STUDIES ON NEUROTRANSMITTER FUNCTION OF ACETYLCHOLINE IN EXPERIMENTAL HYPERAMMONEMIA

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DOCTOR OF PHILOSOPHY

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To my parents and grand mother

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CERTIFICATE

This is to certify that Ms. R.P. Rukmini Devi has carried out the work in the present thesis under my guidance for a full period prescribed under the Ph.D. ordinance of this University. I recommend her thesis entitled "Studies on neurotransmitter function of acetylchohne in experimental hyperammonemia" for submission for the degree of Doctor of Philosophy of this University.

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ABBREVIATIONS

AA	Ammonium acetate
AAA	Aromatic amino acids
AChE	Acetylcholinesterase
ATP	Adenosine 5'-triphosphate
BBB	Blood-brain barrier
BCAA	Branched-chain amino acids
BCKA	Branched-chain keto acids
BS	Brain stem
BUI	Brain uptake index
CAT	Choline acetyltransferase
CC	Cerebral cortex
CE	Cerebellum
CNS	Central nervous system
DAG	Diacyl glycerol
DTNB	5,5'-Dithiobis-(2-nitrobenzoic acid)
GABA	y- Aminobutyric acid
GDH	Glutamate dehydrogenase
GFAP	glial fibrillary acidic protein
HAUS	High affinity uptake system
HE	Hepatic encephalopathy
HEPES	N-[2-hydroxyethyl)piperizine-N-2-[ethanesulphonic acid]
IPSP	Inhibitory' post synaptic potential
LAUS	Low affinity uptake system
a-KG	a- Ketoglutaric acid
mAChR	Muscarinic acetylcholine receptor
MSI	Methionine sulfoximine

nAChR	Nicotinic acetylcholine receptor
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced)
NMDA	N-methyl-D-aspartate
4-NMPB	N-methyl-4-piperidyl benzilate
PCA	Perchloric acid
PChE	Pseudocholinesterase
POPOP	2,2'-P-Phenyl-bis(4-methyl-5-phenyloxazole)
PPO	2,5-Diphenyloxazole
QNB	Quinuclidinyl benzilate
TCA	Trichloroaceric acid
TCA cycle	Tricarboxylic acid cycle
TEAC	Tetraethyl ammonium chloride

PART I

INTRODUCTION

Hyperammonemia - an overview

Ammonia is a chief metabolite of nitrogen metabolism, produced and utilized in many biochemical reactions of the body. Though it is a common metabolite, increased systemic ammonia levels are neurotoxic. Since blood and brain ammonia levels are in dynamic equilibrium, increased blood ammonia levels are rapidly reflected in brain. Elevated blood and brain ammonia levels have been reported in certain pathological states which are associated with deranged cerebral function. Ammonia is believed to be chief culprit in neurological disorders associated with liver inadequacy (Mousseau and Butterworth, 1994; Raabe, 1990; Rao *et al,* 1992).

Enhanced blood ammonia levels are seen in neurological disorders associated with hepatic inadequacy, inherited disorders of urea cycle enzymes and of amino acid metabolism. The most common disorders among them are fulminant hepatic failure, Reye's syndrome, chronic liver dysfunction, cirrhosis etc. (Delong and Glick, 1982; Huttenlocher *et al,* 1969). Fulminant hepatic failure is sudden in its onset and is mostly due to hepatitis 'B' infection (Papas-venegeleu *et al,* 1984). Reye's syndrome is an acute encephalopathy of childhood with associated fatty infiltration of the liver (Reye *et al.,* 1963). The disorder has characteristic onset 3-5 days after a viral illness, often influenza B and less frequently Varicella (Corey *et al.,* 1977; Linneman *et al.,* 1974). "Hepatic Encephalopathy" (HE) is the name given to spectrum of neurological changes associated with liver dysfunction. At initial stages, personality and emotional disturbances have been reported and confusion, drowsiness, hyperthermia and coma have been observed as the disease progresses. In advanced stages, decerebrate rigidity, decortical posture, convulsions and finally death have been reported (Gilberstadt *et al.*, 1980; Levy *et al,* 1987; Pomier-Layrargues *et*

al., 1991; Schenker *et al.*, 1974). These changes were also observed in experimental models developed by intraperitoneal administration of methionine sulfoximine (MSI) (Jyothi, 1994) and galactosamine (Reeba, 1995). In all these disorders, ammonia acts either as a sole neurotoxin or in conjunction with other toxins like false neurotransmitters, mercaptans and short chain fatty acids (Bruton *et al.,* 1970; Butterworth *et al.*, 1987; Cooper and Plum, 1987; Fischer and Baldessarini, 1971; Lockwood *et al.*, 1991; Zieve and Brunner, 1985; Zieve *et al.,* 1983).

Hyperammonemia is also observed in some clinical conditions like valproate therapy and portacaval anastomosis (Wartor *et al.,* 1983) and other conditions such as congestive heart failure (Valero *et al.,* 1974), birth asphyxia (Goldberg *et al.,* 1979) and shock (Nelson and Seligson, 1953). Hyperammonemic conditions prevail even in some neuropsychiatric disorders like Alzheimer's disease (Branconnier *et al.,* 1986; Fisman *et al.,* 1985; 1989; Hoyer *et al.,* 1990; Seiler, 1993). Available evidence shows that ammonia may also play a pathogenetic role in Alzheimer's disease (Seiler, 1993). It has been suggested that astrocytic damage by ammonia might mediate pathologic events of Alzheimer's disease (Frederickson, 1992). An important function of astrocytes and microglia is the production of inflammatory cytokines (Benvensite, 1992). Microglia act as scavenger cells in brain, phagocytosing cell debris (Thomas, 1992). They are involved in the removal of β-amyloid fibrils and aggregates (Frackowiak *et al.,* 1992; Shigematsu *et al.*, 1992), thus the phagocytic activity of microglia is also considered important in Alzheimer's pