regime was used in two brain regions to compare and contrast the effects of ethanol on the efflux of taurine and several other amino acids that were analyzed. The NAc was examined due to its association drugs of abuse and influence on ethanol consumption and the striatum was chosen as a control region due to the proximity to NAc and lose association with

drugs of abuse. Brain ethanol concentration was also measured to compare against taurine release. In addition, ethanol was applied locally to further investigate its potential to induce taurine efflux due to osmoregulation.

The second aim of my research was to examine the mechanism of ethanol-induced taurine efflux (CHAPTER 4). Taurine could be released due to neuronal depolarization and/or osmoregulation. Taurine efflux due to osmoregulation is believed to occur through a common channel found in several cell types (VSOAC). Known inhibitors of this channel were assessed in their ability to block osmotic induced taurine efflux *in vivo* and the most effective inhibitor was used to block the osmoregulation contribution of ethanol-induced taurine efflux. Neuronal depolarization of ethanol-induced taurine efflux was characterized by using inhibitors of neurotransmission (TTX and Ca^{2+} -free ACSF with EGTA).

The third aim of my research was to examine the physiological significance of ethanol-induced taurine efflux (CHAPTER 5). Acute exposure to ethanol (i.p.) induces taurine efflux, however there is a lack of data on ethanol self-administration producing taurine efflux. These studies were undertaken to ascertain if low doses of ethanol due to self-administration could induce an increase in taurine efflux. The implications of this study are that ethanol-induced efflux may not occur to dosages that are physiological relevant. In this experiment animals were taught to self-administer ethanol and the taurine efflux was examined. These results were compared to the results from CHAPTER 3.

The hypothesis tested indicated that the increase of taurine due to ethanol would be dose-dependent, regionally selective, and local administration would have no effect, which would indicate that taurine is released due to neuronal depolarization. In addition,

ethanol-induced efflux would be physiologically significant, as taurine would be increased in the self-administration studies similar to the degree seen in the intraperitoneal studies. The mechanism by which ethanol induces taurine efflux would be due to neuronal depolarization and would display similar characteristics of neurotransmitter release. In contrast, osmotic inhibitors would have no effect on the taurine efflux.

CHAPTER 2
GENERAL METHODS

Animals

Male Sprague-Dawley rats, 70-90 days of age, (Harlan, Indianapolis, IN) weighing 250-350 g were housed 2-3 per cage in a temperature and humidity controlled environment with a 12-hr normal phase light/dark cycle. All testing and training was conducted during the light phase. The subjects had *ad libitum* access to food and water throughout the experiment unless otherwise indicated. All procedures were conducted in strict adherence to the *National Institute of Health Guide for the Care and Use of Laboratory Animals*

Chemicals

NaCl, $CaCl_2$, KCl, $MgSO_4$, o-phthaldialdehyde (OPA), β-mercaptoethanol (BME), sodium tetraborate, and hydroxypropyl-β-cyclodextrin (HPBCD), sodium dodecyl sulfate (SDS), Ethylene-bis (oxyethylenenitrilo) tetraacetic acid (EGTA), amino acids, sodium borate, 4,4'-Diisothiocyanatostilbene-2,2'-disulfonic acid disodium salt hydrate (DIDS), 4-Acetamido-4'-isothiocyanato-2,2'-stilbenedisulfonic acid disodium salt hydrate (SITS), 5-nitro-(3-phenylpropylamino)benzoic acid (NPPB), veratrine, niflumic acid and tetrodotoxin 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. Napthalene-2,3-dicarboxaldehyde (NDA) was received from Molecular Probes (Eugene, OR). 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.

Surgeries for CHAPTER 3

Each rat was anesthetized with 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 the CPU or NAc using the following coordinates: CPU, +0.8 anteroposterior, +3.0 lateral to bregma, -4.0 dorsoventral to skull; NAc, +1.6 anteroposterior, +1.7 lateral, -6.2 dorsoventral. The guide cannula was secured to the skull with dental cement anchored by three stainless steel screws. Following surgery, each rat was placed on a heat pad to maintain core body temperature and was closely monitored for full recovery from the procedure, as visualized by locomotor activity. Each rat was housed separately and allowed to recover for 5- 7 days with daily handling to ensure patency of the guide cannula and to minimize handling stress during subsequent measurements.

Microdialysis for CHAPTER 3

Microdialysis probes (o.d. 270 μm; active length 2 mm for the NAc and 3 mm for CPU; cellulose membrane, 13,000 molecular weight cut-off) were constructed by the method of Pettit and Justice (Pettit and Justice, 1989). On the day of the experiment, each rat was lightly anesthetized with halothane, moved into the dialysis cage and the guide cannula was cleared for insertion of the dialysis probe. The probe was perfused with artificial cerebrospinal fluid (ACSF; 145 mM NaCl, 2.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$) at 1 μL/min. After probe insertion, each rat was placed in a clear polyurethane test box (9 3/8" x 18" x 10” deep) with bedding, water bottle and food.

Following this procedure, samples were taken every 11 seconds for 4-6 minutes (20-30 samples) and this was repeated every 15 minutes for the first ninety minutes after probe implantation. About 2 hrs after probe insertion, saline (1 ml/kg body weight, 0.9% NaCl) was injected i.p. and 285 samples (55 minutes) were taken. Subsequent injections of ethanol (20% w/v in saline, i.p.) occurred in the following order at one-hour intervals: 0.5 g/kg, 1 g/kg, and 2 g/kg. After each injection, 285 samples were collected. The amino acid concentrations in dialysate samples were analyzed on-line by CE-LIF. A calibration curve was generated either before or after the experiment by placing the tip of the dialysis probe in three different concentrations of a standard solution containing glutamate, aspartate, GABA, taurine, dopamine, serine and glycine and collecting 30 samples each. The variability of these samples was approximately 2% standard error. The mean relative fluorescence of these samples was converted to concentration through the use of the calibration curve (a plot of peak height versus standard concentration).

After microdialysis, rats were killed by rapid decapitation. Brains were removed and frozen at -20°C and 40 μm sections were taken to verify probe placement.

Microdialysis and Surgeries for Anesthetized Rats

Microdialysis probes (o.d. 270 μm; active length 2 mm; cellulose membrane, 13,000 molecular weight cut-off) were constructed by the method of Bowser and Kennedy (2001). The anesthetic was changed in the experiments conducted in Michigan (CHAPTER 4) due to the fact the surgeries were approved on Dr. Kennedy's protocol. Each rat was anesthetized with an i.p. injection of ketamine/medetomidine (approximately 65 mg/kg and 0.5 mg/kg) and placed in a stereotaxic apparatus for surgical implantation of the microdialysis probe. The probe was positioned in the left side

of the NAc using the following coordinates: +1.6mm anteroposterior, +1.7mm lateral, -6.2mm dorsoventral. The microdialysis probe was perfused with normal ACSF (145 mM NaCl, 2.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$) at 1 μL/min (CMA 102 microdialysis pump; 1mL Hamilton series 1001 gastight syringe) and then was inserted into the NAc. The rats remained anesthetized in the stereotaxic apparatus for the duration of the experiment. In the first series of experiments the amount of Na^+ was varied in the ACSF to characterize the osmotic response. After 90 minutes to allow for stabilization of amino acids levels (Smith et al., 2003), basal levels were sampled for 30 minutes (60 electropherograms) followed by a change in the Na^+ concentration of the ACSF after which another 60 electropherograms were collected. After the hypoosmotic shock, normal ACSF was perfused through the probe for 30 minutes. This procedure was repeated two more times. Once conditions were determined that induced a selective, reproducible two-fold increase in taurine efflux, the effects of osmotically-activated channel inhibitors were tested. After 90 minutes of equilibration after probe insertion, basal levels were measured for 30 minutes (normal ACSF), followed by 30 minutes of recording under hypoosmotic conditions and then 30 minutes again of normal ACSF. After this, an osmotic inhibitor dissolved in normal ACSF was perfused through the probe for 30 minutes followed by 30 minutes of hypoosmotic ACSF with the osmotic inhibitor present.

Microdialysis and Surgeries for Freely-moving Animals in CHAPTER 4

Each rat was anesthetized with ketamine/medetomidine as before 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 the NAc using

the following coordinates: +1.6mm anteroposterior, +1.7mm lateral, -6.2mm dorsoventral. The guide cannula was secured to the skull with dental cement anchored by three stainless steel screws. After surgery, each rat was housed separately and allowed to recover for 7-10 days with daily handling to facilitate probe insertion. At the conclusion of each experiment, the rats were killed and the brains removed to verify probe placement.

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 (Pettit and Justice, 1989). The probe was perfused with ACSF, lowered into NAc, and allowed to equilibrate for 90 minutes as before. Rats were randomly divided into four groups based on the following manipulations of the ACSF: normal ACSF (control); 1 μM TTX; 4 mM SITS; or 0 mM Ca^{2+} plus 2 mM EGTA. The effect of manipulation of the ACSF on basal levels of amino acids was determined over 30 minutes. Following this, saline (0.9%, i.p., 12.5 ml/kg body weight) was injected and samples were collected for 60 minutes. Next, 2.5 g/kg EtOH (20% w/v in saline, i.p.) was injected and amino acid levels were monitored for 90 minutes. The efficacy of 1 μM TTX was determined by perfusing veratrine through the probe at the end of experiments in the control rats.

CE-LIF for CHAPTER 3

Amino acids were measured by CE-LIF utilizing the procedure established by Bowser and Kennedy (Bowser and Kennedy, 2001). Primary amine moieties in the dialysate were derivatized on-line by mixing with a 1 μL/min stream of OPA solution (10 mM OPA, 40 mM BME, 36 mM borate, 0.81 mM HPBCD, 10% MeOH (v/v), pH 9.5) with a reaction time of approximately 1.5 min. The reaction time did not limit the

temporal resolution of the measurement; however, it did induce a delay between changes occurring at the dialysis probe and their detection by the CE system. Samples of the dialysate were injected onto the capillary using a flow-gate interface as follows. The outlet of the reaction capillary was aligned with the inlet of the CE separation capillary (10 cm long, 10 μm ID, 150 μm OD fused-silica) in a Plexiglas block, leaving a gap of approximately 30 μm. A 1 mL/min cross-flow was applied between the capillaries, which carried the sample solution to waste. To inject a plug of sample, the cross-flow was stopped for 1s allowing the sample to fill the gap between capillaries and the voltage was then raised to -2 kV for 100 ms. After injection, the gating cross-flow was resumed to wash excess dialysate to waste, and the separation voltage was ramped from 2 kV to 20 kV over 500 ms using a CZE1000R high voltage power supply (Spellman High Voltage Co., Hauppauge, NY). Figure 2-1 is a schematic of the microdialysis set-up with CE-LIF.

The OPA labeled amino acids were detected using LIF in a sheath-flow detector cell (Figure 2-2). The outlet of the separation capillary was inserted into a 2 mm square OD, 200 μm square ID quartz cuvette (Mindrum Precision, Inc., Rancho Cucamonga, CA). Grinding the outlet of the separation capillary to a point reduced the dead volume at the detection point. Sheath buffer (40 mM borate, pH = 9.5) was siphoned around the outside of the separation capillary. This arrangement reduces 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 a 90° angle using a 60X, 0.7 N.A. long-working distance objective

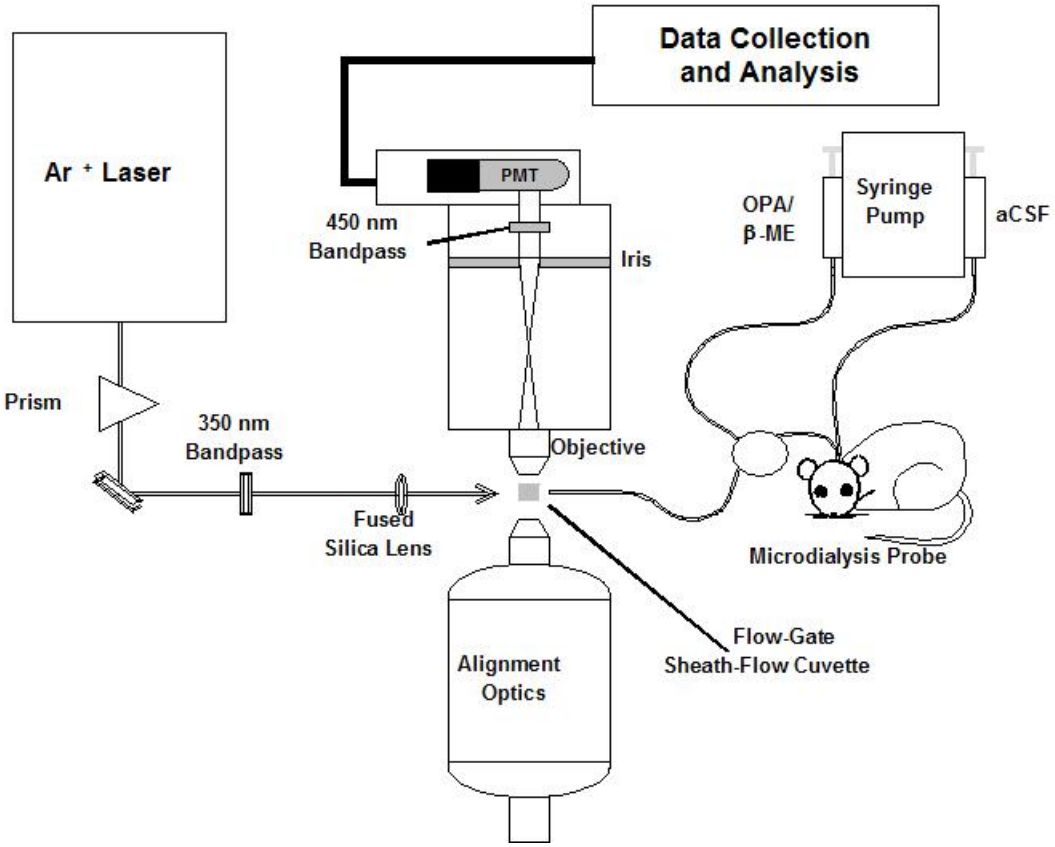

Figure 2-1 A schematic of the microdialysis and instrumentation set-up for the CE-LIF experiments (Bowser and Kennedy, 2001).

(Universe Kogaku Inc., Oyster Bay, NY), spatially filtered using an iris, passed through a 450 ± 25 nm bandpass (Melles Groit, Irvine, CA) and collected on a photomultiplier tube (PMT, R1477; Hamamatsu, Bridgewater, NJ). Current from the PMT was amplified, filtered (10 ms rise-time) and sampled using LabView 5.0 (National Instruments, Austin TX). An electropherogram from basal conditions is seen in Figure 2-3.

CE-LIF for CHAPTER 4

In CHAPTER 4 the instrument used to detect primary amino acids in ACSF was slightly different from the instrument used in CHAPTER 3. 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 3 relies on derivatizating amino acids with OPA/BE, which has fast kinetics although the end product has low fluorescence. Therefore, the instrument in CHAPTER 3 has been constructed to compensate for the low fluorescent product and to achieve low detection limits by utilizing a UV laser, custom made optics, and a sheath flow system (to decrease background noise). In contrast, the instrument in CHAPTER 4 utilized NDA (napthalene-2,3-dicarboxaldehyde)/CN (cyanide) as a derivatizating agent, which has slow reacting

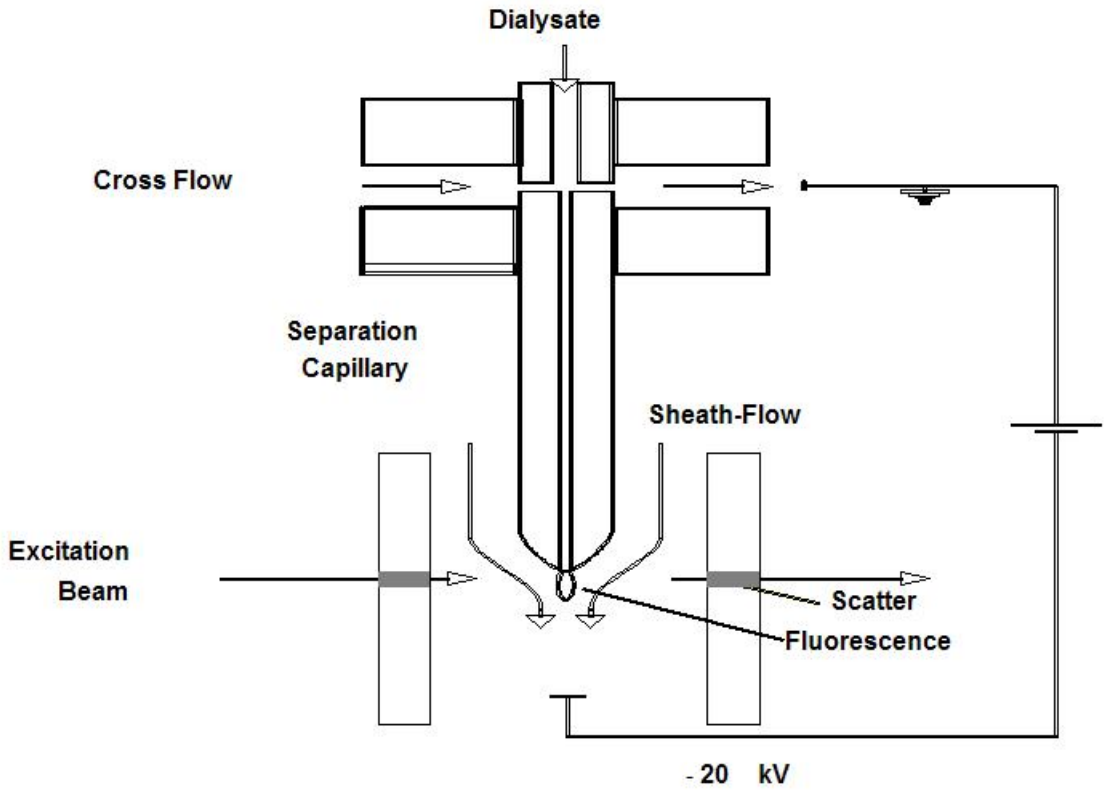

Figure 2-2 Diagram of the flow-gate, separation capillary, and sheath flow (Bowser and Kennedy, 2001).

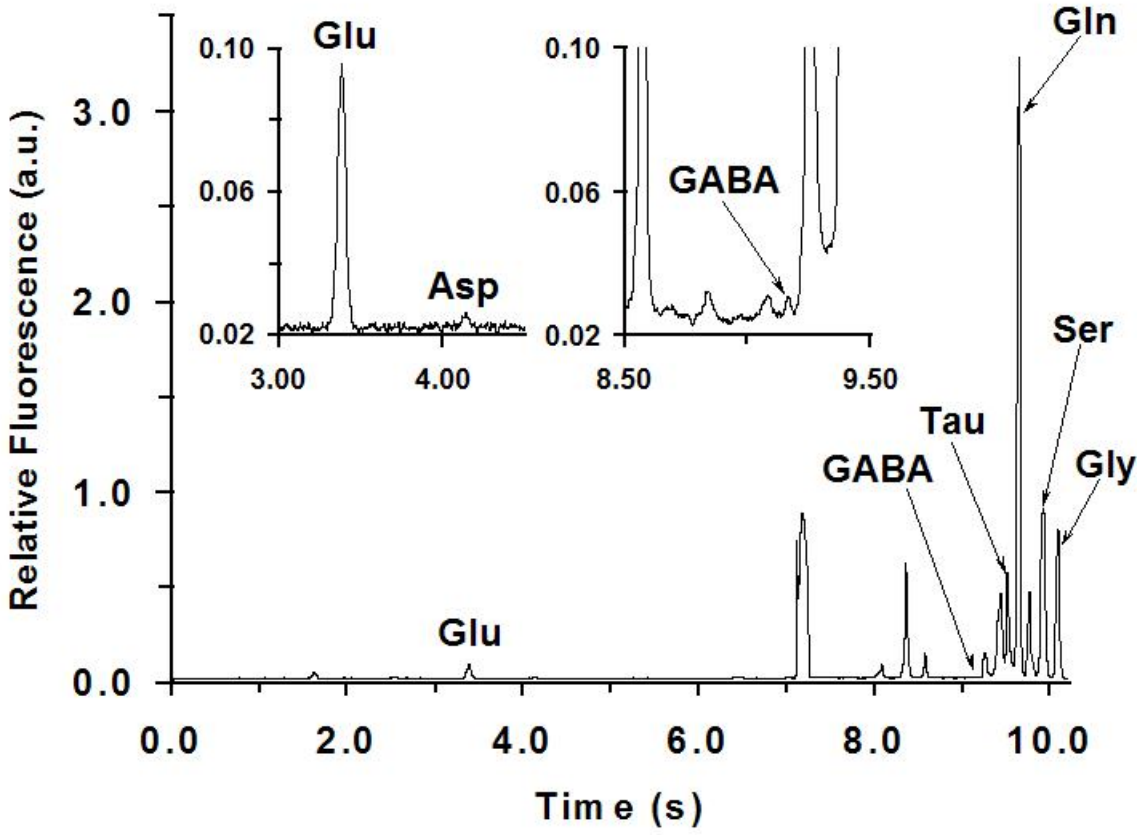

Figure 2-3 A representative electropherograms under basal conditions. Inset are close-ups of the glutamate, aspartate, and GABA peaks.

kinetics but produces a high fluorescent product which enables the instrument to achieve better limits of detection. Due to the high fluorescent end product the instrument in CHAPTER 4 does not need a UV laser, custom optics, or a sheath flow apparatus to achieve similar detection limits. In addition, the ease of operation of the instrument is increased by replacing the complex focusing optics of the instrument in CHAPTER 3 with a commercially available microscope for the instrument in CHAPTER 4.

Another difference between the two systems is the additives to the electrophoresis buffer to obtain separation. Amino acids that have been derivatized have similar electrokinetic mobilities and do not separate very well in simple electophoresis buffers.

In order to achieve separation, additives that form weak complexes with the derivatized amino acids are utilized to allow for separation to occur. The additive for the CE-LIF in CHAPTER 3 was Hydroxypropyl-β-cyclodextrin. However, this additive did not allow for optimal separation with the instrument in CHAPTER 4 that utilized NDA/CN as the derivatizing agent. Another additive, SDS, was added above the critical micelle concentration and this enabled optimal separation by micelle electrokinetic chromatography for the instrument in CHAPTER 4. However, due to the different additives the separation time is different between the two instruments.

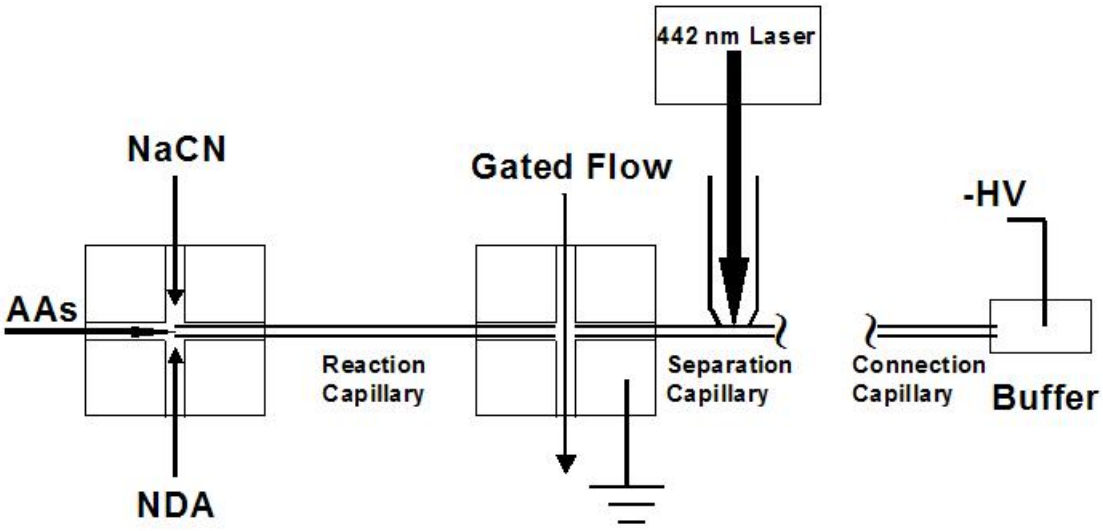

Figure 2-4 A schematic of the instrument used in CHAPTER 4. The set-up is very similar to the previous instrument with the exception of the sheath-flow cuvette (Shou et. al., 2004).

The instrument in CHAPTER 3 has a separation time of 11 seconds where as the instrument in CHAPTER 4 have a separation time of 30 seconds. A diagram of the instrument used in CHAPTER 4 is in Figure 2-4.

Flow-gated Interface and CE System

All separations were performed using 10 μm i.d. by 360 μm o.d. fused silica (Polymicro Technology, Phoenix, AZ) as the separation capillary. The total length of the separation capillary was 10 cm and the length from the inlet to the detection window was 8 cm. A -13 kV potential was applied as the separation voltage from a 1000R CZE power supply (Spellman, Plainview, NY). The separation buffer consisted of 15 mM NaH_2PO_4 and 30 mM SDS adjusted to pH 8.0 by 1 M NaOH. These conditions allowed amino acid derivatives to be resolved in less than 30 seconds, which enabled injection intervals to be set at 30 seconds. Each day, capillaries were rinsed for 15 min with 0.1 M NaOH and 20 min with separation buffer prior to experiments. Injections were performed using a flow-gated interface described in detail previously (Bowser and Kennedy, 2001). Instrument control and data analysis was accomplished using a combination of DAC board (National Instruments) and Lab-View 6.1 (National Instruments). The DAC board measures the signals from the instrument and transfers the data into the computer and the Lab-View 6.1 is the operational software for the DAC board.

Neurotransmitter Derivatization

Amino acid standards were mixed and dissolved in ACSF. For all experiments, NDA was dissolved in a 50:50 mixture (v:v) of CH_3CN and 15 mM borate at pH 9.2, and

cyanide was dissolved in 15 mM borate at pH 9.2. For on-line derivatization, ACSF, 5 mM NDA, and 10 mM cyanide were loaded into separate syringes and pumped into separate arms of a 0.25 mm bore PEEK micro-volume cross (Valco, Houston, TX) at 1.0, 0.7, 1.3 μL/min respectively (total flow rate was 3.0 μL/min) using two CMA/102 microdialysis pumps. The reaction mixture flowed through the fourth arm of the cross into a 100 cm long, 100 μm (i.d.) by 360 μm (o.d.) capillary that served as the reaction capillary. The reaction time was approximately 3 minutes. This capillary was connected to the on-line injection system (flow-gate) for direct interface to the CE system.

Laser-induced Fluorescence Detection System

The LIF detector was a commercial Axioskop 20 fluorescence microscope (Carl Zeiss, Hanover, MD) equipped with a DCP-2 microscope photometer system (CRG Precision Electronics, Houston, TX). This detector is similar to that described previously except for the wavelengths utilized (Lada and Kennedy, 1996). The 442 nm line of a 12 mW He-Cd laser (Liconix, Santa Clara, CA) was used as the excitation source. The excitation beam was reflected by a 480 nm dichroic mirror through the objective and focused onto the capillary through a 40x, 1.30 numerical aperture Fluar oil immersion lens (Carl Zeiss, Hanover, MD). The emitted fluorescence was collected by the objective, passed through the dichroic mirror and filtered with a 490 ± 10 nm emission filter (Chroma Technology, Rochingham, VT). Alignment of the laser and focusing onto the capillary were performed as described previously (Hernandez et al., 1991; Lada and Kennedy, 1996). A sample electropherogram under control conditions is shown in Figure 2-5 and ethanol-stimulated conditions is shown in Figure 2-6.

HPLC

For some experiments, dialysate was collected at 15-min intervals (flow rate = 2 μl/min), frozen, and then taurine content was measured at a later date using HPLC with electrochemical detection (Eppler and Dawson, 2001). Samples were derivatized before HPLC by reacting 20 μl dialysate and 180 μl mobile phase with 175 μl of a 9 mM OPA/0.5% BME solution in a 0.1 M disodium borate solution (pH 10.0) for exactly 2 min. The derivatization reaction was terminated by adding 25 μl 0.4 M iodoacetamide which also served to diminish electrochemical interference by OPA. Derivatized dialysate (250 μl) was injected onto a reversed-phase column (Microsorb-MV, 4.6 mm

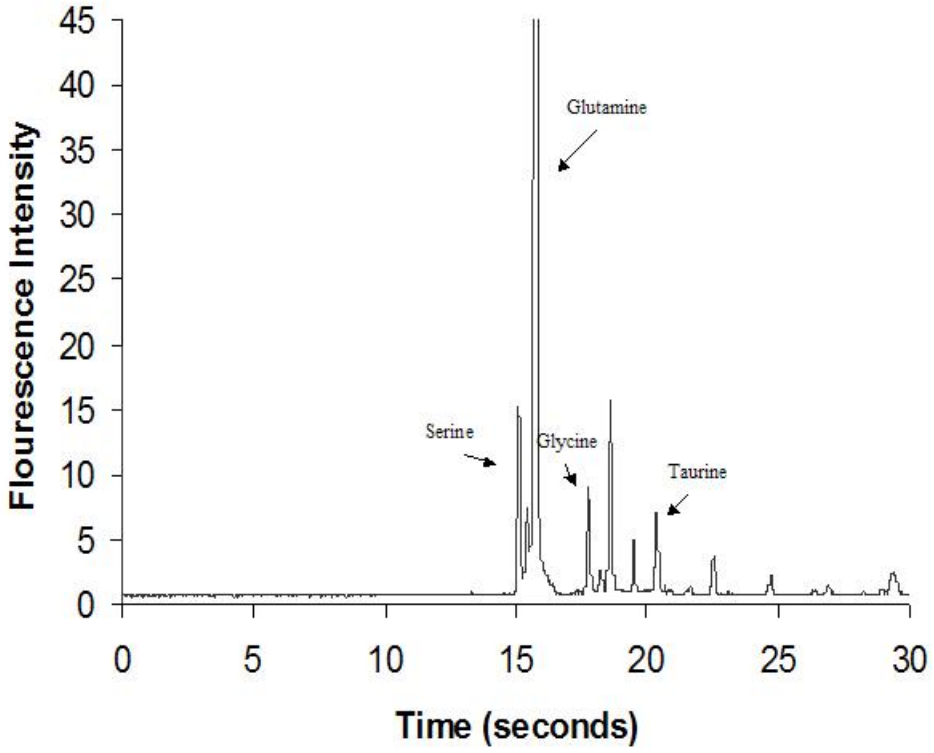

Figure 2-5 A typical electropherogram from dialysate of an ethanol-naïve rat.

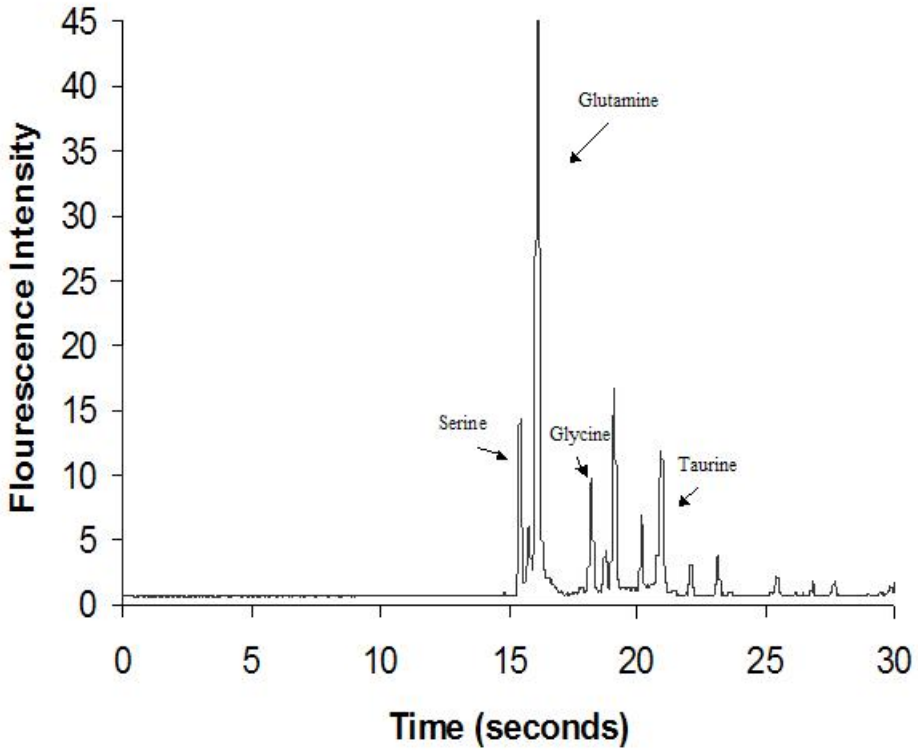

Figure 2-6 A electropherogram from a rat 10 minutes after i.p. 2.5 g/kg ethanol.

ID x 10 cm, C_{18} with 3 μm packing material; Rainin, Woburn, MA) and mobile phase was pumped at 0.7 ml/min (PM-11, Bioanalytical Systems, West Lafayette, IN). Derivatized taurine was detected using a Ag/AgCl reference electrode and a glassy carbon working electrode at an applied voltage of 0.725 V (LC-4B, Bioanalytical Systems, West Lafayette, IN). Sample taurine concentrations were calculated by a Hewlett Packard integrator (Model 3390A, Avondale, PA) based on external standards obtained from Sigma Chemicals (St. Louis, MO) and 1mM stock solutions in ddH_20 were prepared. The stock solutions were diluted with mobile phase and injected daily.

Dialysate Ethanol Determinations

Dialysate ethanol content was measured using headspace gas chromatography with an automated solid phase microextraction (SPME) procedure. A Varian 2800 gas chromatograph was equipped with a Supelcowax-10 megabore column (15 mm x 0.53 mm; 0.5 μm film). The injector was set to 175^{o}C; the column oven was 65^{o}C; the flame ionization detector was 220^{o}C. Samples were heated to 65^{o}C for 15 min before analysis. The SPME of the headspace was carried out in an 8200 autosampler using 3 min absorption and 2 min desorption. The SPME fiber was coated with carboxenpolydimethylsiloxane (75 μm). Ethanol concentrations were quantified by comparison to external standards. The lowest standard run routinely was 0.3125 mM which gave a signal to noise ratio of 10.

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, 1982). The criteria for probe selection is that the majority of the 2mm probe length must be contained within the NAc. A similar criterion was utilized for the striatum study (3mm probe length must be contained within the striatum). In addition, upon sectioning for probe placement if any pathology was observed the subject was not included in the study.

Data Analysis

The first twenty samples collected in each CE-LIF experiment (pre-injection) were used to calculate a baseline of 100%. Subsequent data were expressed as a percent of this baseline and are depicted in each figure as the mean ± SEM (standard error mean). For statistical purposes, the mean of each 5 samples was calculated and subjected to a

threeway ANOVA with unweighted-means solution for unequal N using brain region, dose and time period as variables for each analyte. To follow-up significant time interactions, data were divided into first 20 minutes, second 20 minutes and last 12 minutes after injection and analyzed separately. A significance level of $p<0.05$ was used for all statistical analyses. Only those subjects with accurate probe placements were included in the analysis. The statistics were performed on data that were percentages which may not exhibit a normal distribution and valid for subsequent parametric testing. To confirm significance reported from using data as percentages the data was subjected to log transformation and analysis of variance were performed. In CHAPTER 3 overall significant brain region X dose X time interaction were confirmed for taurine, glutamate and serine. Conversely, glycine did not display significant overall brain region X dose X time interaction. In CHAPTER 4 significant group X time interactions were confirmed for taurine, serine and glycine. These results indicate that the significance reported for the percent baseline data was legitimate.

CHAPTER 3
DIFFERENTIAL INCREASE IN TAURINE LEVELS BY LOW DOSE ETHANOL IN THE NUCLEUS ACCUMBENS AND CAUDATE-PUTAMEN REVEALED BY MICRODIALYSIS WITH ON-LINE CAPILLARY ELECTROPHORESIS

Introduction

Approximately 100,000 people die each year due to alcohol-related disease or injury (Narconon, 2004). The continued use of ethanol despite these consequences indicates the strong positive reinforcement caused by its consumption. The ability of ethanol to increase dopamine signaling in the nucleus accumbens (NAc) is thought to be one of the neurobiological possibilities of this positive reinforcement (Brodie and Appel, 1998; Brodie et al., 1990; Di Chiara, 2002; Nevo and Hamon, 1995). Support for this possibility is that disruption of this response reduces alcohol self-administration (El-Ghundi et al., 1998; Gonzales and Weiss, 1998; Phillips et al., 1998). Acamprosate (a calcium homologue of the naturally-occurring amino acid, taurine) and homotaurine (a taurine precursor) also decrease ethanol self-administration and ethanol-induced dopamine efflux (Olive et al., 2002) although the mechanism for this effect is not known.

Ethanol affects many different neural systems as it has been shown to activate inhibitory GABA receptors (Allan and Harris, 1987; Harris, 1999; Proctor et al., 2003) and inhibit excitatory glutamate receptors (Fitzgerald and Nestler, 1995; Lovinger et al., 1989). Similarly, taurine has been shown to positively modulate GABA receptor function (Bureau and Olsen, 1991; del Olmo et al., 2000; Liljequist, 1993; Quinn and Harris, 1995) and glycine receptor function (Han et al., 2001; Mori et al., 2002a; Sergeeva and

Haas, 2001). In addition, glutamate receptor agonists increase taurine release (Lehmann et al., 1985; Magnusson et al., 1991; Oja and Saransaari, 2000; Saransaari and Oja, 2000). Changes in levels of neurotransmitters affected by ethanol could lead to the pathological state of alcoholism (Nevo and Hamon, 1995; Tsai, 1998). In the NAc, acute low dosages of ethanol have been shown to increase glutamate efflux, whereas higher dosages decrease glutamate efflux (Dahchour et al., 1994; Dahchour et al., 1996; De Witte et al., 1994; Heidbreder and De Witte, 1993; Moghaddam and Bolinao, 1994). In contrast, acute dosages of ethanol do not change GABA efflux in NAc (Dahchour et al., 1994; Dahchour et al., 1996; De Witte et al., 1994; Heidbreder and De Witte, 1993; Moghaddam and Bolinao, 1994) but may alter taurine. In the NAc, taurine efflux is increased by ethanol (Dahchour et al., 1994)(Dahchour et al., 1994), and this effect is enhanced by repeated ethanol exposure (Dahchour and De Witte, 2000a; Dahchour et al., 1994; Dahchour et al., 1996). Taurine alters the reinforcing properties of ethanol (Aragon and Amit, 1993; Quertemont et al., 1998) and rats genetically selected for differences in ethanol self-administration exhibit alterations in both ethanol-induced taurine and dopamine efflux in the NAc (Dahchour et al., 2000; Katner and Weiss, 2001; Quertemont et al., 2002). While this evidence is suggestive for a role of taurine in ethanol reinforcement, significant gaps exist in our knowledge of how this can occur. For example, self-administering animals will normally not ingest more than 0.5 g/kg of ethanol (Bienkowski et al., 2001; Samson, 1986; Vacca et al., 2002); yet taurine levels have not been demonstrated to be altered by such low doses. In addition, the temporal pattern of taurine efflux relative to ethanol administration has not been sufficiently characterized to determine if it could play a primary role in mediating the reinforcing

effects of ethanol. Finally, the selectivity of taurine changes has not been directly compared across brain regions. If ethanol affects taurine in a similar fashion throughout the brain, a specific neuronal role of taurine in mediating the reinforcing effects of ethanol is less likely.

Most of the prevailing *in vivo* data have utilized microdialysis coupled with HPLC-based analysis in which samples are collected every 10-15 minutes. Due to the large sample size (20-30μL) and corresponding sampling time (10-15 minutes) the relationship between the release of taurine with that of dopamine, GABA or glutamate cannot be determined. Capillary electrophoresis coupled with laser-induced fluorescence detection (CE-LIF) has recently been adapted to measuring *in vivo* extracellular levels of amino acids with much higher temporal resolution (Bowser and Kennedy, 2001; Lada and Kennedy, 1996). For example, this instrumentation can analyze primary amines in dialysate (70nL sample volume) every 10 seconds and provides the means to examine neuronal processes (Lada et al., 1998). Due to the small volumes utilized by CE-LIF a lower perfusion rate for sampling can be used. This provides the ability to measure primary amines from smaller regions of the brain, like the NAc, without the concern that other regions are providing primary amines to the dialysate. A potentially powerful method, microdialysis with rapid on-line CE-LIF had not previously been used to show the effects of ethanol in freely moving animals.

Methods

Animals

Male Sprague-Dawley rats, 70-90 days of age, (Harlan, Indianapolis, IN) weighing 250-350 g were housed 2-3 per cage in a temperature and humidity controlled environment with a 12-hr normal phase light/dark cycle. All testing and training were

conducted during the light phase. The subjects had *ad libitum* access to food and water throughout the experiment unless otherwise indicated. All procedures were conducted in strict adherence to the *National Institute of Health Guide for the Care and Use of Laboratory Animals*

Chemicals

NaCl, $CaCl_2$, KCl, $MgSO_4$, o-phthaldialdehyde (OPA), β-mercaptoethanol (BME), sodium tetraborate, and hydroxypropyl-β-cyclodextrin (HPBCD) were purchased from Sigma Chemical Co. (St. Louis, MO). Ethanol (95%) was obtained from Aaper Alcohols and Chemicals, Shelbyville KY. Saline was purchased from Baxter Healthcare Corporation, Deerfield, IL.

Surgeries

Each rat was anesthetized with 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 the CPU or NAc using the following coordinates: CPU, +0.8 anteroposterior, +3.0 lateral to bregma, -4.0 dorsoventral to skull; NAc, +1.6 anteroposterior, +1.7 lateral, -6.2 dorsoventral. The guide cannula was secured to the skull with dental cement anchored by three stainless steel screws. After surgery, each rat was housed separately and allowed to recover for 5- 7 days with daily handling to ensure patency of the guide cannula and to minimize handling stress during subsequent measurements.

Microdialysis

Microdialysis probes (o.d. 270 μm; active length 2 mm for the NAc and 3 mm for CPU; cellulose membrane, 13,000 molecular weight cut-off) were constructed by the

method of Pettit and Justice (Pettit and Justice, 1989). On the day of the experiment, each rat was lightly anesthetized with halothane, moved into the dialysis cage and the guide cannula was cleared for insertion of the dialysis probe. The probe was perfused with artificial cerebrospinal fluid (ACSF; 145 mM NaCl, 2.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$) at 1 μL/min. After probe insertion, each rat was placed in a clear polyurethane test box (9 3/8" x 18" x 10" deep) with bedding, water bottle and food. Following this procedure, samples were taken every 11 seconds for 4-6 minutes (20-30 samples) and this was repeated every 15 minutes for the first ninety minutes after probe implantation. About 2 hrs after probe insertion, saline (1 ml/kg body weight, 0.9% NaCl) was injected i.p. and 285 samples (55 minutes) were taken. Subsequent injections of ethanol (20% w/v in saline, i.p.) occurred in the following order at one-hour intervals: 0.5 g/kg, 1 g/kg, and 2 g/kg. After each injection, 285 samples were collected. The amino acid concentrations in dialysate samples were analyzed on-line by CE-LIF. A calibration curve was generated either before or after the experiment by after placing the tip of the dialysis probe in three different concentrations of a standard solution containing glutamate, aspartate, GABA, taurine, dopamine, serine and glycine and collecting 30 samples each. The mean relative fluorescence of these samples was converted to concentration through the use of the calibration curve (a plot of peak height versus standard concentration).

After microdialysis, rats were killed by rapid decapitation. Brains were removed and frozen at -20°C and 40 μm sections were taken to verify probe placement.

CE-LIF

Amino acids were measured by CE-LIF utilizing the procedure established by Bowser and Kennedy (Bowser and Kennedy, 2001). Primary amine moieties in the dialysate were derivatized on-line by mixing with a 1 μL/min stream of OPA solution (10 mM OPA, 40 mM BME, 36 mM borate, 0.81 mM HPBCD, 10% MeOH (v/v), pH 9.5) with a reaction time of approximately 1.5 min. The reaction time did not limit the temporal resolution of the measurement; however, it did induce a delay between changes occurring at the dialysis probe and their detection by the CE system. Samples of the dialysate were injected onto the capillary using a flow-gate interface as follows. The outlet of the reaction capillary was aligned with the inlet of the CE separation capillary (10 cm long, 10 μm ID, 150 μm OD fused-silica) in a Plexiglas block, leaving a gap of approximately 30 μm. A 1 mL/min cross-flow was applied between the capillaries, which carried the sample solution to waste. To inject a plug of sample, the cross-flow was stopped for 1s allowing the sample to fill the gap between capillaries and the voltage was then raised to -2 kV for 100 ms. After injection, the gating cross-flow was resumed to wash excess dialysate to waste, and the separation voltage was ramped from 2 kV to 20 kV over 500 ms using a CZE1000R high voltage power supply (Spellman High Voltage Co., Hauppauge, NY).

The OPA labeled amino acids were detected using LIF in a sheath-flow detector cell. The outlet of the separation capillary was inserted into a 2 mm square OD, 200 μm square ID quartz cuvette (Mindrum Precision, Inc., Rancho Cucamonga, CA). Grinding the outlet of the separation capillary to a point reduced the dead volume at the detection point. Sheath buffer (40 mM borate, pH = 9.5) was siphoned around the outside of the

separation capillary. This arrangement reduces 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 a 90° angle 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 (Melles Groit, Irvine, CA) and collected on a photomultiplier tube (PMT, R1477; Hamamatsu, Bridgewater, NJ). Current from the PMT was amplified, filtered (10 ms rise-time) and sampled using LabView 5.0 (National Instruments, Austin TX).

HPLC

For some experiments, dialysate was collected at 15-min intervals (flow rate = 2 μl/min), frozen, and then taurine content was measured at a later date using HPLC with electrochemical detection (Eppler and Dawson, 2001). Samples were derivatized before HPLC by reacting 20 μl dialysate and 180 μl mobile phase with 175 μl of a 9 mM OPA/0.5% BME solution in a 0.1 M disodium borate solution (pH 10.0) for exactly 2 min. The derivatization reaction was terminated by adding 25 μl 0.4 M iodoacetamide which also served to diminish electrochemical interference by OPA. Derivatized dialysate (250 μl) was injected onto a reversed-phase column (Microsorb-MV, 4.6 mm ID x 10 cm, C_{18} with 3 μm packing material; Rainin, Woburn, MA) and mobile phase was pumped at 0.7 ml/min (PM-11, Bioanalytical Systems, West Lafayette, IN). Derivatized taurine was detected using an Ag/AgCl reference electrode and a glassy carbon working electrode at an applied voltage of 0.725 V (LC-4B, Bioanalytical

Systems, West Lafayette, IN). Sample taurine concentrations were calculated by a Hewlett Packard integrator (Model 3390A, Avondale, PA) based on external standards prepared and injected daily.

Dialysate Ethanol Determinations

Dialysate ethanol content was measured using headspace gas chromatography with an automated solid phase microextraction (SPME) procedure. A Varian 2800 gas chromatograph was equipped with a Supelcowax-10 megabore column (15 mm x 0.53 mm; 0.5 μm film). The injector was set to 175^{o}C; the column oven was 65^{o}C; the flame ionization detector was 220^{o}C. Samples were heated to 65^{o}C for 15 min before analysis. The SPME of the headspace was carried out in an 8200 autosampler using 3 min absorption and 2 min desorption. The SPME fiber was coated with carboxenpolydimethylsiloxane (75 μm). Ethanol concentrations were quantified by comparison to external standards. The lowest standard run routinely was 0.3125 mM which gave a signal to noise ratio of 10.

Data Analysis

The first twenty samples collected in each CE-LIF experiment (pre-injection) were used to calculate a baseline of 100%. Subsequent data were expressed as a percent of this baseline and are depicted in each figure as the mean ± SEM (standard error mean). For statistical purposes, the mean of each 5 samples was calculated and subjected to a threeway ANOVA (provided by a program developed in house) with unweighted-means solution for unequal N using brain region, dose and time period as variables for each analyte. To follow-up significant time interactions, data were divided into first 20 minutes, second 20 minutes and last 12 minutes after injection and analyzed separately.

A significance level of $p<0.05$ was used for all statistical analyses. Only those subjects with accurate probe placements were included in the analysis.

Results

Ethanol-induced Taurine Efflux

Acute injection of ethanol increased taurine levels in the CPU and NAc in a dose-dependent manner. In the NAc (Figure 3-1), the largest increase in taurine levels (90±15% above baseline) occurred 5 to 15 minutes after the 2 g/kg injection after which taurine levels gradually stabilized to 40±7% above basal until the end of the sampling period. The 1 g/kg dose increased taurine levels for about 15 minutes with a maximal increase of 28±8%. A slightly lower magnitude of increase (20±9%) was observed after the 0.5g/kg dose with a somewhat shorter duration. There was a slight elevation in taurine levels for 5 minutes after saline injection, never reaching a 10% increase from basal values. However, taurine efflux in response to the saline injection decreased over time but never significantly deviated from basal levels.

The CPU was less sensitive to the ethanol injection (Figure 3-2). The highest dose of ethanol maximally increased taurine levels by only 25±9% above baseline for 30 minutes. Similarly, 0.5 and 1 g/kg ethanol i.p. increased taurine by a maximum of only 15±5% above baseline levels. The saline injection also increased taurine levels by 15±5% but only for about 5 minutes after which taurine levels returned to a stable baseline for the duration of sampling.

These observations were supported by the results of a 3-way ANOVA as follows. An overall significant brain region X dose X time interaction ($F(156,1560) = 1.85$; $p< 0.001$) was detected. During the first twenty minutes of sampling, there was a significant brain region X dose X time interaction ($F(57,570) = 3.65$; $p < 0.001$) which was primarily

due to a significant region X time interaction at the 2 g/kg dose ($F(19,190) = 6.17$; $p < 0.001$) but not after any of the other injections. There were significant dose X time interactions for both the NAc ($F(57,342) = 11.6$; $p < 0.001$) and CPU ($F(57,228) = 3.43$; $p < 0.001$) and significant main effects of time after all injections in both brain regions (NAc: $F(19,114)$ ranged from 2.22 to 21.6; CPU: $F(19,76)$ ranged from 2.91 to 6.11; p at least < 0.05). During the twenty to forty minutes after injection, ANOVA revealed only significant two-way interactions of region X dose ($F(3,30) = 4.52$; $p < 0.01$) and region X time ($F(19,190) = 5.63$; $p < 0.001$) which was primarily due to a significant main effect of dose only in the NAc ($F(3,18) = 17.5$; $p < 0.001$). Follow-up analyses indicated a significant main effect of region after the 2 g/kg dose ($F(1,10) = 6.11$; $p < 0.05$) but not after saline, 0.5 or 1 g/kg ethanol. During the last 12 minutes after injection, the interaction between brain region and dose was no longer significant ($F(3,30) = 2.74$; $p = 0.06$).

Ethanol-induced Glutamate Efflux

When the effect of cumulative ethanol injections on glutamate levels was measured, and compared to taurine a very different time course of changes was seen. That is, no effect occurred at any dose until at least 10-15 minutes after injection in both brain regions (Figure 3-3). At this point, 2 g/kg ethanol began to decrease glutamate levels in both the NAc and the CPU by 10-15% whereas 0.5 g/kg ethanol increased glutamate levels, particularly in the CPU. Specifically, 0.5 g/kg increased glutamate levels in the CPU (Figure 3-4) starting at 20 minutes to reach a maximum increase of60$\pm$30% above baseline by the end of the sampling period. However, this large magnitude increase occurred in only 2 out of 5 subjects, explaining the large SEMs. The 0.5 g/kg dose of ethanol also caused a small increase in glutamate in the NAc,

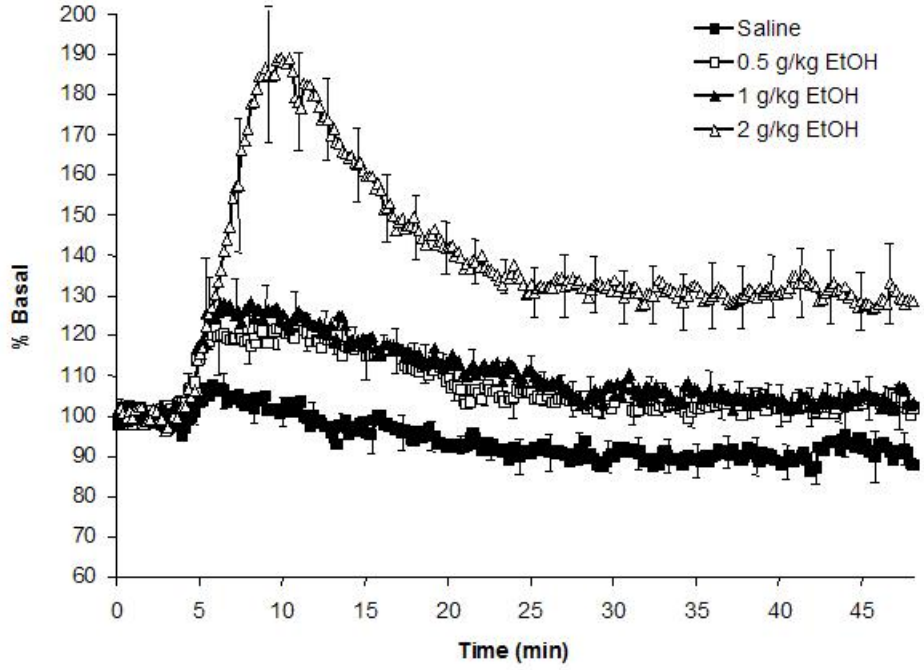

Figure 3-1 Ethanol-induced taurine efflux. The change in taurine efflux in microdialysate from the NAc (N=7) of Sprague-Dawley rats after saline or 0.5, 1.0 or 2.0g/kg i.p. ethanol injections given in a cumulative fashion where each increasing dose of ethanol follows the next after an hour has elapsed (0.5g/kg, hour elapsed, 1.0g/kg, hour elapsed, 2.0g/kg, hour elapsed). Microdialysate was sampled for one hour after each injection. The injection occurred at the start of each 1-hr sampling period but there was approximately a 5 minute delay for dialysate to travel from the probe tip to the flow-gate. Data were calculated as a percent of the mean ± SEM relative fluorescence of the first twenty sample (basal efflux).

which was primarily due to larger magnitude increases in 3 out of 7 rats tested. There was no change in glutamate levels after the saline or 1 g/kg injection for either region. These observations were supported by an overall significant brain region X dose X time

interaction ($F(156,1560) = 1.54$; $p < 0.001$). The glutamate data from the first twenty, second twenty and last twelve minutes of sampling were subjected to a 3-

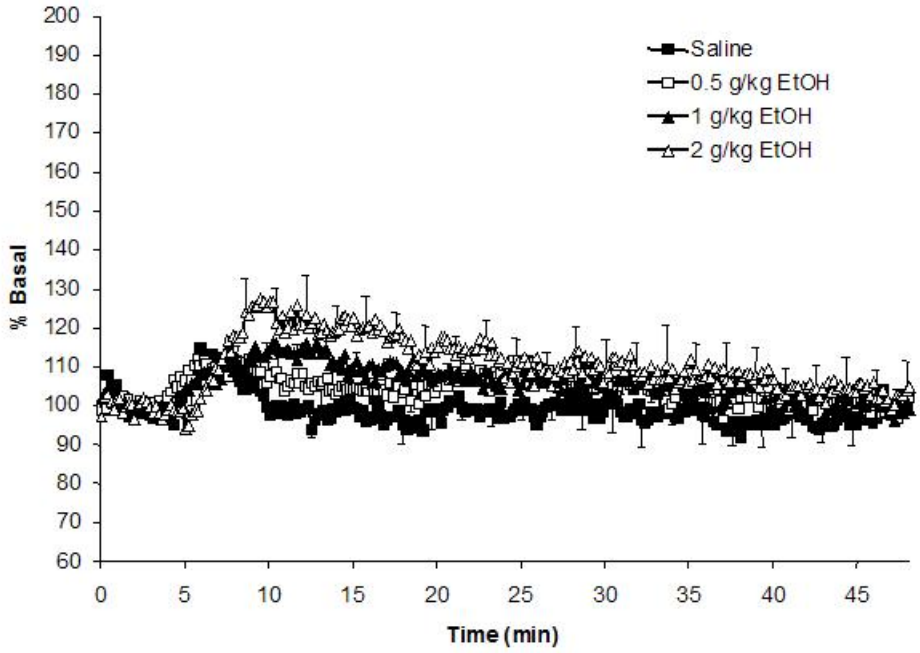

Figure 3-2 Ethanol-induced taurine efflux. The change in taurine efflux in microdialysate from the CPU (N=5) of Sprague-Dawley rats after saline or 0.5, 1.0 or 2.0g/kg i.p. ethanol injections given in a cumulative fashion.

way ANOVA. During the first twenty minutes after injection, there was an overall dose X time interaction ($F(57,570) = 2.15$; $p < 0.001$) which was due to a main effect of time after the 2 g/kg injection ($F(19,190) = 10.8$; $p < 0.001$) . There was no significant effect of brain region. On the other hand, there were significant region X dose X time interactions during the second twenty minutes ($F(57,570) = 1.95$; $p < 0.001$) and last

twelve minutes ($F(33,330) = 1.61$; $p < 0.05$) which were due to an effect of dose in the CPU ($F(3,12) = 4.20$; $p < 0.05$) but not the NAc, and an effect of time after only the 0.5 and 2 g/kg injections ($F(19,190) = 1.95$ and 2.02, respectively; $p < 0.01$).

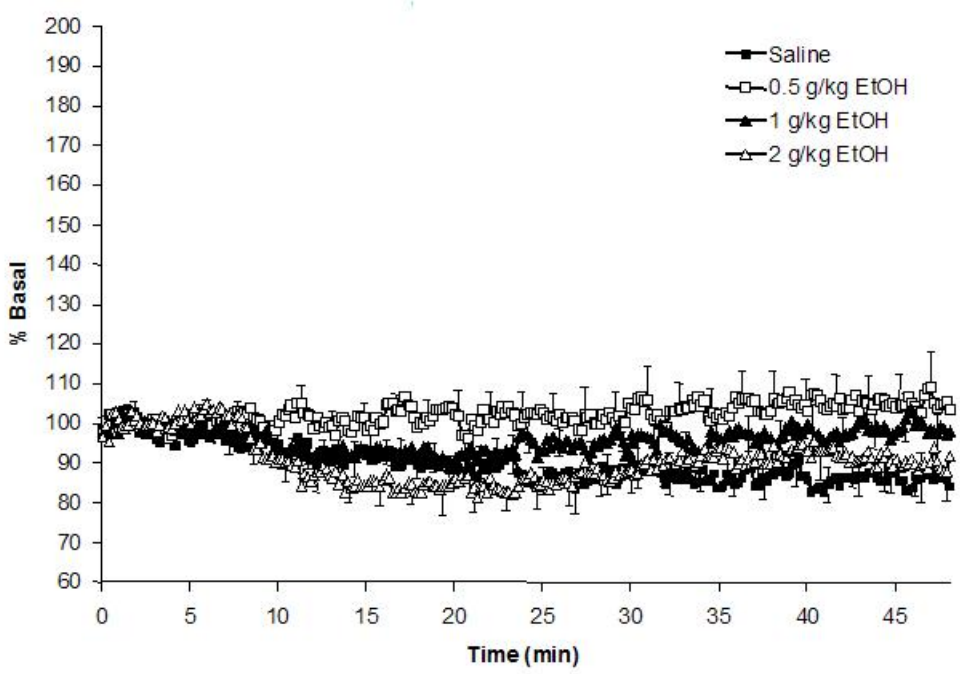

Figure 3-3 Ethanol-induced changes in glutamate efflux. The change in glutamate efflux in microdialysate from the NAc (N=7) of Sprague-Dawley rats after saline or 0.5, 1.0 or 2.0 g/kg i.p. ethanol injections given in a cumulative fashion. Microdialysate was sampled and data calculated as described for Figure 3-1.

Ethanol-induced Glycine Efflux

Acute ethanol slowly decreased glycine levels in a dose-related manner in both the NAc and the CPU (Figure 3-5,3-6). The 2 g/kg dose maximally decreased glycine by 25-30% after 45 minutes whereas 1 g/kg decreased glycine by 10-15% over time and neither

saline nor 0.5 g/kg affected glycine levels. Overall there was no significant effect of brain region X dose X time ($F(156,1560) = .67$; $p < 0.99$). However, there was a significant dose X time interaction ($F(57,570) = 2.74$; $p < 0.001$) during the first twenty

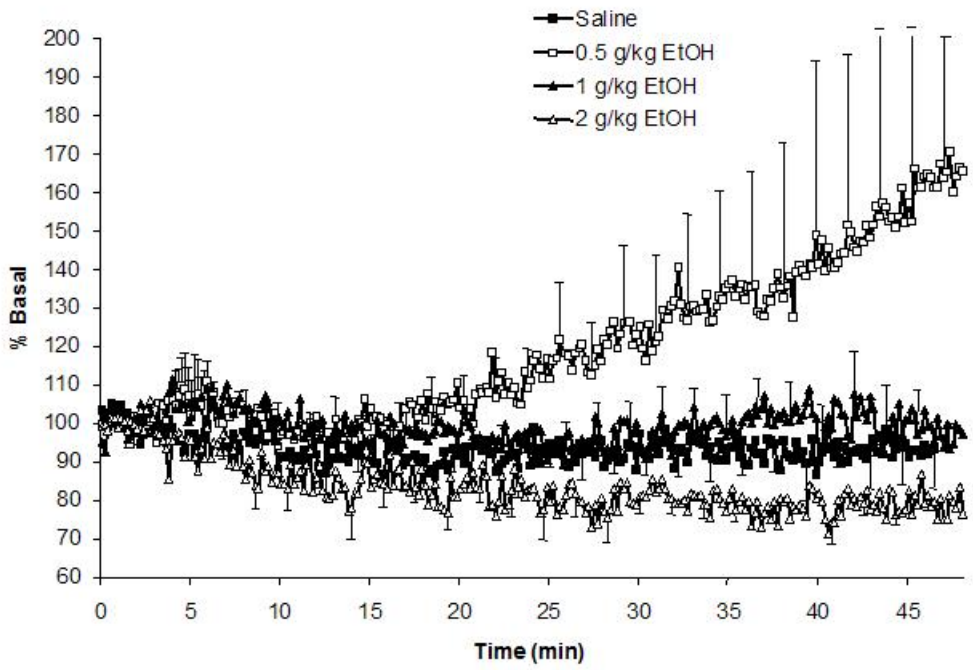

Figure 3-4 Ethanol-induced changes in glutamate efflux. The change in glutamate efflux in microdialysate from the CPU (N=5) of Sprague-Dawley rats after saline or 0.5, 1.0 or 2.0 g/kg i.p. ethanol injections given in a cumulative fashion. The drastic increase in glutamate efflux observed in the 0.5 g/kg dose is attributed to two out of the five rats whose glutamate efflux was larger.

minutes after injection, and by significant dose main effects during the second twenty minutes ($F(3,30) = 6.40$; $p < 0.01$), and the last twelve minutes ($F(3,30) = 5.43$; $p < 0.01$). There was a region X time interaction during the first twenty minutes ($F(57,570) = 3.65$; $p < 0.001$) which was due to a greater overall effect of time in the NAc than the CPU.

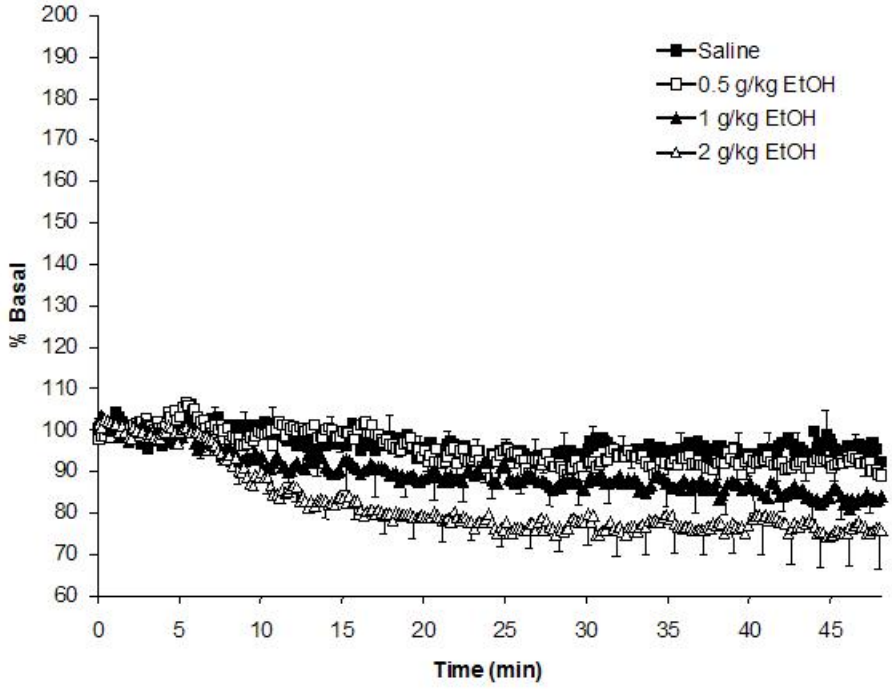

Figure 3-5 Ethanol-induced decreases in the efflux of glycine. The change in glycine in microdialysate from the NAc (N=7) of Sprague-Dawley rats after saline or 0.5, 1.0 or 2.0 g/kg i.p. ethanol injections given in a cumulative fashion. Microdialysate was sampled and data calculated as in Figure 3-1.

Ethanol-induced Serine Efflux

To further understand the mechanism of ethanol-induced amino acid efflux, serine was examined due to its involvement in osmoregulation and lack of neurotransmission qualities. Ethanol slowly decreased serine levels by about 10-20% in the NAc and CPU (Figure 3-7,3-8) especially at the higher doses tested. These observations were supported by a significant overall brain region X dose X time interaction ($F(156,1560) = 1.28$; $p<$

0.05). There was a significant dose X time interaction ($F(57,570) = 3.19$; $p < 0.001$) during the first twenty minutes after injection and by significant dose effects during the

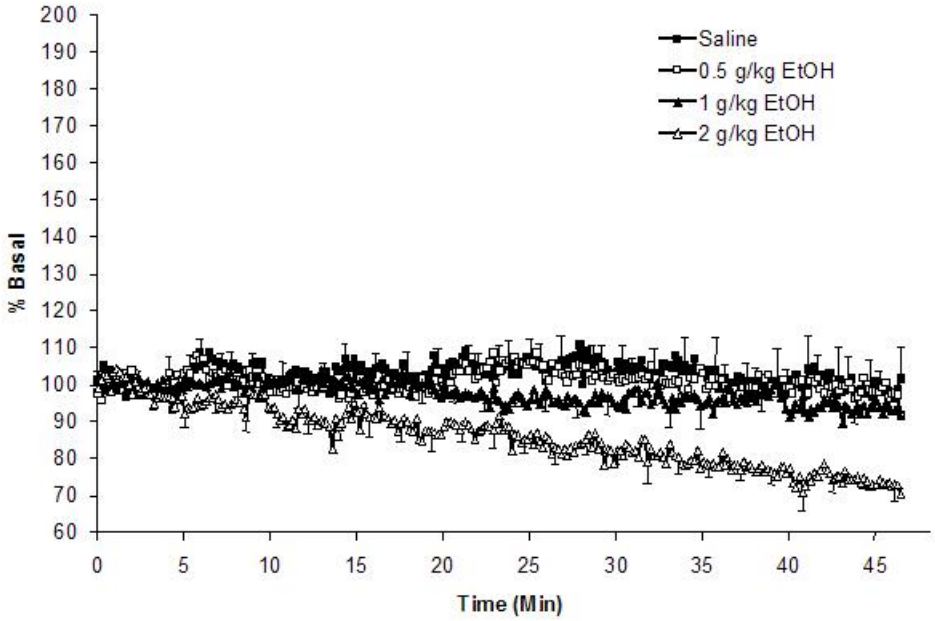

Figure 3-6 Ethanol-induced decreases in the efflux of glycine. The change in glycine in microdialysate from the CPU (N=5) of Sprague-Dawley rats after saline or 0.5, 1.0 or 2.0 g/kg i.p. ethanol injections given in a cumulative fashion.

second twenty minutes ($F(3,30) = 4.20$; $p < 0.05$) and the last twelve minutes ($F(3,30) = 6.12$; $p < 0.01$). There were no main effects or interactions involving brain region. There were no systematic effects of ethanol on GABA levels in the NAc and a small inhibitory effect (about 15%) of the highest ethanol dose in the CPU (data not shown). However,

ANOVA of these data did not indicate any significant main effects or interactions during any of the time periods after injection.

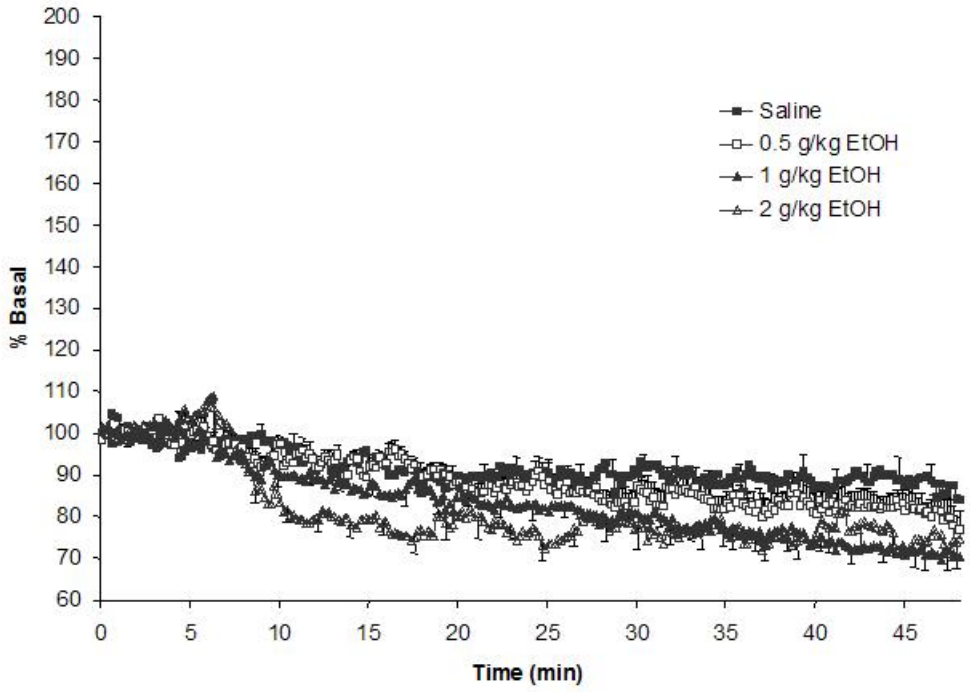

Figure 3-7 Ethanol-induced decreases in the efflux of serine. The change in serine efflux in microdialysate from the NAc (N=7) of Sprague-Dawley rats after saline or 0.5, 1.0 or 2.0 g/kg i.p. ethanol injections given in a cumulative fashion. Microdialysate was sampled and data were calculated as described in Figure 3-1.

Basal Efflux

As mentioned previously, the first twenty samples from each treatment (saline, 0.5, 1 and 2g/kg) were taken to represent the basal values of that particular treatment. After the first twenty samples, the data was converted to percent of the basal mean for

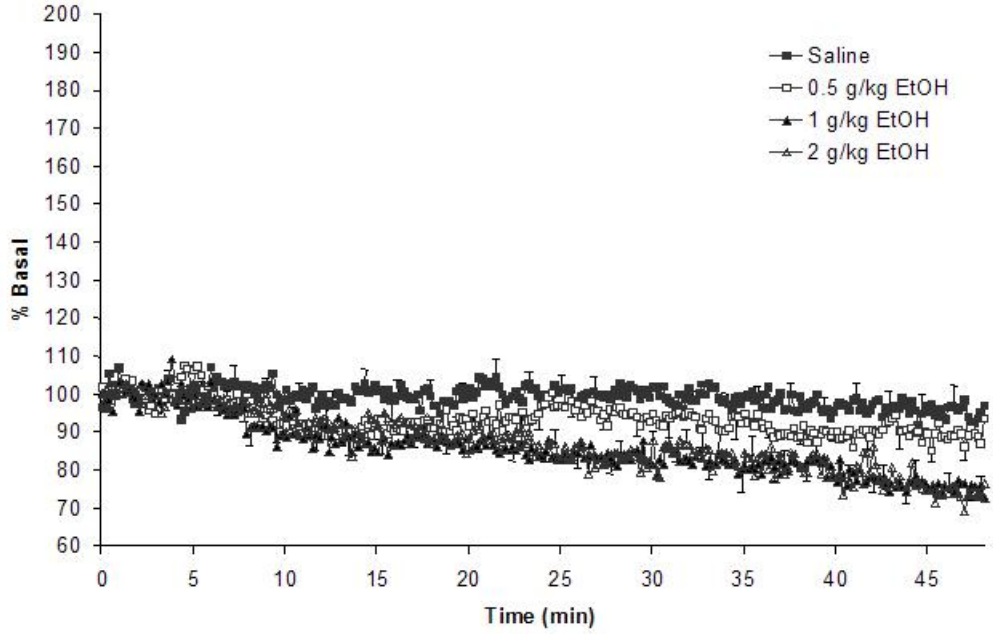

Figure 3-8 Ethanol-induced decreases in the efflux of serine. The change in serine efflux in microdialysate from the CPU (N=5) of Sprague-Dawley rats after saline or 0.5, 1.0 or 2.0 g/kg i.p. ethanol injections given in a cumulative fashion.

that treatment. In order to determine whether there were residual effects from one treatment to the next (i.e. saline mean basal might be smaller than the ethanol group's mean basal values), the mean basal levels of amino acids in dialysate were compared. Figure 3-9 is the mean amino acid concentration calculated from the first twenty 11-sec samples (i.e., the first 3.5 minutes of sampling) for taurine, glycine, glutamate and GABA in microdialysate from the NAc (Figure 3-9) and CPU (Figure 3-10) following each injection. Basal values for the treatments in the NAc did not significantly deviate from one treatment to the next. Likewise, the CPU basal values for the amino acids

measured did not significantly change from treatment to treatment. It was also apparent from these data that all amino acids had reached stable levels by 2 hrs after probe insertion.

Ethanol-induced Taurine Efflux Measured by HPLC

In order to compare the sensitivity of the CE-LIF assay to conventional microdialysis methods, we injected rats with saline or 1 g/kg ethanol (in a counter-balanced fashion) and collected dialysate samples from CPU every 15 minutes that were subsequently analyzed for taurine using HPLC. Taurine levels in the striatum were significantly elevated by both saline and ethanol injections (Figure 3-12). The 1 g/kg dose of ethanol increased taurine above basal levels at the 15 min interval, as confirmed by a significant main effect of time ($F(17,85)=3.39$, $p<0.001$). The saline injection also increased taurine above basal levels at the 15 min interval ($F(5,25)=2.82$, $p<0.05$). However, there was no significant main effect or interaction involving the type of injection (saline vs ethanol).

Ethanol-levels in Dialysate

In order to compare the dialysate ethanol concentrations between the two types of ethanol exposure (four cumulative escalating doses versus one 1 g/kg dose), we compared dialysate ethanol levels in the animals used in Figure 3-11 with those of an additional three animals experiencing the series of cumulative injections as used in Figures 3-1 to 3-8 but with dialysate collected every 10 minutes. The 1 g/kg ethanol injection, when given alone, increased brain dialysate ethanol levels to about 10 mM within the first 15 minutes after injection which then took approximately 3 hours to

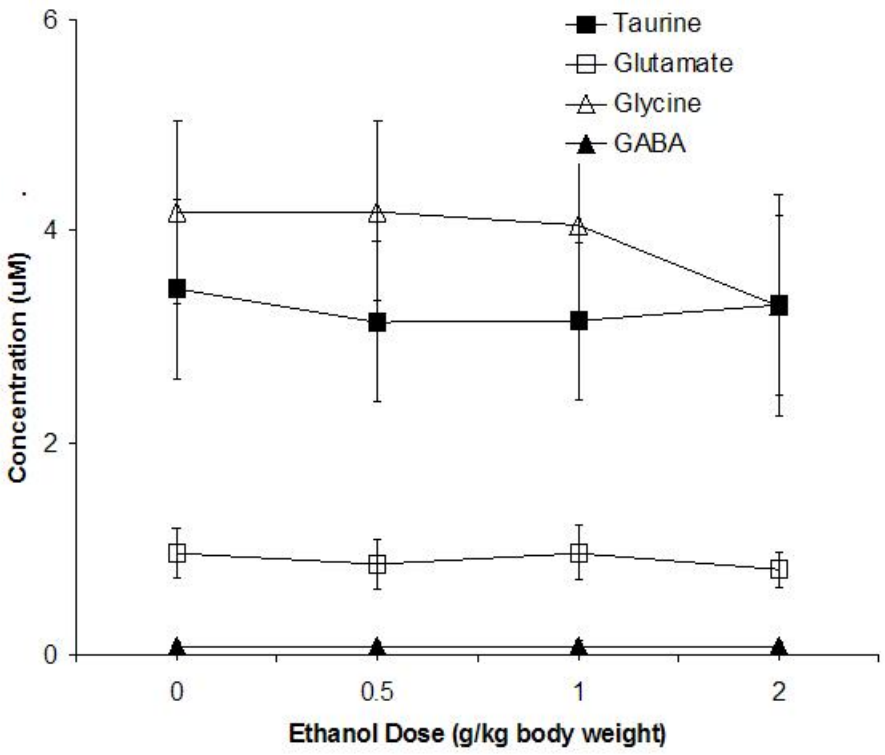

Figure 3-9 Basal dialysate levels of amino acids. The mean basal concentrations for taurine, glycine, glutamate and GABA in microdialysate from the NAc (N=7) of Sprague-Dawley rats after saline or 0.5, 1.0 or 2.0 g/kg i.p. ethanol injections given in a cumulative fashion. The mean basal values were calculated from the first twenty samples at the beginning of each 1-hr sampling period.

return to basal levels (Figure 3-12 and data not shown). The first cumulative injection of 0.5 g/kg ethanol increased brain ethanol levels to about 7 mM, which then decreased to about 4 mM just prior to the second cumulative injection of 1.0 g/kg. This injection raised brain dialysate ethanol to about 14 mM where it remained fairly constant until the final ethanol injection of 2 g/kg. This injection further raised brain ethanol levels to

about 30 mM where they remained stable for the duration of the experiment. The metabolism of ethanol, which is attributed to the non-inducible alcohol

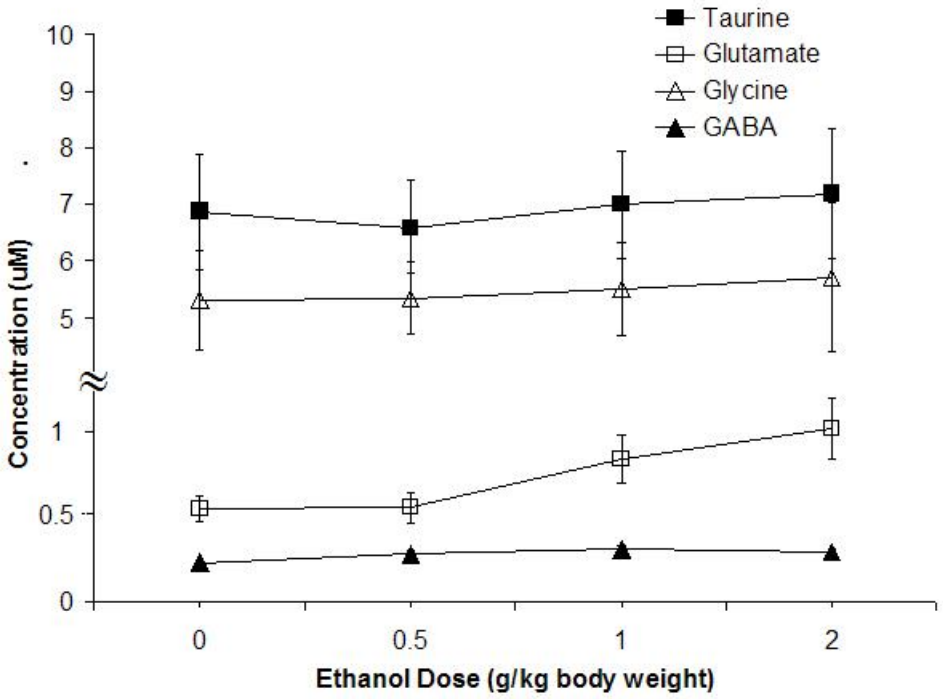

Figure 3-10 Basal dialysate levels of amino acids. The mean basal concentrations for taurine, glycine, glutamate and GABA in microdialysate from the CPU (N=5) of Sprague-Dawley rats after saline or 0.5, 1.0 or 2.0 g/kg i.p. ethanol injections given in a cumulative fashion.

dehydrogenase, displays zero order kinetics (time dependent) (Martin et al., 1984). The elimination rate of ethanol in rats is 1.0 g/kg per hour (Lim et al., 1993) and explains the slight decrease in brain ethanol levels at the lower doses of ethanol. When the subsequent higher doses of ethanol are given the constant ethanol elimination rate is harder to observe due to the amount of ethanol already in the brain. Due to the constant metabolism of ethanol, the brain ethanol levels due to 2.0 g/kg would be higher than if 2.0 g/kg were

given acutely, due to the ethanol still present from the 1.0 g/kg given one hour before the 2.0 g/kg dose.

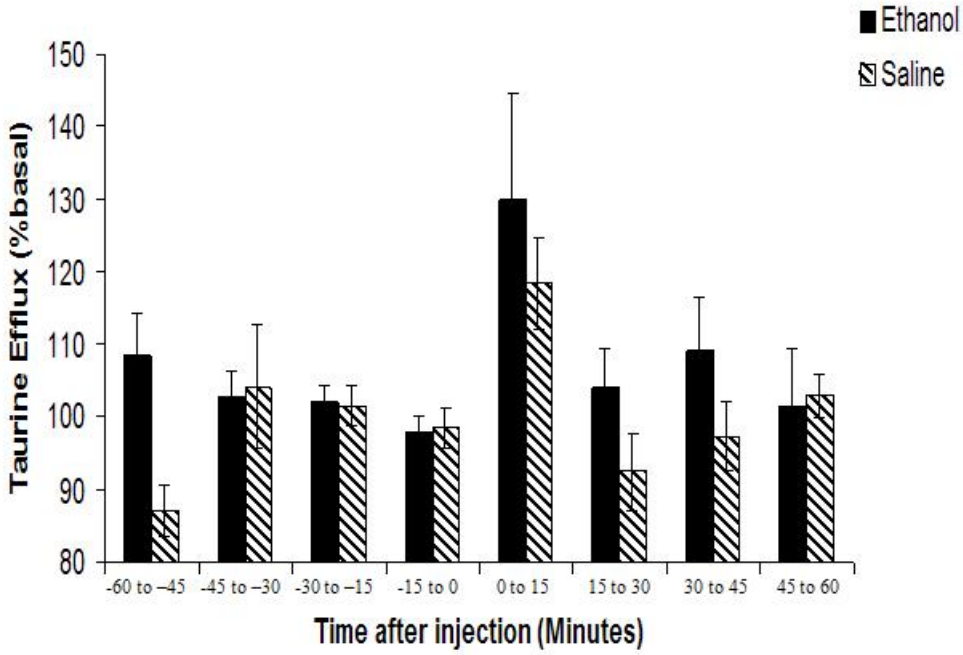

Figure 3-11 HPLC determination of ethanol-induced taurine efflux. The level of taurine in microdialysis samples from CPU of rats after saline or ethanol injections. Samples were collected every 15 minutes and subjected to HPLC analysis. Shown are means±SEMs for N=7.

Direct Perfusion of Ethanol

To ascertain whether the ability of ethanol to affect amino acid levels was a direct effect of ethanol on the cells surrounding the microdialysis probe, ethanol was perfused directly into the NAc. In this CE-LIF experiment, a series of concentrations (50 mM, 500 mM, 1 M) of ethanol was perfused (1µL/min) by reverse dialysis into the NAc and

samples were collected every 11 seconds for 30 min at each concentration (Figure 3-13). Increasing the concentration of ethanol in the ACSF had no effect on taurine levels but caused a concentration-dependent increase in both glutamate and glycine levels by about

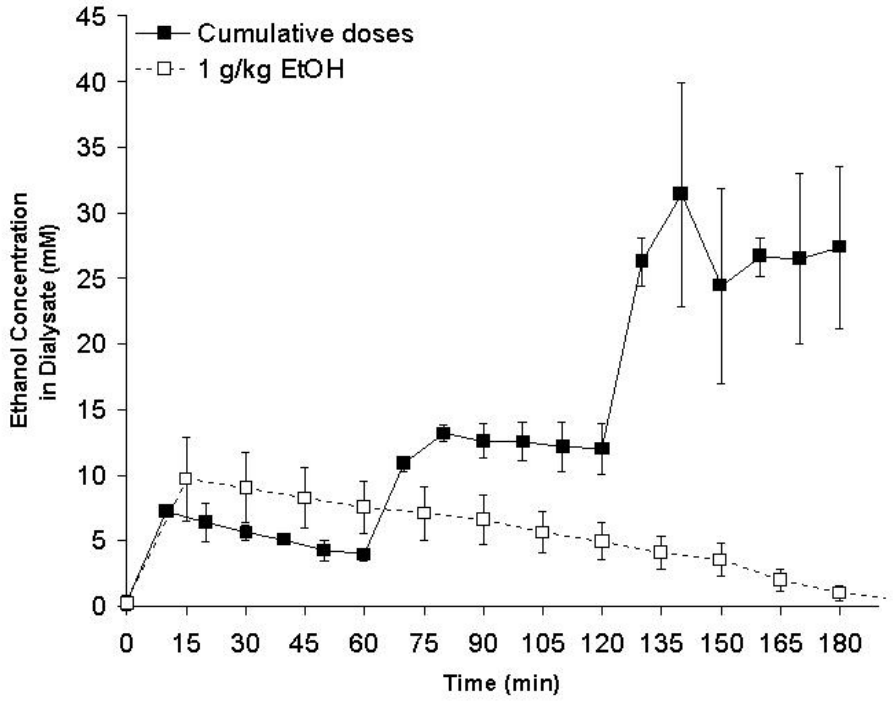

Figure 3-12 Brain dialysate ethanol levels. The level of ethanol in microdialysis samples from striatum of rats after one injection of ethanol (1 g/kg) or after cumulative ethanol injections as described for figures 3-1 to 3-8. Shown are means±SEMs for N=3-5.

30-40% above baseline. On the other hand, 50 and 500 mM ethanol tended to decrease serine levels. However none of the effects caused by including ethanol in the ACSF resulted in amino acid levels that were significantly different from levels under control conditions.

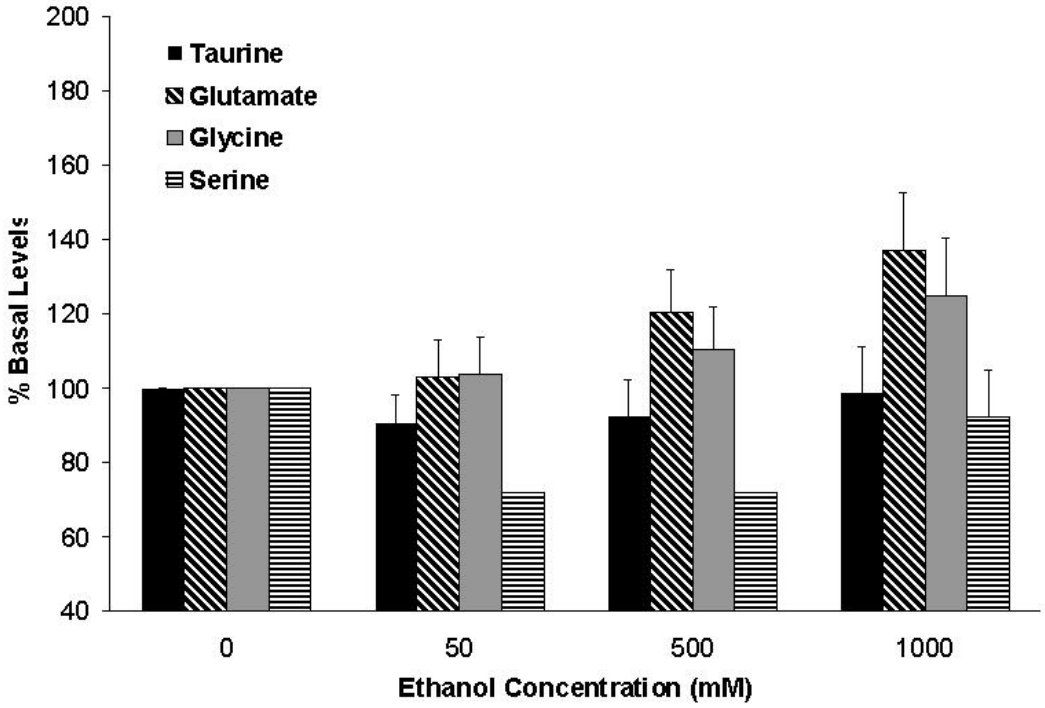

Figure 3-13 Taurine, glutamate and glycine levels in NAc after direct exposure of NAc to ethanol using reverse dialysis. Basal levels were measured for 20 minutes prior to switching to ACSF containing one of three concentration of ethanol. Data are expressed as a percent of the basal samples for each treatment. Shown are means ± SEMS for N = 4.

Discussion

These results provide novel support for the hypothesis that ethanol exposure causes a selective interaction with taurine levels in different regions of the brain. For the first time, increases in taurine were observed at a dose that animals will self administer and at a greater magnitude in brain regions known to be activated during positive reinforcement (NAc) as compared to nearby motor regions (CPU). Furthermore an early

rapid increase followed by a later more persistent elevation in taurine levels was observed suggesting a complex release mechanism. Taurine changes involved a systemic effect of ethanol rather a local effect, which argues against this efflux being attributed solely to osmotic changes caused by local ethanol exposure. Although the amount of local exposure to ethanol could not be verified, the largest dose of direct perfusion (1M) did not affect taurine efflux. These novel observations were made possible by the first high temporal resolution monitoring of multiple amino acids in the brains of freely moving animals using microdialysis coupled to rapid on-line CE-LIF analysis.

Systemic administration of ethanol produced dose-related biphasic increases in taurine. In the NAc, 2 g/kg dosage of ethanol increased taurine levels almost two-fold during the first twenty minutes after injection where it remained elevated by 30% for at least one hour. The dose-related increase in taurine seen after injections of the two higher doses of ethanol confirms previous reports in NAc (Dahchour et al., 1994; Dahchour et al., 1996). However, the significant dose-related increase in taurine seen in CPU, and the increases in both CPU and the NAc after 0.5 g/kg ethanol have not previously been reported. Similarly, previous reports did not indicate a biphasic nature of the response of taurine to higher doses of ethanol. The primary difference between this work and previous studies is the decrease in sampling interval to 11 s from 15 min. The conclusion that the 82-fold improvement in temporal resolution enabled these novel observations is supported by comparison of data from experiments in which HPLC analysis failed to detect an effect of 1 g/kg ethanol in CPU that was significantly different from the saline response. Additionally, the increased statistical power afforded by the larger number of samples gathered by CE-LIF, compared to only 4 samples per hour collected every 15

minutes with HPLC techniques, may have contributed to significant effects observed upon exposure to both 0.5 and 1 g/kg ethanol in CPU. Thus, the use of CE-LIF in freely-moving animals allowed for the detection of taurine levels evoked by clinically and behaviorally relevant doses of ethanol. Similar effects on taurine were found when an acute dosage of 2.5 g/kg of ethanol was given alone in comparison to the cumulative dosage regime (Smith et al., in press) (see CHAPTER 4). Additionally, preliminary data indicate that self-administered doses as low as 0.4 g/kg ethanol increase taurine levels in NAc by about 20% as measured by on-line CE-LIF (Smith et al., 2003) (see CHAPTER 5). Ethanol is self-administered in the range of 0.5 g/kg to 0.95 g/kg (Bienkowski et al., 2001; Samson, 1986; Vacca et al., 2002) and a place preference effect can be observed with 1.0 g/kg i.p. (Bozarth, 1990). In addition, lower to moderate doses (0.4 g/kg to 1.7 g/kg) of self-administered ethanol will increase responding for reward brain stimulation (Bain and Kornetsky, 1998). Conversely, higher doses of ethanol (1.5 g/kg and higher) have been shown to have aversive properties (Bienkowski et al., 1997; Froehlich et al., 1998). From this perspective, lower to moderate doses are more rewarding than higher doses. If taurine was released in response to the rewarding effects of ethanol then taurine efflux would be much greater for the lower to moderate doses of ethanol. This is not the case as taurine displays a dose response relationship with ethanol. Dopamine plays an integral part in the reward pathway (described in CHAPTER 1) yet several studies have shown that ethanol can increase dopamine efflux in the NAc in a dose-dependent manner (Imperato and Di Chiara, 1986; Yoshimoto et al., 1992). The precise cause of ethanol-induced taurine efflux remains unknown.

The role of taurine in the brain and the regulation of its efflux have not yet been fully characterized. Taurine is one of the most abundant amino acids in the brain (Huxtable, 1992; Pow et al., 2002), and is present in neurons as well as glia (Huxtable, 1989; Huxtable, 1992). Many biological roles have been proposed for taurine including neurotransmission, osmoregulation, Ca^{2+} modulation, membrane stabilization and antioxidation (Huxtable, 1992; Wright et al., 1986), all of which might contribute to alterations in extracellular taurine levels. Although only a small percentage of taurine is found in synaptic vesicles, it has been proposed as an inhibitory neurotransmitter (Huxtable, 1992; Oja et al., 1990; Okamoto et al., 1983a). In support of this, depolarization of neuronal populations initiate taurine release (Rogers et al., 1991; Schousboe et al., 1990a; Schousboe et al., 1990b; Shain and Martin, 1990), however, this release is Ca^{2+}-independent, Cl^--dependent, and lower in magnitude compared to other neurotransmitters (Rogers et al., 1991; Schousboe et al., 1990b). Taurine efflux can also occur via a swelling-activated channel activated by hypo-osmotic conditions (Bres et al., 2000; Sanchez-Olea et al., 1991; Solis et al., 1990). Additionally, extracellular taurine levels are regulated by the activity of a high affinity taurine transporter (Smith et al., 1992). All of these mechanisms might contribute to altered levels of extracellular taurine caused by ethanol.

At least a portion of ethanol-induced taurine efflux has been shown to be due to osmoregulation (Lallemand et al., 2000; Quertemont and De Witte, 2001; Quertemont et al., 2003). However, our data indicating the regional selectivity of ethanol's effects argue against this mechanism. Also contrary to the osmoregulatory hypothesis is our data that local exposure of the NAc to 1000 mM ethanol increased taurine by only 10% above

baseline which is much less than the two-fold increase seen after the 2 g/kg dose (resulting in 30 mM dialysate ethanol). Similar studies that utilized the direct perfusion of ethanol have estimated that the amount of ethanol in 50μm layer of tissue adjacent to the microdialysis probe to be greater than 30% of the perfusate concentration (Gonzales et al., 1997). This would indicate that the direct perfusion of 1000mM would result in an ethanol concentration of at least 300mM in tissue adjacent to the probe. The definitive evidence for determining whether the mechanism for ethanol-induced taurine efflux is neuronal or osmotic requires testing whether this efflux is dependent on signaling-activated Ca^{2+} and sodium channels or whether it is more dependent on osmotically activated chloride currents.

Although our data can only imply a neuronal origin, it is likely that ethanol-induced taurine increases, regardless of the source, could have a neuronal impact. Post-synaptically, taurine stimulates Cl^- conductance to hyperpolarize membranes (Okamoto et al., 1983b; Ye et al., 1997), presumably as an agonist at glycine and $GABA_A$ receptors (Bureau and Olsen, 1991; Chattipakorn and McMahon, 2002; Haas and Hosli, 1973; Han et al., 2001; Horikoshi et al., 1988; Mori et al., 2002b; Quinn and Harris, 1995; Schmieden et al., 1992). Since there is evidence for expression of both $GABA_A$ and glycine receptors in the NAc (Martin and Siggins, 2002), taurine might alter neuronal activity via these receptors after ethanol exposure. However, the amount of taurine increased by ethanol might not be physiologically significant. From the previous experiments, the basal levels of taurine in the NAc are approximately 4μM, which is confirmed from previous *in vivo* studies (Semba et al., 1995). This would indicate that taurine levels during the 2.0 g/kg would be close to 8 μM. Taurine has been shown to

inhibit muscimol binding with an IC_{50} (inhibitory concentration 50%) of 50μM and enhance flunitrazepam binding with an EC_{50} (effective concentration 50%) of 10μM (Bureau and Olsen, 1991). In neurons freshly isolated from nucleus accumbens of young rats, taurine has been shown to elicit chloride currents with an EC_{50} of 1.25mM (Jiang et al., 2004). From these studies it would seem that the even at the highest concentrations, approximately 8 μM, achieved by the 2.0 g/kg i.p. ethanol, taurine would be ineffective. However, these studies were not conducted *in vivo* and application of effective taurine concentrations *in vitro* might be different given the dynamics of an *in vivo* system. Conversely, several studies have indicated that taurine can modify ethanol-induced behavior (Aragon et al., 1992; Ferko, 1987). A problem with these studies is that brain taurine concentration was not measured, which makes a correlation to behavior modification difficult. Taurine can modify several neurotransmitter systems and behavioral effects associated with ethanol, however the concentration at which taurine functions in this manner is still questionable. Ethanol can induce a two-fold increase in taurine efflux in the NAc, which could be high enough to significantly alter several receptors in this area.

Given taurine's proposed role as an inhibitory neurotransmitter and our ability to view (in almost real-time) a multitude of amino acids in response to ethanol elicits the question of a possible interaction of these neurotransmitter systems. Along these lines, the higher ethanol doses significantly decreased glutamate, glycine, and serine levels, a finding that has not before been reported (Dahchour et al., 1994; Dahchour et al., 1996; Quertemont et al., 2000). The time course of these changes indicates that the inhibitory effect of ethanol occurred after taurine levels were increased by ethanol. These same

decreases in glutamate, serine and cysteine were observed when animals received only 1 injection of 2.5g/kg of ethanol (Smith et al., in press) as compared to the cumulative injection scheme used presently. It will be interesting to test whether the decreases in these amino acids are dependent on ethanol-induced increases in taurine inhibition.

The effect of the lowest dose of ethanol on glutamate was much more complicated. In both regions, but particularly in CPU, the 0.5 g/kg dose caused a marked elevation in glutamate levels in only about half of the individual rats tested for each region. There is only one previous report that low dose ethanol administration might increase glutamate in the NAc (Moghaddam and Bolinao, 1994) compared to reports indicating no effect of 1, 2 or 3 g/kg ethanol on glutamate (e.g., (Dahchour et al., 1994; Dahchour et al., 1996)). Instead, increases in glutamate are generally associated with withdrawal from ethanol (Dahchour and De Witte, 1999; Dahchour and De Witte, 2000b; Dahchour et al., 1998; Quertemont et al., 2002). It is possible that the increase in glutamate found in the present study is more related to decreasing brain ethanol levels occurring in the one hour following the 0.5 g/kg dose (from 7 to 4 mM). Dialysate ethanol concentrations remained fairly level following the higher doses and perhaps longer observation would have revealed a slow increase in glutamate as brain ethanol levels started to fall. It is unclear why this response would be more robust in CPU versus NAc or why it would be variable between animals. Although it has been shown that taurine can block glutamate efflux during withdrawal (Dahchour and De Witte, 2000b), each of the individual animals that exhibited increases in glutamate levels after the low dose of ethanol had robust increases in ethanol-induced taurine efflux. On the other hand, the animals with unchanged glutamate levels had fairly average increases in

taurine. Thus the ability of low doses of ethanol to increase glutamate levels does not appear to be dependent on the amount of ethanol-induced taurine release in individual animals.

Ethanol craving is regulated by its ability to increase neuronal firing in the mesolimbic dopamine pathway (Hodge et al., 1997; Koob et al., 1998). This effect of ethanol does not occur in all dopaminergic pathways, since ethanol decreases dopamine transmission in CPU (Budygin et al., 2001). Such regional specificity may indicate a relationship between the magnitude of ethanol-induced taurine efflux and the ability of ethanol to increase dopamine transmission. Therefore, it would also be significant to simultaneously track dopamine and taurine changes on a high resolution time scale. However, dopamine could not be accurately measured in the present experiments due to poor isolation of dopamine from several other endogenous substances under the conditions used.

In summary, our novel observations have opened up several new lines of possible investigation including the temporal relationship between changes in taurine, dopamine and glutamate as well as the mechanisms of taurine release caused by ethanol. The ability of CE-LIF to measure the temporal relationship between changes in these neurotransmitters, coupled with its ability to measure subtle changes in neurotransmitter levels in awake animals self-administering ethanol, should provide a tremendous opportunity to better understand the alterations in signaling that underlie the development of alcoholism.

CHAPTER 4
ETHANOL-INDUCED TAURINE EFFLUX IN THE NUCLEUS ACCUMBENS IS MEDIATED VIA CALCIUM-DEPENDENT OSMOTICALLY ACTIVATED CHANNELS

Introduction

Microdialysis studies have revealed that ethanol increases the efflux of taurine in several brain areas (Dahchour and De Witte, 1999; Dahchour and De Witte, 2000; Dahchour et al., 2000; Dahchour et al., 1994; Dahchour et al., 1996; De Witte et al., 1994; Quertemont et al., 1998a; Quertemont et al., 2002), an effect that has been suggested to reflect ethanol's ability to alter osmotic conditions (Dahchour and De Witte, 1999; Dahchour and De Witte, 2000; Dahchour et al., 2000; Dahchour et al., 1994; Dahchour et al., 1996; De Witte et al., 1994; Quertemont et al., 1998a; Quertemont et al., 2002). Recently, advancements in microdialysis techniques have revealed that ethanol-induced taurine efflux is most sensitive in the nucleus accumbens (NAc; Smith et al., 2003; Smith et al., 2004), an area of the brain closely associated with the addiction/drug reinforcement processes (Di Chiara, 2002; Di Chiara and Imperato, 1988; Joseph et al., 2003; McBride et al., 1999; Smith et al., 2003). This regionally selective increase in taurine caused by ethanol suggests a dependence of a specific neuronal circuitry and may indicate more than one mechanism for efflux in NAc compared to that in other brain regions.

Besides being one of the most abundant amino acids in neuronal tissue (Huxtable, 1989; Jacobson and Hamberger, 1984), taurine also plays a role in neuroprotection (Foos

and Wu, 2002; Huxtable, 1989; Huxtable, 1992; Saransaari and Oja, 2000b) and osmotic homeostasis (Hussy et al., 2000; Huxtable, 1989; Huxtable, 1992; Pasantes-Morales et al., 2002; Schaffer et al., 2000). Taurine may also have neuronal signaling functions since it can modulate GABA (Bureau and Olsen, 1991; del Olmo et al., 2000; Haas and Hosli, 1973; Horikoshi et al., 1988; Liljequist, 1993; Wahl et al., 1992), NMDA (Kurachi et al., 1983; Lehmann et al., 1984) and glycine receptors (De Saint Jan et al., 2001; Flint et al., 1998; Hussy et al., 1997; Jiang et al., 2004; Martin and Siggins, 2002; Mori et al., 2002; Sergeeva and Haas, 2001). Recently, a change in osmolarity has been shown to release taurine selectively in the supraoptic nucleus (SON), upon which it inhibits glycine receptor function, thereby controlling the release of vasopressin (Hussy et al., 2001). These findings indicate that alterations in osmotic conditions can affect neurotransmission in brain regions in a selective manner.

Since ethanol has been shown to increase taurine efflux selectively in an area of the brain closely associated with addiction, taurine could be a key step in the alcohol addiction process because of its ability to alter neurotransmission, particularly that of GABA and glutamate. Taurine can alter several of the effects of ethanol such as ethanol-induced sleep time (Boggan et al., 1978; Ferko, 1987; Ferko and Bobyock, 1988; Mattucci-Schiavone and Ferko, 1985; McBroom et al., 1986), locomotor activity (Aragon et al., 1992), taste aversion (Aragon and Amit, 1993) and reinforcement, both positive and negative (Quertemont et al., 1998b). In support of this possibility, taurine analogs reduce both ethanol intake (Boismare et al., 1984; Czachowski et al., 2001; Olive et al., 2002; Stromberg et al., 2001) and ethanol-stimulated dopamine release (Olive et al., 2002). Thus, the importance of understanding the mechanism of ethanol-induced taurine

efflux may lead to the understanding of taurine's capability of altering the neurochemical environment in the NAc during ethanol exposure thereby resulting in a change in addiction susceptibility.

The first step in understanding the effects of taurine in the NAc is to explore the mechanism by which ethanol stimulates taurine release. The exact mechanism of ethanol-induced taurine efflux is unknown; however taurine release is known to occur in response to two different stimuli: osmoregulation or neuronal depolarization. Osmotic release of taurine occurs via a volume sensitive organic anion channel (VSOAC)(Kirk, 1997; Kirk et al., 1992; Strange et al., 1996; Strange and Jackson, 1995). A VSOAC is responsible for the efflux of several dissimilar organic solutes. In addition, the channel displays a similar pharmacological profile, in terms of being sensitive to several anion transport inhibitors, in several different cell types (Bothwell et al., 2002; Bres et al., 2000; Jackson and Strange, 1993; Sanchez-Olea et al., 1996). On the other hand, several studies have indicated classical neurotransmitter characteristics of taurine efflux such as Ca^{2+}-dependency of potassium-stimulated efflux (Bernardi et al., 1977; Kontro and Oja, 1987b; Philibert et al., 1988; Philibert et al., 1989a; Philibert et al., 1989b) as well as tetrodotoxin (TTX) sensitivity (Jacobson and Hamberger, 1984; Semba et al., 1995; Singewald et al., 1993).

In this chapter, the possible mechanisms of taurine efflux in the NAc induced by 2.5g/kg ethanol injections, administered intraperitoneally (i.p.), were examined using capillary electrophoretic separation with laser-induced fluorescence detection (CE-LIF). This methodology allows high temporal resolution of changes in analyte content of microdialysate and therefore might reveal different temporal components of ethanol-

induced taurine efflux. The possibility of ethanol-induced taurine efflux being due to neuronal depolarization was examined by delivering tetrodotoxin (TTX) via reverse dialysis to inhibit voltage-activated sodium channels. In separate experiments, Ca^{2+}-free ACSF containing EGTA was used to block vesicular neurotransmitter release. The contribution of osmotic influences of ethanol and the resulting osmoregulation via taurine efflux was investigated by perfusing known osmotic channel inhibitors.

Methods

Animals

Male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 250-350 grams were housed 2-3 per cage in a temperature and humidity-controlled environment with a 12-hr normal phase light/dark cycle. All testing and training was conducted during the light phase. The subjects had *ad libitum* access to food and water throughout the experiment unless otherwise indicated. All procedures were conducted in strict adherence to the *National Institute of Health Guide for the Care and Use of Laboratory Animals.*

Chemicals

Napthalene-2,3-dicarboxaldehyde (NDA) was received from Molecular Probes (Eugene, OR). Sodium phosphate and potassium cyanide were from Fisher Scientific (Pittsburgh, PA). Ethanol was obtained from Aaper Alcohols and Chemicals (Shelbyville, K.Y.). Sodium dodecyl sulfate (SDS), NaCl, $CaCl_2$, KCl, $MgSO_4$, Ethylene-bis (oxyethylenenitrilo) tetraacetic acid (EGTA), amino acids, sodium borate, 4,4’-Diisothiocyanatostilbene-2,2’-disulfonic acid disodium salt hydrate (DIDS), 4-Acetamido-4'-isothiocyanato-2,2'-stilbenedisulfonic acid disodium salt hydrate (SITS), veratrine, niflumic acid and tetrodotoxin were obtained from Sigma-Aldrich (St. Louis,

MO). All aqueous solutions were prepared with deionized water (Milli-Q system, Millipore, Milford, MA) and filtered through a 0.2 μm nylon membrane filter.

Neurotransmitter Derivatization

Amino acid standards were mixed and dissolved in ACSF. For all experiments, NDA was dissolved in a 50:50 mixture (v:v) of CH_3CN and 15 mM borate at pH 9.2, and cyanide was dissolved in 15 mM borate at pH 9.2. For on-line derivatization, ACSF, 5 mM NDA, and 10 mM cyanide were loaded into separate syringes and pumped into separate arms of a 0.25 mm bore PEEK micro-volume cross (Valco, Houston, TX) at 1.0, 0.7, 1.3 μL/min respectively (total flow rate was 3.0 μL/min) using two CMA/102 microdialysis pumps. The reaction mixture flowed through the fourth arm of the cross into a 100 cm long, 100 μm (i.d.) by 360 μm (o.d.) capillary that served as the reaction capillary. The reaction time was approximately 3 minutes. This capillary was connected to the on-line injection system (flow-gate) for direct interface to the CE system.

Flow-gated Interface and CE System

All separations were performed using 10 μm i.d. by 360 μm o.d. fused silica (Polymicro Technology, Phoenix, AZ) as the separation capillary. The total length of the separation capillary was 10 cm and the length from the inlet to the detection window was 8 cm. A -13 kV potential was applied as the separation voltage from a 1000R CZE power supply (Spellman, Plainview, NY). The separation buffer consisted of 15 mM NaH_2PO_4 and 30 mM SDS adjusted to pH 8.0 by 1 M NaOH. These conditions allowed amino acid derivatives to be resolved in less than 30 seconds, which enabled injection intervals to be set at 30 seconds. Each day, capillaries were rinsed for 15 min with 0.1 M NaOH and 20 min with separation buffer prior to experiments. Injections were

performed using a flow-gated interface described in detail previously (Bowser and Kennedy, 2001). Instrument control and data analyses were accomplished using a combination of DAC board (National Instruments) and Lab-View 6.1 (National Instruments). Electropherograms were batch post-processed using Cutter software to determine analyte peak heights (Shackman et al., 2004). Peaks were identified from previous experiments based on migration times and spiking of samples; analyte concentrations were determined via comparison against standards' calibration curves.

Laser-induced Fluorescence Detection System

The LIF detector was a commercial Axioskop 20 fluorescence microscope (Carl Zeiss, Hanover, MD) equipped with a DCP-2 microscope photometer system (CRG Precision Electronics, Houston, TX). This detector is similar to that described previously except for the wavelengths utilized (Hernandez et al., 1991; Lada and Kennedy, 1996). The 442 nm line of a 12 mW He-Cd laser (Liconix, Santa Clara, CA) was used as the excitation source. The excitation beam was reflected by a 480 nm dichroic mirror through the objective and focused onto the capillary through a 40x, 1.30 numerical aperture Fluar oil immersion lens (Carl Zeiss, Hanover, MD). The emitted fluorescence was collected by the objective, passed through the dichroic mirror and filtered with a 490 ± 10 nm emission filter (Chroma Technology, Rochingham, VT). The light was spatially filtered using the diaphragm on the photometer. Alignment of the laser and focusing onto the capillary were performed as described previously (Lada and Kennedy, 1996). A sample electropherogram is shown in Figure 4-1, both under control and ethanol-stimulated conditions.

Microdialysis and Surgeries for Anesthetized Rats

Microdialysis probes (o.d. 270 μm; active length 2 mm; cellulose membrane, 13,000 molecular weight cut-off) were constructed by the method of Bowser and Kennedy (Bowser and Kennedy, 2001). Each rat was anesthetized in accordance with animal care guidelines established for Dr. Kennedy' lab with an i.p. injection of ketamine/medetomidine (approximately 65 mg/kg and 0.5 mg/kg) and placed in a stereotaxic apparatus for surgical implantation of the microdialysis probe. The probe was positioned in the left side of the NAc using the following coordinates: +1.6mm anteroposterior, +1.7mm lateral, -6.2mm dorsoventral. The microdialysis probe was perfused with normal ACSF (145 mM NaCl, 2.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$) at 1 μL/min (CMA 102 microdialysis pump; 1mL Hamilton series 1001 gastight syringe) and then was inserted into the NAc. The rats remained anesthetized in the stereotaxic apparatus for the duration of the experiment. In the first series of experiments the amount of Na^+ was varied in the ACSF to characterize the osmotic response. After 90 minutes to allow for stabilization of amino acids levels (Smith et al., 2003), basal levels were sampled for 30 minutes (60 electropherograms) followed by a change in the Na^+ concentration of the ACSF after which another 60 electropherograms were collected. After the hypoosmotic shock, normal ACSF was perfused through the probe for 30 minutes. This procedure was repeated two more times.

Once conditions were determined that induced a selective, reproducible two-fold increase in taurine efflux, the effects of osmotically-activated channel inhibitors were tested. After 90 minutes of equilibration after probe insertion, basal levels were measured for 30 minutes (normal ACSF), followed by 30 minutes of recording under hypoosmotic

conditions and then 30 minutes again of normal ACSF. After this, an osmotic inhibitor dissolved in normal ACSF was perfused through the probe for 30 minutes followed by 30 minutes of hypoosmotic ACSF with the osmotic inhibitor present.

Microdialysis and Surgeries for Freely-moving Animals

Each rat was anesthetized with ketamine/medetomidine as before 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 the NAc using the following coordinates: +1.6mm anteroposterior, +1.7mm lateral, -6.2mm dorsoventral. The guide cannula was secured to the skull with dental cement anchored by three stainless steel screws. After surgery, each rat was housed separately and allowed to recover for 7-10 days with daily handling to facilitate probe insertion. At the conclusion of each experiment, the rats were killed and the brains removed to verify probe placement.

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 (Pettit and Justice, 1989). The probe was perfused with ACSF, lowered into NAc, and allowed to equilibrate for 90 minutes as before. Rats were randomly divided into four groups based on the following manipulations to the ACSF: normal ACSF (control); 1 μM TTX; 4 mM SITS; or 0 mM Ca^{+2} plus 2 mM EGTA. The effect of manipulation of the ACSF on basal levels of amino acids was determined over 30 minutes. Following this, saline (0.9%, i.p., 12.5 ml/kg body weight) was injected and samples were collected for 60 minutes. Next, 2.5 g/kg EtOH (20% w/v in saline, i.p.) was injected and amino acid

levels were monitored for 90 minutes. The efficacy of 1 μM TTX was determined by perfusing veratrine through the probe at the end of experiments in the control rats.

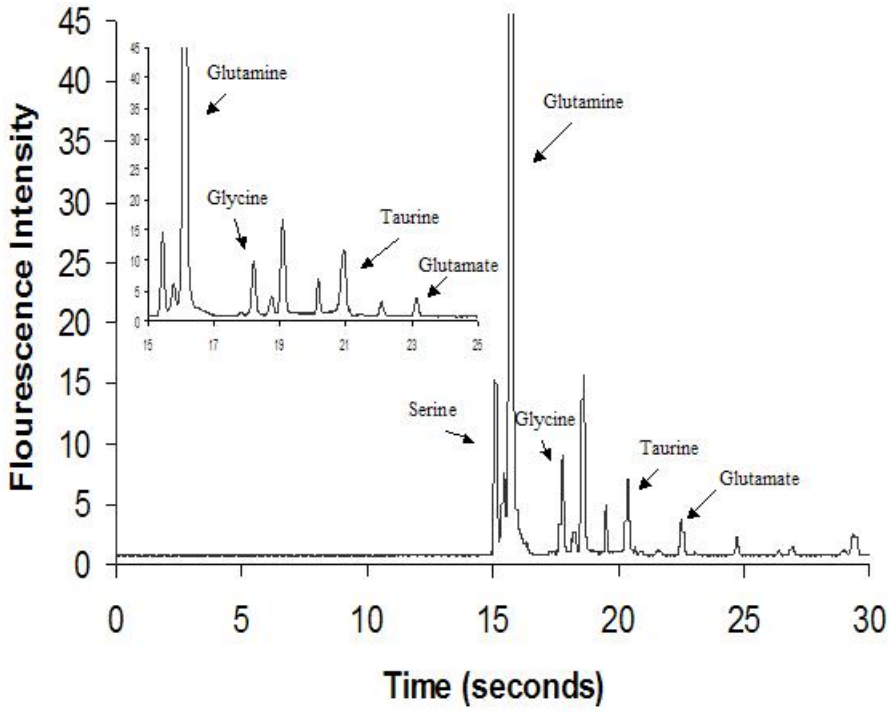

Figure 4-1 A typical electropherogram from dialysate of an ethanol-naïve rat. Inset, is a representative electropherogram from rat 10 minutes after i.p. 2.5 g/kg ethanol

Results

Efflux Due to Change in Na^+

Our first experiments examined the effect of osmotic disturbance, caused by decreasing Na^+ in the ACSF, on the efflux of taurine. When the Na^+ concentration was decreased from 145 mM to 125 mM, a two-fold increase in taurine efflux was observed (Figure 4-2), while other amino acids were unaffected (Figures 4-2 and 4-3). When Na^+

concentration was further reduced to 100 mM, a 10-fold increase in taurine efflux occurred but glycine efflux was also increased 65% above baseline and serine efflux was decreased by 20% (Figure 4-3). Glutamate and aspartate remained unchanged. A 25-fold increase in taurine efflux was observed when the Na^+ concentration was lowered to 75 mM. Similarly, glycine efflux increased to 100% above baseline. In addition, glutamate and aspartate efflux increased to 25% and 20% above baseline, respectively. However, serine efflux did not change from the basal levels. When the Na^+ concentration was reduced to 50 mM, taurine efflux was approximately 25 times the level measured at 145 mM Na^+ while glycine efflux increased 2-fold and glutamate efflux increased by 50% above basal values. No significant change was observed in the other amino acids. Interestingly, at the lower Na^+ concentrations of ACSF (100 mM, 75 mM, 50 mM) a spike of glutamate efflux preceded the taurine efflux (Figure 4-4 and Figure 4-5). The glutamate spike was directly proportional in magnitude to the taurine efflux at the lower Na^+ concentrations and preceded the taurine efflux by one run (30 seconds), which could indicates a possible involvement glutamate-taurine interaction (see discussion).

Efflux Due to 125mM Na^+

Previous studies have indicated that ethanol injections can induce a selective increase in taurine efflux and at the higher dosage tested (2 g/kg) this efflux was approximately two-fold (Smith et al., 2004) (see CHAPTER 3), therefore the effects of slightly hypo-osmotic conditions (125 mM Na^+) appeared to be the most similar in magnitude and selectivity as the ethanol-induced response. The reproducibility of this response was next examined. The 125 mM Na^+ concentration was perfused through the dialysis probe during three consecutive periods separated by perfusion with normal ACSF. The mean of last twenty samples of the first basal run were used to convert

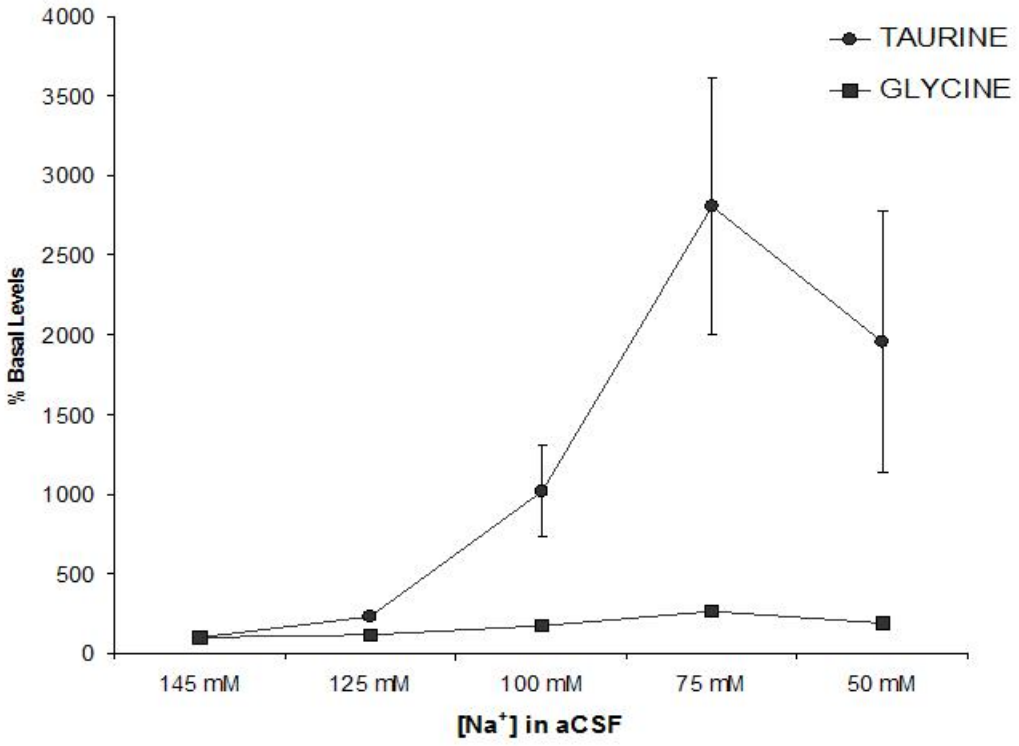

Figure 4-2 Taurine and glycine efflux in response to variations in ACSF osmolarity. Alteration of sodium in ACSF to 125mM produced taurine efflux 228± 25% of basal levels (N= 2-4). In contrast, glycine efflux was unaffected by the 125mM ACSF (110± 4.2% of basal levels, N= 2-4). Reducing sodium caused a larger but non-selective increase in both amino acids. Twenty values were taken from the plateau to represent the max change. Data are expressed as a percent of basal relative fluorescence (the last twenty samples of the first basal run).

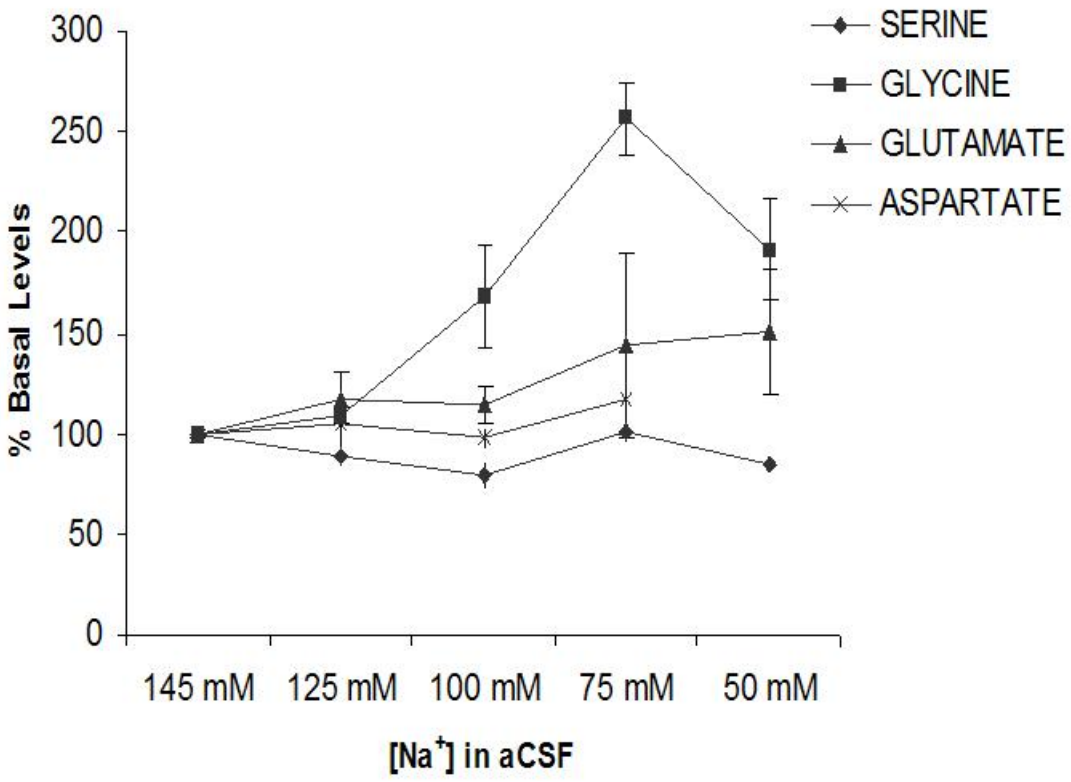

Figure 4-3 The efflux of aspartate, glycine, and serine in response to the alterations in the sodium in ACSF. The efflux due to the change in osmolarity was not as great as that observed in the taurine efflux. Microdialysate was sampled and data calculated as in Figure 4-2.

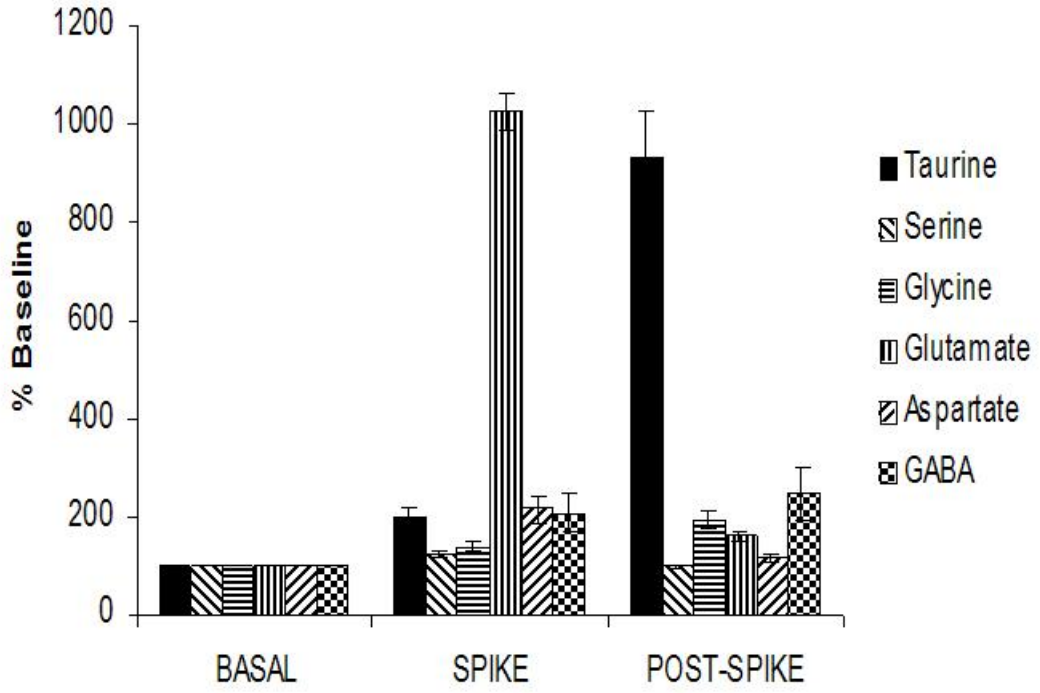

Figure 4-4 Glutamate spike seen due to hypoosmotic ACSF containing 100mM Na^+. Data are represented as % of basal. Basal was calculated from the first 8-10 samples after the change of ACSF from normal to hypoosmotic. The spike of glutamate is the average of two electropherograms, the spike of glutamate usually occurs for one electropherogram. Post-spike is the average of 8-10 electropherograms immediately after the rapid glutamate increase, which lasted at most for two electropherograms. Further decreasing the Na^+ produced a greater increase in the glutamate spike which was directly proportional to the taurine efflux (N=4).

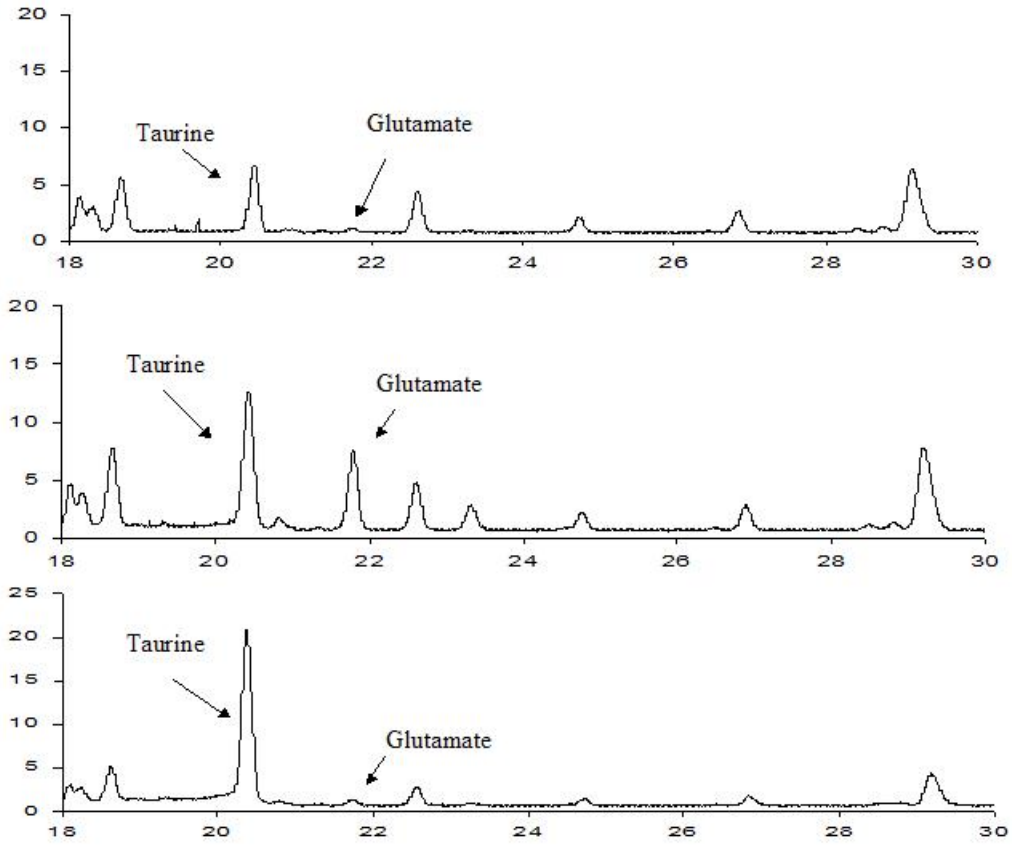

Figure 4-5 Representative electropherograms of the glutamate spike. Each electropherograms are 30 sec apart.

the relative fluorescence into percent of basal levels. This mean value corresponded very well with the basal values obtained when normal ACSF was being perfused (between hypo-osmotic conditions). All three periods of hypo-osmolarity produced consistent two-fold increases of taurine efflux (Figure 4-6) with no effect on other amino acids (see Figure 4-3 at 125mM). Based on these characteristics, the 125 mM Na^+ concentration was chosen as the osmotic challenge in all subsequent studies testing the ability of different osmotic inhibitors to block taurine efflux.

The Effect of Osmotic Inhibitors on Efflux

Osmotic inhibitors were characterized based on their ability to block hypo-osmotically-induced taurine efflux while added to the dialysate media. 4-Acetamido-4'-isothiocyanato-2,2'-stilbenedisulfonic acid disodium salt hydrate (SITS) at 4 mM blocked

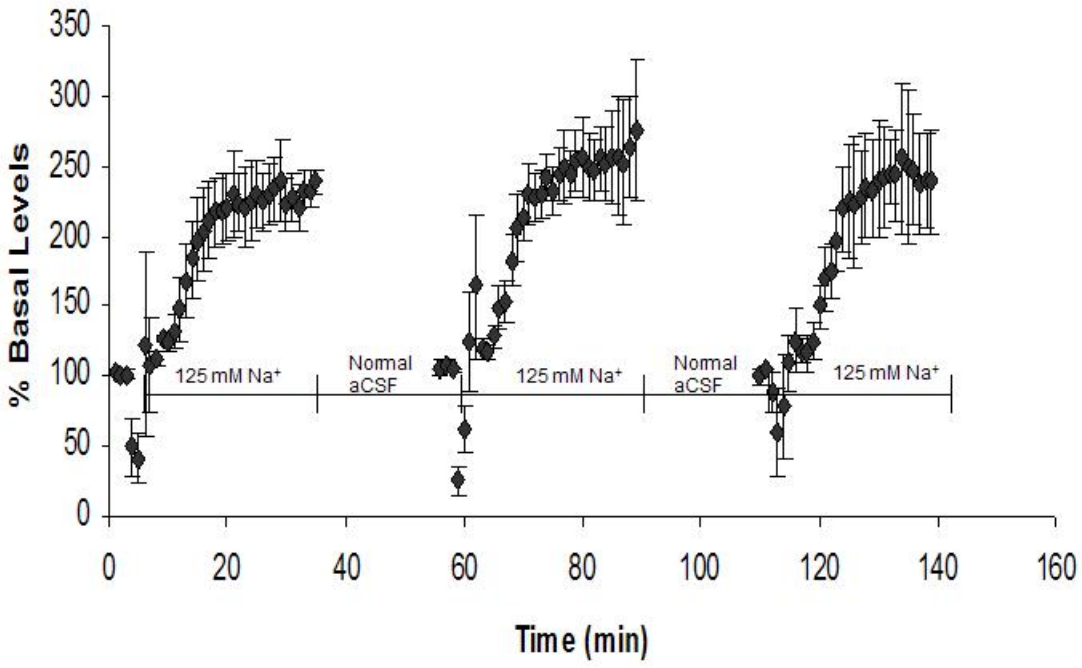

Figure 4-6 The response of taurine efflux to repeated exposure of reduced sodium (125mM) ACSF in anesthetized Sprague-Dawley rats. Between each perfusion of 125mM ACSF, normal (145mM sodium) ACSF was perfused for 40 minutes, N=4. Data are expressed as a percent of basal relative fluorescence (the last twenty samples of the first basal run).

the hypo-osmotic release of taurine without affecting the basal efflux (Figure 4-7) of taurine, glycine, aspartate, serine or glutamate. However, a 20% increase in glycine efflux and a 150% increase in aspartate efflux were seen in response SITS during hypo-

osmotic conditions (Figure 4-7). Glutamate efflux in response to hypo-osmotic media was blunted when SITS was added although not significantly. ANOVA revealed a significant increase in taurine caused by the hypo-osmotic condition that was decreased by SITS ($F(2,8) = 83$; $p < 0.001$).Higher dosages of SITS (4 or 6 mM) also increased basal taurine levels (data not shown). Other osmotic inhibitors tested were not as effective as SITS. Niflumic acid at 500 μM caused at 350% increase in the basal release of taurine and a 450% increase in the hypo-osmotically stimulated release of taurine (data not shown). DIDS, which is similar in structure to SITS, was ineffective at blocking hypo-osmotic stimulated release of taurine and at higher concentrations stimulated the basal release of taurine (data not shown).

Ethanol-induced Efflux with SITS,TTX or Ca^{2+}-free Conditions

Acute injection of 2.5 g/kg i.p. of ethanol increased taurine levels significantly over time (Figure 4-8; $F(109,436) = 14.10$; $p<0.001$). Approximately seven minutes after the ethanol injection, taurine levels begin to rise reaching a maximum of 200% above basal levels by 10 minutes after the injection. Taurine levels gradually descended to pre-injection levels by about 30 minutes after ethanol injection. Other amino acids were only slightly affected by the ethanol injection, although the 10-15% decreases in serine (Figure 4-9) and glycine (Figure 4-10) over time were statistically significant ($F(109,436) =$ 11.14 and 12.52, respectively; $p<0.001$). Glutamate (Figure 4-11) and glycine (Figure 4-10) also increased slightly approximately 5 minutes after the ethanol injection but this effect was not different from that seen after a saline injection (see below). Next, ethanol-induced taurine efflux was measured in the presence of SITS, TTX or Ca^{2+}-free conditions (Figure 4-8). 4 mM SITS reduced the maximal

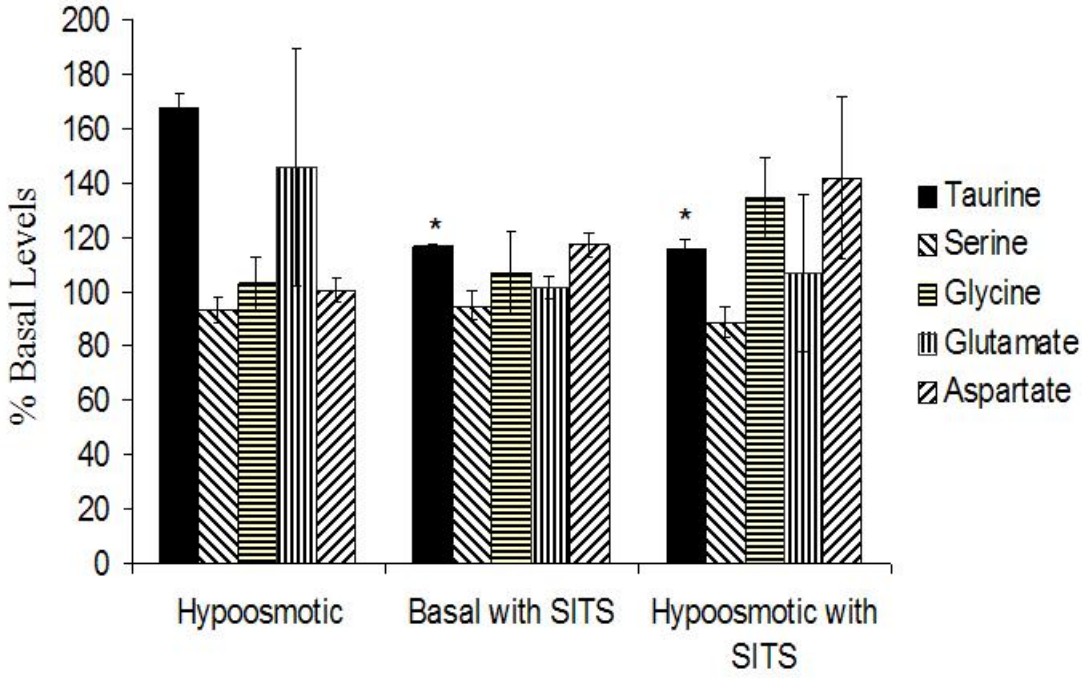

Figure 4-7 The effects of osmotic inhibitors on taurine, serine, glycine, glutamate, and aspartate efflux in the NAc to 125 mM NaCl in the ACSF in anesthetized Sprague-Dawley rats. After 90 minutes of perfusing normal ACSF, the sodium content of the ACSF was reduced to 125mM which caused an increase in taurine efflux two-fold. This was followed by 30 minutes of perfusion of normal ACSF which was followed by 30 minutes of perfusion of normal ACSF with SITS 4mM. The basal perfusion with SITS was followed by perfusion with 125mM reduced sodium ACSF with drug. Twenty values were taken from the plateau of each perfusion to represent the max change. Data are expressed as a percent of basal relative fluorescence (the last twenty samples of the first basal run), N=2-3. A significant ($p<0.001$) difference is observed between the hypoosmotic efflux of taurine and the basal with SITS and hypoosmotic with SITS.

ethanol-evoked taurine efflux by 50% 7 minutes after injection and 80% 10 minutes after injection ($F(109,872) = 1.58$; $p<0.001$). Ca^{2+}-free conditions also significantly reduced the ethanol-induced taurine efflux ($F(109,654) = 2.85$; $p<0.001$) with a similar time course as did SITS. TTX at 1 μM did not affect the ethanol-induced efflux of taurine. The efficacy of this concentration of TTX in the probe was confirmed in separate experiments comparing the effects of veratrine alone in the probe and veratrine plus TTX. Veratrine caused a 35-fold increase in glutamate efflux, which was completely blocked by the addition of the TTX (table 4-1). Veratrine stimulated taurine release was also reduced by the addition of TTX (table 4-1).

The different ACSF conditions had little effect on the ethanol-induced changes in other amino acids besides taurine. TTX and 4 mM SITS caused a steady decrease in efflux of serine (Figure 4-9) and glycine (Figure 4-10) to about 70-80% of basal. However, the Ca^{2+}- free ACSF did not affect the efflux of glycine or serine, which remained very close to control values. In contrast, glutamate efflux (Figure 4-11) was not affected by TTX and was increased only slightly in response to the 4 mM SITS. However, the Ca^{2+}- free ACSF did decrease glutamate efflux significantly ($F(109,763) = 0.28$; $p<0.001$).

Saline injections (Figure 4-12) did not produce any significant changes in amino acid levels. Approximately 5 minutes following the saline injection, a slight increase in the efflux of every amino acid in each condition was observed (less than 10% increase). During collection of the saline data (1 hour) SITS and Ca^{2+}-free ACSF with EGTA did not change the efflux of taurine, glutamate, glycine, or serine. However, TTX did cause a gradual non-significant decrease in the efflux of taurine, glycine and serine.

Probe placements in all experiments were confirmed by visual inspection of coronal sections (25um) and verified by the atlas of Paxinos and Watson (Paxinos and Watson, 1982)(Figure 4-13).

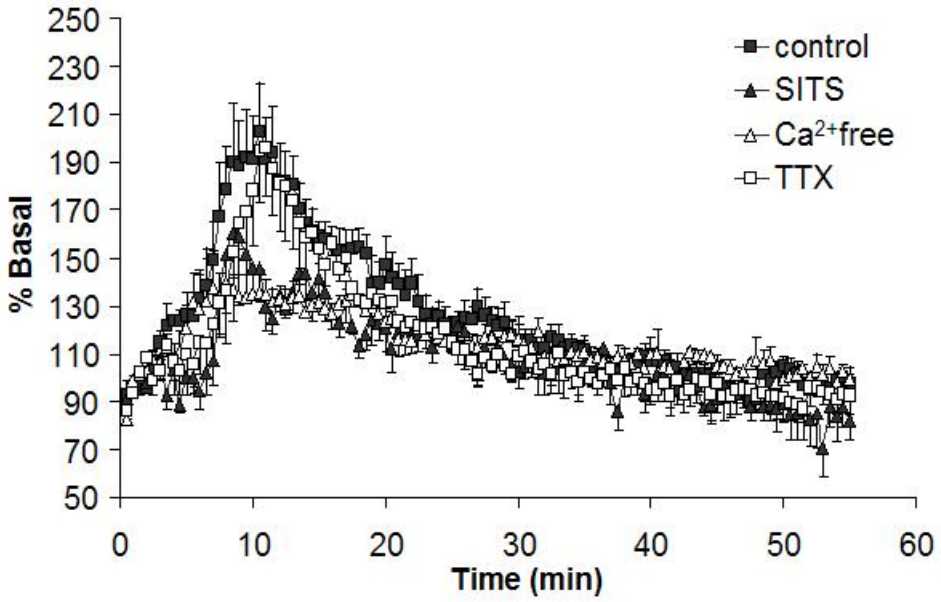

Figure 4-8 Taurine levels in NAc after a 2.5 g/kg injection of ethanol in freely-moving Sprague-Dawley rats with either normal ACSF (N = 5) or Ca-free ACSF with 2mM EGTA (N=3) or 1uM TTX (N=4) or 4mM SITS (N=4) in the dialysis probe. Data are expressed as a percent mean ± SEM relative fluorescence of the first 10-15 samples for each treatment (basal). There was a 7.5-min delay after injection for dialysate from NAc to reach the end of the capillary for detection. A significant ($p<0.001$) interaction over time with the control taurine group, as well as a significant ($p<0.001$) interaction with control versus SITS and Ca^{2+}- free ACSF groups.

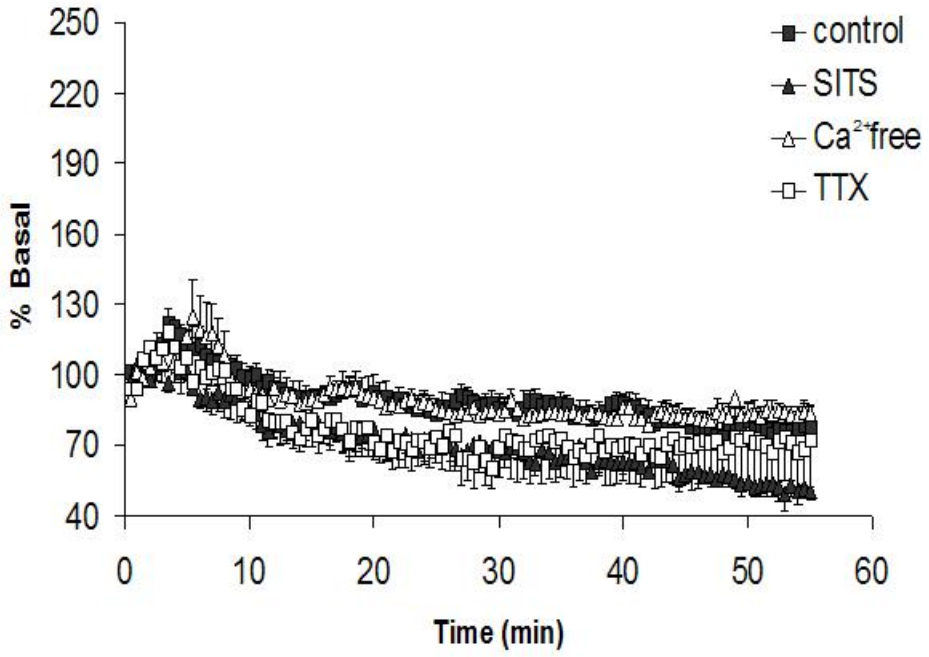

Figure 4-9 Serine levels in NAc after a 2.5 g/kg injection of ethanol in freely-moving Sprague-Dawley rats with either normal ACSF (N = 5) or Ca-free ACSF with 2mM EGTA (N=3) or 1uM TTX (N=4) or 4mM SITS (N=4) in the dialysis probe. Microdialysate was sampled and data calculated as in Figure 4-8.

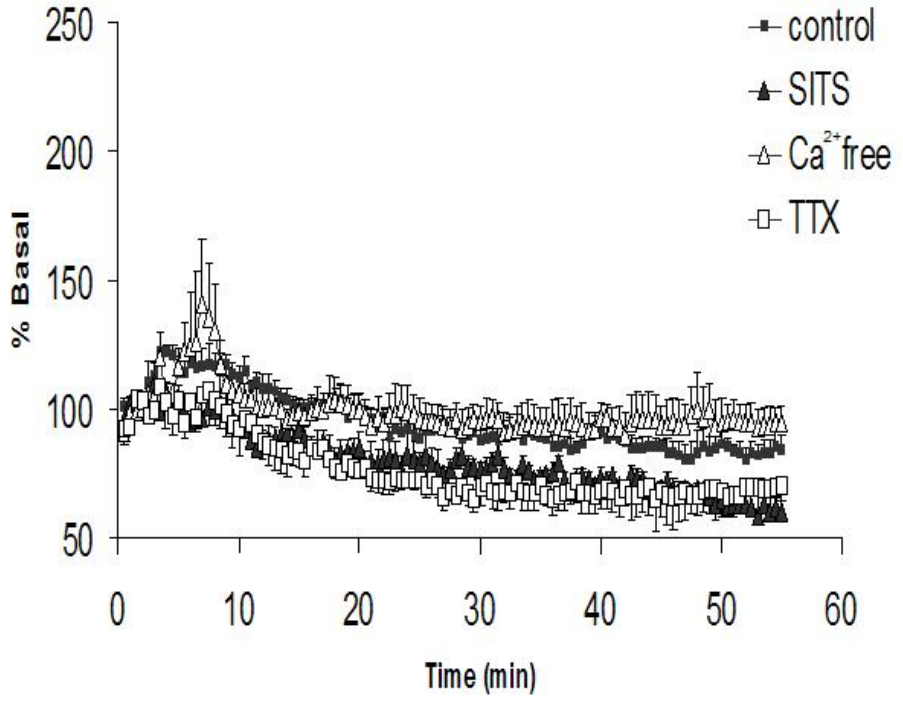

Figure 4-10 Glycine levels in NAc after a 2.5 g/kg injection of ethanol in freely-moving Sprague-Dawley rats with either normal ACSF (N = 5) or Ca-free ACSF with 2mM EGTA (N=3) or 1uM TTX (N=4) or 4mM SITS (N=4) in the dialysis probe. Microdialysate was sampled and data calculated as in Figure 4-8.

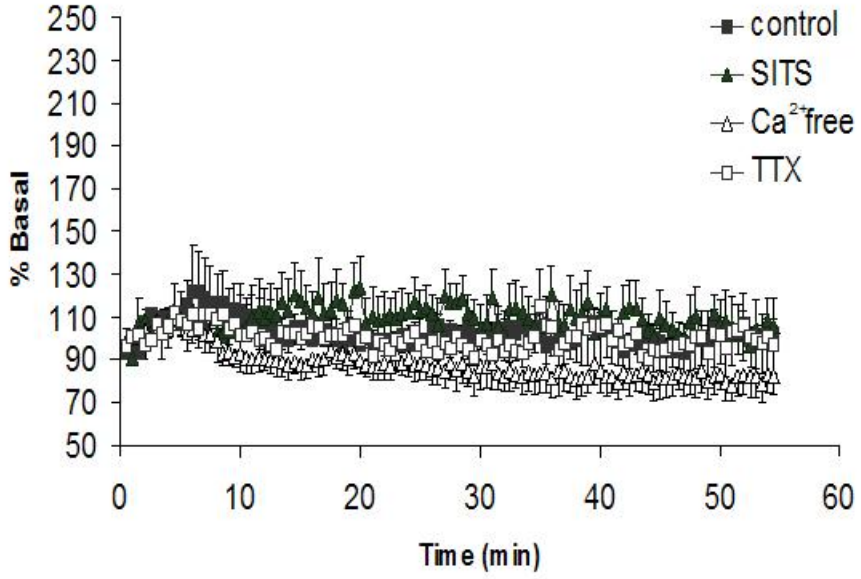

Figure 4-11 Glutamate levels in NAc after a 2.5 g/kg injection of ethanol in freely-moving Sprague-Dawley rats with either normal ACSF (N = 5) or Ca-free ACSF with 2mM EGTA (N=3) or 1uM TTX (N=4) or 4mM SITS (N=4) in the dialysis probe. Microdialysate was sampled and data calculated as in Figure 4-8. Ca-free ACSF with 2mM EGTA caused a significant decrease in glutamate efflux ($F(109,763) = 0.28$; $p<0.001$).

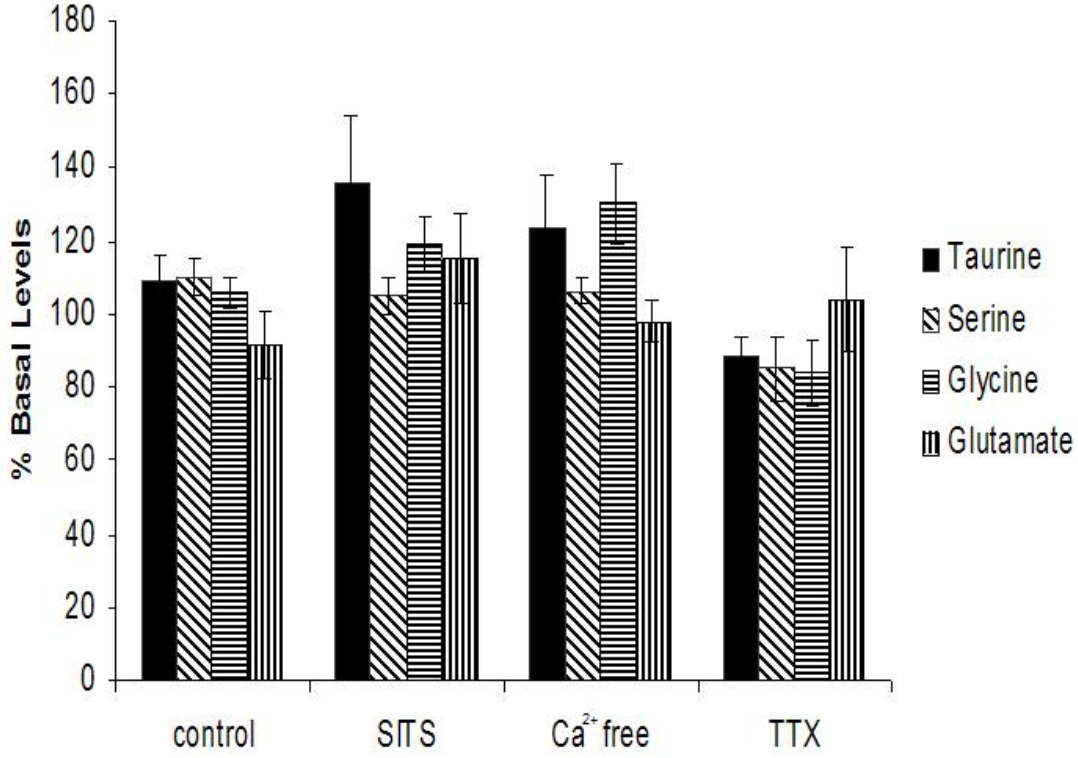

Figure 4- 12 Amino acid levels after saline injection in freely-moving Sprague-Dawley rats with either normal ACSF (N = 5) or Ca-free ACSF with 2mM EGTA (N=3) or 1uM TTX (N=4) or 4mM SITS (N=4) in the dialysis probe. Data were calculated as a percent of the mean ± SEM relative fluorescence of the first 10-15 samples. The average of 50 electropherograms (from 20 to 70) were used to represent the change in efflux.

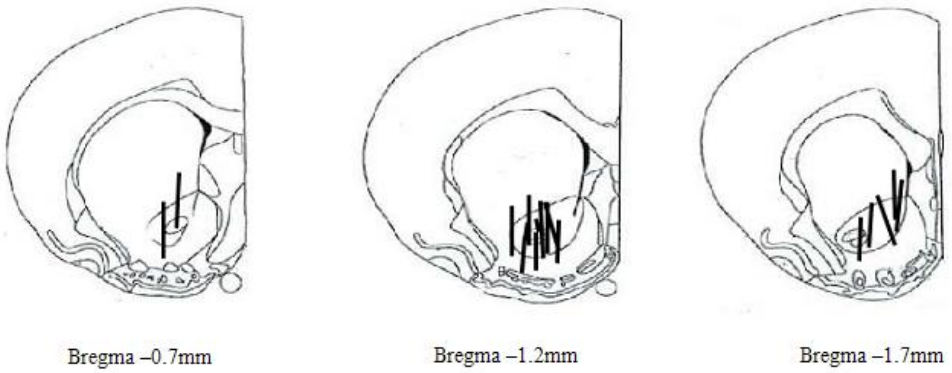

Figure 4-13 Coronal sections showing probe placements in the NAc (N=15).

Amino Acids	Basal	Veratrine	TTX
Taurine	103.9±5.0	402.9 ±22.2	210.4 ±24.5
Glutamate	104.1 ±4.9	2362.4 ±71.3	157.3 ±43.7
Serine	103.8 ±3.0	113.6 ±8.3	50.8 ±4.5
Glycine	103.5 ±3.8	196.5 ±11.3	71.7 ±4.7

Table 4-1 The efflux of several amino acids due to veratrine (200ug/ml) and TTX (1uM). Normal ACSF was perfused for 1 hour before being switched to ACSF containing veratrine and 30 minutes (60 electropherograms) were collected. Next, the ACSF was switched to veratrine with TTX and 30 minutes (60 electropherograms) were collected. Twenty samples were taken from the plateau of the veratrine and veratrine/TTX perfusion. Data were calculated as a percent of the mean ± SEM relative fluorescence of the first 6-10 samples (N=3).

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Discussion

We have recently reported that a cumulative dosing regimen of ethanol evoked a regionally selective efflux of taurine in the NAc (Smith et al., 2004) (see CHAPTER 3). In the present study, the mechanism of ethanol-induced efflux of taurine and other amino acids in the NAc was studied by employing CE-LIF analysis which offers considerably higher temporal resolution than HPLC-based assays (Shou et al., 2004). In response to a 2.5 g/kg ethanol injection, taurine efflux increased nearly two-fold while the levels of glutamate, glycine and serine were unaffected or slightly decreased by ethanol injection. Saline injection did not change the levels of any of the amino acids measured in the same animals. These results agree with previous experiments in which larger volume samples were analyzed by HPLC (Dahchour et al., 2000; Dahchour et al., 1994; Dahchour et al., 1996; De Witte et al., 1994; Quertemont et al., 2000). The mechanism of ethanol-induced taurine efflux is unknown and further experiments in this study were designed to ascertain if taurine efflux is caused by neuronal depolarization or an osmotically-mediated event.

Ethanol could be affecting neuronal depolarization rates thereby subsequently affecting the release of any of a number of neurotransmitters in NAc. Even though there is a lingering debate as to whether taurine fulfills the criteria of a neurotransmitter, several studies have shown that taurine can be released by neuronal depolarization (Bockelmann et al., 1998; Dutton et al., 1991; Huxtable, 1989; Kontro and Oja, 1987a; Oja and Saransaari, 2000; Philibert et al., 1989a; Saransaari and Oja, 1994; Schousboe et al., 1990; Shain and Martin, 1990). However, TTX did not affect ethanol-induced taurine efflux. This result would seem to indicate that ethanol-induced taurine

efflux is not due to depolarization of neurons resulting in taurine release by synapses in the vicinity of the probe.

None of the other amino acids examined in this study were affected by TTX, regardless of whether efflux was measured under basal conditions or after i.p. injection of either ethanol or saline. Previous studies have also concluded that TTX does not affect the basal release of amino acids when measured using microdialysis (Lada et al., 1998; Semba et al., 1995; Timmerman et al., 1999). Several studies have also concluded that blockade of voltage dependent Na^+ channels failed to decrease extracellular glutamate levels (Baker et al., 2002; Westerink et al., 1987). These results can be explained by the fact that although a vesicular component contributes to basal glutamate levels, it is not in response to action potentials and that the major contributor is from non-vesicular release from the cystine-glutamate antiporter (Baker et al., 2002). Furthermore, In order to test whether this was due to methodological constraints of reverse dialysis of the TTX, we confirmed that reverse dialysis of veratrine into NAc caused a tremendous increase in glutamate efflux and a smaller increase in glycine and taurine (Holopainen et al., 1989; Jacobson and Hamberger, 1984; Patterson et al., 1995; Semba et al., 1995). The addition of TTX reduced veratrine-stimulated amino acid levels to their basal values. These results indicate that the dose of TTX used was capable of blocking neuronal depolarization. Consequently, ethanol-induced taurine release appears to be TTX insensitive.

Although taurine has numerous functions, it is predominately known as a osmolyte (Huxtable, 1989; Moran et al., 1994; Olson and Li, 2000; Pasantes-Morales et al., 1990; Solis et al., 1988a). When cells are confronted with hypotonic media, initial swelling occurs followed by the efflux of many organic osmolytes, a process known as

regulatory volume decrease (Hoffmann, 2000; Kimelberg and Frangakis, 1985). The efflux of osmolytes that are sensitive to anion channel inhibitors (Kirk, 1997) occurs through VSOAC (Jackson and Strange, 1993). Isotonic ethanol has been shown to induce cell swelling (Aschner et al., 2001; Kimelberg et al., 1993) and the subsequent release of several amino acids involved in cell volume regulation (Aschner et al., 2001), specifically taurine (Aschner et al., 2001; Kimelberg et al., 1993). Therefore, we tested the hypothesis that the ethanol-induced taurine release observed *in vivo* was due to a response to ethanol-induced hypo-osmolarity and mediated by VSOAC.

The osmotically-dependent release of taurine is believed to occur through the VSOAC because several pharmacological agents that block this channel also block the osmotic release of taurine in cell lines (Bothwell et al., 2002; Bres et al., 2000; Jackson and Strange, 1993; Kimelberg et al., 1990; Lambert and Hoffmann, 1994; Sanchez-Olea et al., 1996; Strange et al., 1996). Several *in vivo* studies have also concluded that many Cl^- channel blockers can inhibit the osmotic release of taurine (Estevez et al., 1999; Solis et al., 1988b). From these studies, it appeared that the stilbene disulfonates (SITS, DIDS) and niflumic acid would be the most effective, if ethanol-stimulated efflux occurred via VSOAC.

In order to test the efficacy of these inhibitors under microdialysis conditions, taurine efflux was stimulated by reducing the Na^+ concentration in the ACSF. As Na^+ was reduced, a dose-dependent increase in the release of taurine was observed consistent with several studies showing that the osmotic release of taurine displays a dose-dependency (Deleuze et al., 1998; Olson and Li, 2000; Pasantes-Morales et al., 1990; Solis et al., 1988a). The reduction of Na^+ from the media could alter other systems that rely on Na^+

balance such as the taurine transporter. However, when sucrose was utilized to change the osmolarity of the media, the hypo-osmotic efflux of taurine was very similar to that observed when Na^+ is reduced (Bres et al., 2000). The osmotic release of taurine is believed to be Na^+ independent (Pasantes-Morales and Schousboe, 1997). When Na^+ concentration was reduced from 145mM to 125mM, taurine efflux increased approximately two-fold which correlated well with the magnitude of taurine release in response to a 2.5g/kg ethanol i.p. From previous studies the reduction of Na^+ from 145mM to 125mM correlates well with a reduction in osmotic pressure by approximately 15% (Sakai and Tosaka, 1991). This reduction in osmotic pressure is physiologically relevant, although borderline a pathological condition (Deleuze et al., 1998). Several studies that examine the release of osmolytes from the supraoptic nucleus use a similar reduction in osmotic pressure (Bres et al., 2000). In addition, at this concentration, taurine was reliably and selectively released compared to the other amino acids, similar to findings of other studies that measured the taurine response to stimuli that mildly affect the osmotic status of cells (Miyata et al., 1997).

Interestingly, a transient glutamate spike proportional in magnitude to the taurine efflux occurred reproducibly about 30 seconds preceding the increase in taurine. The glutamate spike quickly returned to basal levels after which taurine levels began to rise, which would seem to indicate a possible involvement of glutamate in controlling taurine levels in response to osmotic events. Several studies have indicated a possible glutamate receptor activation that modifies taurine release (Bianchi et al., 1996; Del Arco and Mora, 1999; Magnusson et al., 1991; Menendez et al., 1989; Menendez et al., 1990; Oja and Saransaari, 2000; Saransaari and Oja, 2000a; Segovia et al., 1997; Shibanoki et al., 1993).

However, our findings are the first indication of such a short-lived glutamate response to altered osmolarity that might then lead to an increase in taurine levels. Clearly, these findings would not be possible without the high temporal resolution provided by the CE-LIF methodology presently used. An alternative explanation for the spike in glutamate followed by a rise in taurine could be that removing the Na^+ from the ACSF could have caused neuronal depolarization in addition to a change in osmotic conditions. A pathway that could be involved in this scenario would be the excitatory glutamatergic input to the medium spiny neurons of the NAc, which arises from the ventral CA1/subiculum region of the hippocampus (Groenewegen et al., 1987; Brog et al., 1993). If this were the case however, it would be expected that other neurotransmitters, such as GABA and glycine, might also have been released which was not the case.

The VSOAC is assumed to be the channel involved in the release of taurine due to osmotic stress; therefore, known inhibitors that block the efflux of organic solutes through this channel should be able to block taurine efflux in the NAc. In the current findings, the anion channel inhibitor most effective for blocking hypo-osmotically induced taurine efflux was 4 mM SITS. However, other potent inhibitors of VSOAC such as DIDS (Bothwell et al., 2002; Estevez et al., 1999; Hoffmann, 2000; Sanchez-Olea et al., 1991), NPPB (Bres et al., 2000; Nilius et al., 1997; Okada, 1997; Sanchez-Olea et al., 1996) and niflumic acid (Estevez et al., 1999; Sanchez-Olea et al., 1996) did not inhibit the osmotically-induced efflux in our experiments. The reason for this discrepancy in action of the VSOAC inhibitors is not clear. DIDS and SITS are structurally similar (disulfonic stilbenes) and have a similar method of action by steric hindrance and covalent modification of the Cl^- channel (Cabantchik and Greger, 1992). In addition,

SITS and DIDS have also been shown to block ATP-sensitive K^+ channels in mycocytes (Furukawa et. al., 1993) and modify the calcium release in muscle (Hill and Sitsapesan, 2002). More importantly SITS and DIDS are primary used as potent inhibitors of band-3-mediated anion exchange (Aper, 1991), with an IC_{50} that spans from 0.8µM to 500µM (Cabantchik and Greger, 1992). Despite this similarity in action, DIDS was ineffective at reducing hypo-osmotic taurine efflux at any concentration. It is possible that DIDS was not delivered to the brain region as effectively as SITS due to degradation, absorption to the instrument components, or mass transport differences through the brain tissue. It is also possible that molecular variants of the VSOAC with different pharmacological properties are responsible for the selective response to SITS in the NAc *in vivo*, however no evidence exists for molecular variants of the VSOAC in the NAc. A similar observation was made where the osmodependent taurine permeable channels of SON astrocytes displayed different pharmacological properties from VSOAC (Bres et al., 2000). In the same study, they concluded that perhaps VSOAC had different subunit expression and continued to explore gene expression of potential candidates that might encode VSOAC. In a recent study the ClC-3, a member of the ClC voltage-dependent Cl^- channels, was disrupted in transgenic mice (Yamamoto-Mizuma et al., 2004). It was concluded that the VSOAC was still very similar to the wild-type, however VSOAC did display several differences to blockade by ClC-3 antibodies and insensitive to intracellular ATP and Mg^+. Experiments should be designed to address the identity of this channel by using molecular techniques to study the constituents involved as well as a more fully developed pharmacological profile. Nevertheless, it is concluded that SITS is

effective at blocking the selective taurine concentration increase seen in response to hypo-osmotic challenge through its known action as a VSOAC inhibitor.

The ethanol-induced taurine efflux was found to be Ca^{2+}-dependent (Figure 4-8). Although Ca^{2+}-dependent taurine release in response to depolarization has been well documented (del Rio et al., 1987; Holopainen et al., 1989; Oja and Kontro, 1989; Philibert et al., 1988; Solis et al., 1986), this Ca^{2+}-dependence is not likely to be due to neuronally-derived exocytosis because TTX was ineffective at reducing taurine efflux. The Ca^{2+}-dependence is also not likely due to a direct effect on the volume-sensitive Cl^- channel that is involved in the osmotically-induced release of taurine because this channel has been shown to be Ca^{2+}-independent (Hoffmann, 2000; Pasantes-Morales and Morales-Mulia, 2000); however, a new class of Cl^- channels has been observed to be Ca^{2+}-dependent as well as inhibited by DIDS (Fuller et al., 2001). Therefore, it is reasonable to postulate that a novel volume-sensitive channel exists in the NAc that is Ca^{2+} sensitive and is involved in taurine release. If this hypothesis is correct, it may also explain why certain known inhibitors failed to reduce osmotically-induced taurine efflux in NAc (see above). Ca^{2+} may also be involved more distal to the anion-channel mediated taurine efflux release, which would also explain why certain known inhibitors failed to reduce osmotically-induced taurine efflux in NAc. Recent studies suggest the osmosensitive release of taurine is partially Ca^{2+}-dependent since cytosolic Ca^{2+} increases in response to cell swelling (Morales-Mulia et al., 1998; Pasantes-Morales and Morales-Mulia, 2000) and this Ca^{2+} has been shown to be necessary for optimal taurine efflux (Rosso et al., 2004) via the calmodulin/calmodulin kinase pathway (Cardin et al., 2003).

Previous studies have indicated that basal taurine release is increased in the absence of Ca^{2+} (Rogers et al., 1991) and is diminished in the presence of high potassium in a Ca^{2+} free buffer (Korpi and Oja, 1983). However, the saline injection and basal data indicate that taurine efflux was unaffected by the omission of Ca^{2+} as there was no observable change in the percentage of efflux.

Several studies indicate that ethanol-induced taurine efflux is not solely due to osmoregulation. For example, brain ethanol levels after an ethanol injection failed to correlate with the release of taurine in the hippocampus (Lallemand et al., 2000). Most recently our lab has perfused various concentrations of ethanol directly into the NAc (Smith et al., 2004). Even 1 M ethanol delivered by reverse dialysis did not evoke a change in taurine efflux. Perhaps ethanol-induced taurine efflux is not a direct action of ethanol but rather ethanol exerting its effect elsewhere. A pathway that could be involved is the excitatory input to the NAc from the ventral subiculum of the hippocampus (Groenewegen et al., 1987; Brog et al., 1993). The glutamatergic afferents from the ventral subiculum to the NAc has been shown to influence firing rate and activity of dopamine neurons in ventral tegmental area (Floresco et al., 2001). In this scenario, ethanol could induce these glutamatergic afferents from the ventral subiculum of the hippocampus to the NAc, which would release glutamate. Glutamate could then induce neural cells in the NAc to release taurine via an osmoregulation pathway. Interestingly, a transient glutamate spike proportional in magnitude to the taurine efflux occurred reproducibly about 30 seconds preceding the increase in taurine. The glutamate spike quickly returned to basal levels after which taurine levels began to rise, which would seem to indicate a possible involvement of glutamate in controlling taurine levels in

response to osmotic events. Several studies have indicated a possible glutamate receptor activation that modifies taurine release (Bianchi et al., 1996; Del Arco and Mora, 1999; Magnusson et al., 1991; Menendez et al., 1989; Menendez et al., 1990; Oja and Saransaari, 2000; Saransaari and Oja, 2000a; Segovia et al., 1997; Shibanoki et al., 1993) and in microdialysis experiments the perfusion of NMDA inhibited ethanol-induced taurine efflux in the hippocampus (Lallemand et al., 2000). However, TTX did not block ethanol-induced taurine efflux, indicating a non-neuronal depolarization mechanism. Perhaps an interaction exists between ethanol-induced taurine efflux and glutamate that does not involve the fast-acting sodium channels that TTX blocks. Our findings are the first indication of such a short-lived glutamate response to altered osmolarity that might then lead to an increase in taurine levels. Future experiments will address the nature of this glutamate spike as well as its relationship to the rise in taurine following it.

The current results indicate that taurine is released in response to ethanol due to the osmotic disturbance attributed to ethanol. Taurine efflux was reduced by a known VSOAC inhibitor (SITS) as well as by the removal of Ca^{2+}, which has been shown to be a necessary step in the osmotic signaling cascade. In addition, TTX did not affect ethanol-induced taurine efflux. Previous studies confirm that ethanol can cause a change in cellular osmolarity (Aschner et al., 2001; Sato et al., 1990) and taurine is released in order to regulate cell volume (Kimelberg et al., 1993). An equation to calculate the change in osmolarity attributed by ethanol can be attained by multiplying the concentration of ethanol (mmol/L) by 1.25 (Purssel et al., 2001). From the ethanol elimination study conducted in CHAPTER 3, the amount of ethanol produced by 2.5 g/kg

would be approximately 25-30mM. This value would correspond to a change in fluid osmolarity by 30mOsm to 40mOsm. Interestingly, the change in osmolarity produced by ethanol corresponds well with the manipulation of osmolarity that several of the studies utilized to characterize the hypoosmotic release of taurine (Bres et al., 2000;Olson and Li, 2000). Most recently, whole body osmotic state was manipulated to control hypoosmotic release of taurine caused by ethanol (Quertemont et al., 2003). While these studies support the notion that ethanol-induced taurine release is associated with osmotic changes, they do not adequately explain the regional selectivity in taurine release. Taurine is known to be in the brain in high concentrations (Huxtable, 1989) and perhaps it is not a question of preferential release but abundance in the NAc. However, previous microdialysis studies which measured the basal levels of taurine in different brain regions have shown that, although the exact values differ, taurine is consistently lower in the NAc (Dahchour et al., 1996; Del Arco and Mora, 1999) than other areas such as hippocampus (del Rio et al., 1987; Solis et al., 1986) or striatum (Del Arco and Mora, 1999; Semba et al., 1995). Alternatively, certain regions in the brain are more sensitive to the change in osmolarity as seen in the SON (Bourque et al., 1994) and pharmacological, molecular evidence suggests a novel volume-sensitive anion channel in this area (Bres et al., 2000). Perhaps similar volume-sensitive anion channels are expressed in the NAc making it more sensitive to changes in osmolarity.

We have reported the first time *in vivo* usage of on-line electrophoresis with laser induced fluorescence using NDA/CN as a derivatization agent. Ethanol-induced taurine efflux was observed in response to 2.5 g/kg ethanol and was blocked by SITS and Ca^{2+} -free ACSF. However TTX did not affect ethanol-induced taurine efflux. These results

seem to indicate that at least a portion of the efflux is mediated through volume-sensitive osmotic channels.

CHAPTER 5
ETHANOL-INDUCED TAURINE EFFLUX: SELF-ADMINISTRATION

Introduction

Taurine is one of the most abundant amino acids located in the CNS. The abundant nature of taurine is only surpassed by its versatility. Taurine has been shown to be an antioxidant (Nakamori et al., 1993) and to be involved in osmoregulation (Hussy et al., 2000; Pasantes-Morales and Martin del Rio, 1990; Pasantes-Morales et al., 1990a; Pasantes-Morales et al., 1990b; Solis et al., 1988a) Ca^{2+} modulation(Huxtable and Sebring, 1987; Sawamura et al., 1990; Sebring and Huxtable, 1986) and neurotransmission (Huxtable, 1989). As a neurotransmitter, taurine has been shown to increase Cl^- uptake in a manner similar to GABA (Oja et al., 1990) and is competitively inhibited by muscimol binding (Bureau and Olsen, 1991). In addition, taurine enhances benzodiazepine binding to the GABA receptor (Quinn and Miller, 1992a). Taurine has also been shown to inhibit the NMDA receptor (Kurachi et al., 1983a) and is probably the endogenous ligand for the glycine receptor in some areas of the brain (Mori et al., 2002a). These effects of taurine are similar to ethanol action as an agonist on the GABA and glycine receptors and an antagonist at the NMDA receptor (Heidbreder and De Witte, 1993; Lovinger et al., 1989).

Taurine has been shown to modify ethanol's effects such as sleep time (Ferko, 1987), locomotor activity in an open field (Aragon et al., 1992), and condition taste aversion (Aragon and Amit, 1993). Conversely, ethanol has been shown to effect taurine

efflux. *In vivo* microdialysis studies have shown that an acute injection of ethanol increases taurine efflux in the nucleus accumbens (Dahchour et al., 1994; Dahchour et al., 1996), hippocampus, frontal cortex (Dahchour and De Witte, 1999), and amyglada (Quertemont et al., 1998a). Genetic preference also appears to play a role in taurine efflux. For example, in response to an acute ethanol injection, high-alcohol sensitive rats displayed a reduced taurine efflux in the nucleus accumbens compared to low-alcohol sensitive rats (Quertemont et al., 2002). Sardinian ethanol-preferring rats exhibited a reduced ethanol-induced taurine efflux in comparison to Sardinian ethanol-non-preferring rats (Quertemont et al., 2000). However, these studies measured taurine efflux after sedative doses of ethanol and not the lower doses associated with self-administration of ethanol. In addition, these studies employed traditional HPLC-based detection of amino acids in microdialysate in which samples are collected every 15-20 minutes. This technique fails to resolve any fast neurotransmitter changes that might occur. Recently, by coupling microdialysis with on-line derivatization, capillary electrophoresis (CE) separation and laser-induced fluorescence detection (LIF), temporal resolution and detection of multiple analytes has been improved (Bowser and Kennedy, 2001; Lada et al., 1998b). The end result is a faster resolution (seconds) with a greater capacity to measure a multitude of amino acids. The present microdialysis study was conducted to determine ethanol-induced taurine efflux in the nucleus accumbens after low doses of ethanol such as those that might be self-administered in a voluntary manner by rats.

Methods

Animals and Surgeries

Male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 250-350 g were housed with *ad libitum* access to food and water throughout the experiment unless

otherwise indicated. All procedures were conducted in strict adherence to the *National Institute of Health Guide for the Care and Use of Laboratory Animals.* One week prior to the experiment, each rat was surgically implantated with a guide cannula into the left side of NAc (+1.6 anteroposterior, +1.7 lateral, -6.2 dorsoventral).

Microdialysis

On the day of the experiment, each rat was lightly anesthetized with halothane, moved into the dialysis cage and the dialysis probe was inserted. Microdialysis probes (o.d. 270 μm; active length 2 mm; cellulose membrane, 13,000 molecular weight cut-off) were perfused with artificial cerebrospinal fluid (ACSF; 145 mM NaCl, 2.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$,) at 1μL/min. After the experiment, probe placement was verified histologically and only those subjects with accurate placement were included in the analysis.

CE-LIF

The amino acid concentrations in dialysate samples were analyzed on-line by CE-LIF utilizing the procedure established previously (CHAPTER 3). Briefly, amino acids were derivatized on-line with a 1 μL/min stream of OPA solution with a reaction time of approximately 1 min. Samples of the derivatized dialysate were injected onto the capillary using flow-gate interference. The outlet of the reaction capillary was aligned with the inlet of the CE separation capillary in a Plexiglas block, leaving a gap of approximately 30 μm. A 1 mL/min cross-flow was applied between the capillaries. To inject a plug of sample onto the separation capillary, the cross-flow was stopped for 1 sec allowing the sample to fill the gap between capillaries and the voltage was raised to 2 kV for 100 ms. After injection, the gating cross-flow was resumed to wash excess dialysate

to waste, and the separation voltage was ramped to 20 kV over 500 ms. The flow-gate was held at ground to isolate the animal from the voltage dropped across the separation capillary. Capillary electrophoresis was carried out in a 10 cm long, 10 μm ID, 150 μm OD fused-silica capillary.

The OPA labeled amino acids were detected using LIF in a sheath-flow detector cell. The outlet of the separation capillary was inserted into a 2 mm square OD, 200 μm square ID quartz cuvette (Mindrum Precision, Inc., Rancho Cucamonga, CA, USA). Grinding the outlet to a point reduced the dead volume at the outlet of the separation capillary. Sheath buffer was siphoned around the outside of the separation capillary outlet. Analytes migrated off the end of the separation capillary with a laminar flow profile, which reduces the background signal caused by laser scatter. Background fluorescence was excited using the 351 nm line of an argon-ion laser (Enterprise II 622; Coherent Laser Group, Santa Clara, CA, USA) focused onto the analytes. Emission was spatially filtered through a 450 ± 25 nm bandpass and collected on a photomultiplier tube. Current from the PMT was amplified, filtered (10 ms rise-time) and sampled using Lab-View software. A calibration curve was generated either before or after the experiment by placing the tip of the dialysis probe in three different concentrations of a standard solution containing glutamate, GABA, taurine, serine and glycine and then plotting peak height versus concentration

Self-administration

The procedure to induce ethanol self-administration was based upon the sucrose-fading procedure (Samson, 1986) with some minor modifications. To induce voluntary ethanol consumption, ethanol-naïve rats were initially water-deprived for 24 hr and then

given access to 0.2% w/v saccharin solution for 30 min. After this point, rats were no longer water-deprived and were given 30 min access each day to water and 0.2% w/v saccharin in separate graduated sipper tubes. Every 4 days, the ethanol concentration was gradually increased to 10% while the saccharin concentration was gradually decreased to 0.1%. For the remainder of the experiment, rats had concurrent access to the 10% ethanol: 0.1% saccharin solution and water for 30 min/day at approximately 1300 h.

After rats attained stable daily levels of voluntary ethanol consumption (0.72+0.24 g/kg body weight per half hour over a period of three months), they underwent survival surgery for implantation of guide cannula as described above. After a one-week recovery period, during which they received no ethanol access, rats were implanted with a microdialysis probe as described above at approximately 900 h. The one-week no ethanol access would not be expected to produce any signs of withdrawal as another study has indicated that no withdrawal signs were produced after a 5 day deprivation period (Heyser et al., 1997). However, the one-week no ethanol access would be expected to increase ethanol self-administration due to an ethanol deprivation effect (Heyser et al., 1997; Roberts et al., 2000). Baseline samples were taken for 3 hrs while the animal had food and water ad libitum. At 1300 h, the water bottle was replaced with two bottles: one containing water and the other containing 10% ethanol: 0.1% saccharin solution. Amount of each fluid consumed was recorded each minute. After one hour of sampling, the two bottles were replaced with the normal water bottle. Two hours later, rats were injected with ethanol (1 g/kg; 20% w/v in saline).

Results

Of the five animals tested, two consumed 0.1g/kg ethanol which caused no significant increase in taurine levels in the dialysate (Figure 5-1). In addition, one rat did

not drink at all and subsequently taurine levels were not increased in this animal as well. However, two rats did drink 0.7 g/kg ethanol and this was done during the first five minutes of access. These rats had an average increase of 30% taurine levels, which lasted for about 30 minutes. The 1.0 g/kg ethanol injection, which was given two hours after replacing the ethanol bottles with water, produced very similar effects to previous experiments. The acute ethanol injection increased taurine efflux to approximately 30% of basal and remained above basal for about 30 minutes.

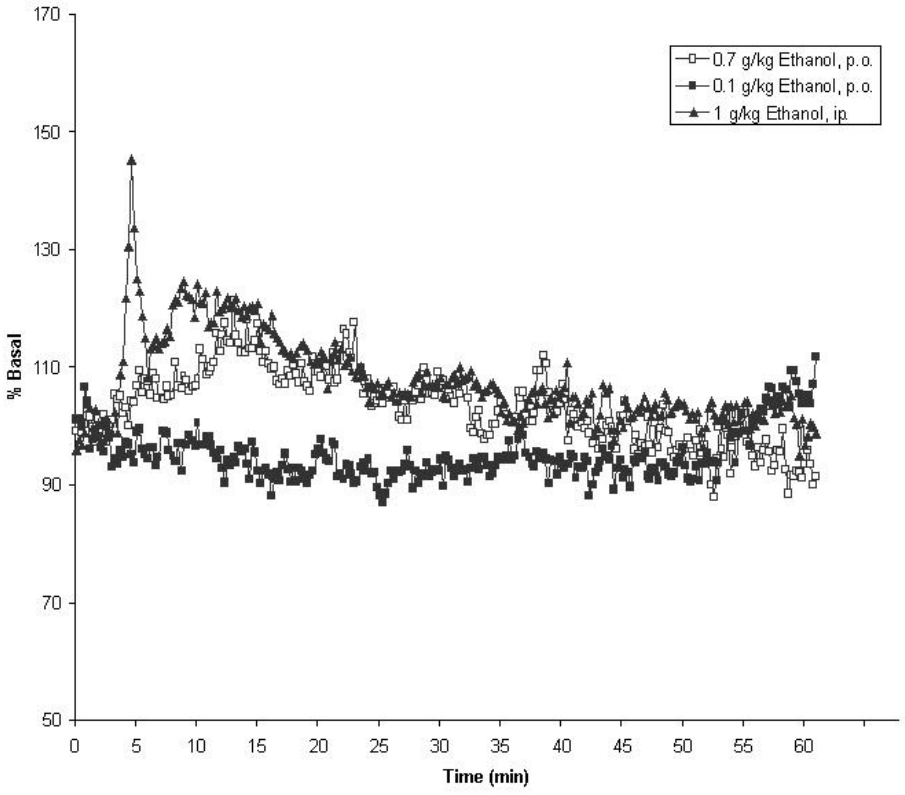

Figure 5-1 Taurine levels in nucleus accumbens in rats after self-administration of 0.1 g/kg ethanol (N = 2) or 0.7 g/kg ethanol (N = 2) or injection of 1 g/kg ethanol i.p (N = 4). Rats were given 1 hr access to two bottles, one containing 10% ethanol v/v and one containing water, and allowed to drink ad libitum. Most of the ethanol was self-administered during the first 5 minutes of the access period.

Discussion

1.0 g/kg ethanol i.p. in rats that had been self-administering ethanol for one hour a day for over a year produced a similar effect in taurine efflux from previous studies (CHAPTER 3). Such an ethanol induced taurine efflux has been observed after i.p. administration of ethanol in genetically ethanol preferring rats (Quertemont et al., 2000). However, we were also able to demonstrate ethanol-induced taurine increases after voluntary ethanol consumption. A fairly low dose of 0.7 g/kg p.o. caused a taurine efflux very similar to that of the 1 g/kg injection i.p.

Thus, the utilization of the CE-LIF enabled observations of taurine efflux caused by a lower dose of ethanol than has been reported previously as well as a maximal ethanol induced taurine efflux occurring within the first 5-7 minutes after ethanol injection. In addition, due to increased sampling permitted by CE-LIF, the taurine efflux caused by 2 g/kg ethanol i.p. displays two possible components of efflux: a large, fast response and a slower response.

CHAPTER 6
GENERAL DISCUSSION

A staggering 14 million Americans abuse alcohol or are alcoholic (Narconon, 2004). Several theories explain the processes of addiction. In general, the manipulations of neurotransmitter functions in several areas of the brain have been widely accepted as key steps in the process of addiction (Koob and Le Moal, 1997; Robinson and Berridge, 1993). One of the most predominant addiction pathways studied is the mesolimibic dopamine pathway (Di Chiara, 1995; Fitzgerald and Nestler, 1995; Jentsch and Taylor, 1999). Dopamine efflux and receptor function is altered in this area due to ethanol exposure (Brodie et al., 1990; Imperato and Di Chiara, 1986; Yim and Gonzales, 2000; Yoshimoto et al., 1992). Similarly, we have shown taurine efflux to be altered in this area due to ethanol (CHAPTERs 3,4,5). These results are in agreement with previous studies (Dahchour et al., 1994; Dahchour et al., 1996; De Witte et al., 1994). However, the purpose of this release is unknown. Taurine has numerous functions but most importantly can modify several neurotransmitter functions (Huxtable, 1989; Malminen and Kontro, 1986; Martin and Siggins, 2002; Ruotsalainen et al., 1996) as well as function as an osmolyte (Bres et al., 2000; Nagelhus et al., 1994; Schaffer et al., 2000). My main focus is to characterize the release of taurine due to ethanol. In particular the mechanism by which taurine is released due to ethanol, which could provide insight to the purpose of this release.

The first studies utilized HPLC analysis, later this was changed to CE-LIF or micellar CE-LIF to analyze primary amines in the dialysate. The CE-LIF systems increased the temporal resolution from 15 minutes (HPLC) to 11 seconds (CE-LIF) and 30 seconds (micellar CE-LIF), which made the majority of the observations possible. For example, we detected an increase in taurine efflux due to low dosages of ethanol (0.5g/kg i.p. CHAPTER 3 and 0.7 g/kg o.p. CHAPTER 5) which had previously not been detected. In addition, the coupling to *in vivo* microdialysis provided an intact neural system in which to examine ethanol-induced taurine efflux. The experiments in this dissertation were the first studies to utilize *in vivo* microdialysis with CE-LIF.

Several results from CHAPTER 3 led to the formation of the hypothesis that ethanol-induced efflux was primarily due to neuronal depolarization. For example, ethanol-induced taurine efflux produced by 2.0 g/kg (CHAPTER 3) and 2.5 g/kg (CHAPTER 4) displayed a rapid, robust two-fold increase during the first 10 minutes. After the first 25 minutes the efflux began to decrease to about 40% above baseline where it remained for the duration of the experiment. There is overwhelming evidence that indicates that taurine is located more in neurons than in glial cells (Huxtable, 1989). The large amount of taurine released due to ethanol would seem to indicate that neuronal cells are the main source of taurine. In addition, taurine efflux from glial cells displays a lag phenomenon where upon stimulation there is a 3-5 minute delay in the efflux of taurine (Philibert et al., 1988). There was a slight reproducible delay from the time ethanol was delivered to detection but this was attributed to the length of the capillary from probe to instrument. Ethanol-induced taurine efflux did not display a lag phenomenon, which would seem to indicate the lack of involvement of glial cells. The

sustained and gradual decrease of taurine efflux probably occurs through the re-uptake of taurine, which has a slow rate of uptake (Km) compared to neurotransmitters (Della Corte et al., 1987; Huxtable, 1989; Lombardini, 1977; Shain and Martin, 1990). Ethanol has been shown to cause an osmotic imbalance that results in the efflux of taurine (Aschner et al., 2001; Kimelberg et al., 1993), however the dosages that we observed that induced taurine efflux were much lower (5 mM brain ethanol level from 0.5 g/kg i.p. CHAPTERs 3 and 0.7 g/kg o.p. CHAPTER 5) than what has been observed to initiate taurine efflux. Regional selectivity and the preferential release of taurine also solidified the hypothesis that ethanol-induced taurine efflux was due to neuronal depolarization. However, results from CHAPTER 4 would question some of the principles used to form the working hypothesis.

Taurine was the only amino acid measured that increased in response to increasing ethanol. If the dosages of ethanol were causing an osmotic disturbance then other amino acids that are osmolytes should be released such as aspartate or glutamate. The selective increase in taurine would seem to argue against osmoregulation being the main mechanism of efflux. In addition, several studies have indicated that taurine is preferentially released due to osmoregulation but other amino acids are released too although not as great in magnitude (Estevez et al., 1999; Kimelberg et al., 1993; Pasantes-Morales et al., 1993a). Since only taurine increased due to ethanol it would seem to suggest that taurine was released due to another mechanism not osmoregulation. However, in other *in vivo* studies (CHAPTER 4) in which the Na^+ was decreased, other amino acids were released but only under extreme osmotic stress (reduction of sodium from 145mM to 100mM, 75mM). A possible explanation for this event could involve

which cell population is more sensitive to osmotic stress. The preferentially release of taurine could be due to glial cells which might be more sensitive to osmotic stress. When the sodium is further reduced, perhaps neurons become affected and begin to release osmolytes. This would explain taurine efflux due to small reductions in sodium and the general release of other osmolytes when the sodium is further reduced. Therefore, osmoregulation cannot be excluded as the mechanism of ethanol-induced taurine efflux.

Comparison of ethanol-induced taurine efflux from the NAc and striatum indicated that there was a regional selectivity; the NAc was more sensitive to ethanol. Several conclusions were made from this result. First, the regional selectivity seems to argue against taurine being released due to osmoregulation. If ethanol were causing an osmotic disturbance it would be expected that taurine would be released in every region equally since ethanol rapidly diffuses across membranes and affects the entire brain (Deitrich et al., 1989). However, it has been shown that certain regions of the CNS are more sensitive to osmotic disturbance; the SON has been shown to be extremely sensitive to osmotic status (Bourque and Oliet, 1997). The SON regulates hormonal release to control body homeostasis (vasopressin) (Engelmann et al., 2001) and taurine is involved in a short negative feedback loop involving the release of this hormone (Hussy et al., 2001). In order to regulate body volume, the SON must be sensitive to minor osmotic change. Perhaps the NAc could contain specialized cells, similar to SON, which would impart heightened sensitivity to osmotic change. However, the NAc is not as sensitive to osmotic change as the SON and a functional reason for heightened sensitivity to osmotic imbalance in the NAc cannot be found.

Secondly, the NAc is a known dopaminergic rich area in the CNS involved with the addiction process and ethanol-induced taurine efflux is heighten in this region compared to other regions suggests the importance of taurine in alcoholism. Since regional selectivity is observed in other neurotransmitter release, these observations support the hypothesis that ethanol-induced efflux is due to neuronal depolarization.

CHAPTER 4 experiments were designed to test the neuronal depolarization and osmoregulation components of ethanol-induced taurine release. In order to test neuronal depolarization of ethanol-induced taurine release, TTX was used to block the fast-acting sodium channels and Ca^{2+}-free ACSF with EGTA was used to inhibit synaptic release. The results from these experiments were mixed. The TTX had no effect on ethanol-induced taurine efflux. In contrast, the Ca^{2+}-free ACSF did blunt ethanol-induced efflux. Ethanol-induced taurine efflux was TTX insensitive but, surprisingly, Ca^{2+}-dependent. This result was unexpected since if taurine were released by neuronal depolarization than both TTX and Ca^{2+}-free ACSF would decrease efflux. However, since only Ca^{2+}-free ACSF decreased efflux another mechanism could be responsible for ethanol-induced taurine efflux.

The osmotic component of ethanol-induced taurine efflux was tested by reverse dialysis of a known inhibitor of VSOAC. The reverse dialysis of SITS decreased ethanol-induced taurine efflux by 80% 10 minutes after injection. These results were very similar to the Ca^{2+}-free ACSF with EGTA studies, which could suggest a similar mechanism. It should be noted that not all known inhibitors of VSOAC channels were able to block osmotic-induced taurine efflux. A similar effect was observed in the SON and was attributed to a variant of this channel, which would impart different pharmacological

properties (Bres et al., 2000). Perhaps the NAc has a variant of VSOAC. However, it was concluded that at least part of ethanol-induced taurine efflux was due to osmoregulation.

Several studies have indicated that ethanol-induced taurine efflux is primarily due to osmoregulation (Kimelberg et al., 1993; Lallemand et al., 2000; Quertemont et al., 2003). My research also indicates that at least part of the mechanism for ethanol-induced taurine efflux occurs due to osmoregulation. A problem with this conclusion is the explanation of how the reduction of Ca^{2+} could blunt ethanol-induced efflux on a channel that has been shown to be Ca^{2+} independent (Cabantchik and Greger, 1992; Kirk, 1997; Strange et al., 1996). Recently, several studies have shown that although the channel might be Ca^{2+} independent, the mechanism of osmotic swelling is not. Cytosolic Ca^{2+} increases in response to cell swelling (Huang et al., 2001; Morales-Mulia et al., 1998; Pasantes-Morales and Morales-Mulia, 2000), which leads to a change in the phosphorylation status of key proteins associated with optimal function of VSOAC (Cardin et al., 2003; Deleuze et al., 2000; Pasantes-Morales et al., 2002). Perhaps removal of Ca^{2+} decreased the functionality of VSOAC, which would explain why Ca^{2+}-free ACSF with EGTA blunted ethanol-induced taurine efflux.

Several observations indicate additional factors could be involved and that ethanol-induced taurine efflux is not solely due to osmoregulation. For example, local exposure of the NAc to 1000 mM ethanol increased taurine by only 10% above baseline, much less than the two-fold increase seen after the 2 g/kg dose resulting in 30 mM dialysate ethanol (CHAPTER 3). If taurine was released in response to cell swelling due to ethanol, than local application of ethanol would also induce cell swelling and taurine efflux. However, this was not the case. Similarly, if ethanol were acting directly on the

NAc to induce neuronal depolarization, than the subsequent taurine efflux would have been observed. This did not occur either. The results from the local application of ethanol indicated that ethanol is inducing taurine efflux (osmotically or depolarization) distal from the NAc. The distal effect of ethanol could explain why the reverse dialysis of TTX did not have an effect. If ethanol caused depolarization in neurons that were not located near the microdialysis probe and therefore unaffected by the TTX, it is possible that these neurons communicated in some way to the cells in the NAc to release taurine.

Another observation that indicates that ethanol-induced taurine efflux is not due to osmoregulation is the comparison of brain ethanol levels to taurine efflux. The reduction of Na^+ to 125mM in CHAPTER 4 induced the osmotic release of taurine approximately two-fold and remained so during the perfusion of the reduced Na^+ ACSF. If ethanol was inducing an osmotic imbalance (as did the reduction of Na^+ in the ACSF), then as long as the concentration of ethanol remains elevated, so does taurine efflux. However, the brain ethanol levels due to the 2.0 g/kg in CHAPTER 3 remained relatively high (25-30mM) for an hour but the taurine efflux decreased. If ethanol was causing an osmotic imbalance, then taurine efflux should be proportional to the concentration of ethanol. Perhaps ethanol exerts its effects on other areas of the brain and these areas innervate the NAc and signal the release of taurine through other amino acids (Figure 6-1).

Glutamate has been shown to induce cell swelling and the subsequent release of taurine (Menendez et al., 1989a; Saransaari and Oja, 2000a). Interestingly, when the Na^+ was reduced to below 125mM a transient glutamate spike preceded taurine efflux and was proportional in magnitude (CHAPTER 4). It is plausible that glutamate could be part of signaling mechanism relating to taurine efflux. A pathway that could be involved in

this scenario, as mentioned in CHAPTER 4, would be the excitatory glutamatergic input to the medium spiny neurons of the NAc, which arises from the ventral CA1/subiculum region

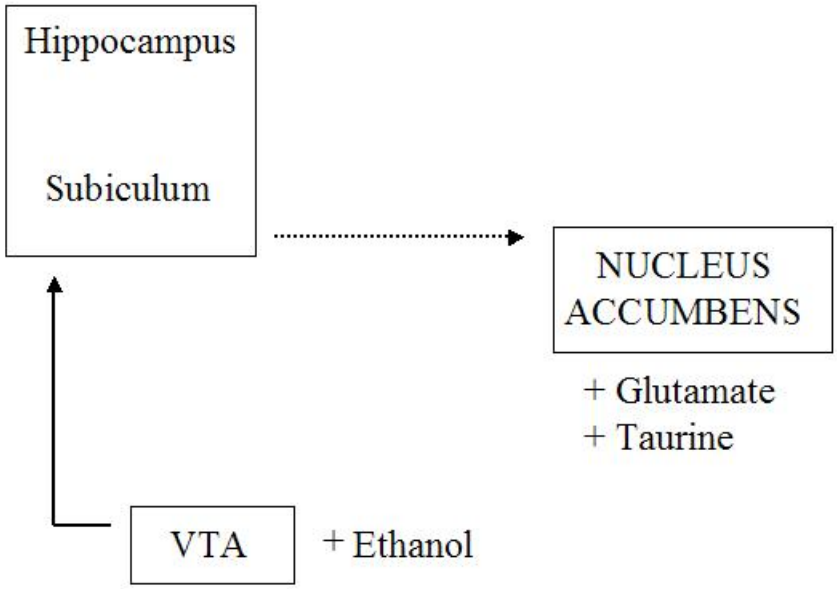

Figure 6-1 A model that might explain how ethanol could induce taurine efflux without directly affecting the NAc. In the diagram ethanol could activate the dopaminergic afferents from the VTA to the hippocampus (solid line). Excitatory glutamatergic efferents (dotted line) from the subiculum of the hippocampus to the NAc could induce glutamate to modify taurine release (Floresco et al., 2001).

of the hippocampus (Groenewegen et al., 1987; Brog et al., 1993). In this scenario, ethanol would increase the firing rate of dopaminergic neurons of the VTA, which innervates the hippocampus. Glutamatergic afferents from the subiculum region to the NAc would increase the release of glutamate, which could increase the efflux of taurine.

However, when taurine efflux was only two-fold the glutamate spike was below our detection limits or was not present. Since 2.0 g/kg (CHAPTER 3) or 2.5 g/kg (CHAPTER 4) of ethanol produced only a two-fold increase in taurine efflux it is plausible that the glutamate spike was below the limits of detection. Therefore it is conceivable that ethanol could induce a similar glutamate spike, which could signal the efflux of taurine. However, the glutamate spike could not be due to neuronal depolarization, as the TTX would have blocked any event involving the fast-acting sodium channels. The origin of the glutamate spike remains a mystery but it is likely due to change in osmolarity since it was consistently observed in the reduced sodium ACSF experiments.

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BIOGRAPHICAL SKETCH

In 1973, Anthony Donald Smith was born in Clarksville, Tennessee, to Don and Barky Smith. Later that year the family would re-locate to Jupiter, Florida. Except for a few years the family spent in North Carolina, he would consider Jupiter his home. He graduated Jupiter High School in 1992 and attended the University of Florida. In 1997 he graduated with a BA in English. After graduation he became a lab technician for Dr. Donna Wielbo. During this time, she convinced him that he should consider graduate school. After a semester of science classes (major in microbiology) he was accepted into the Ph.D. program in the Department of Pharmacodynamics, College of Pharmacy. He feels very fortunate to have received excellent instruction from a very talented faculty and hopes to be able to make them proud with his future endeavors.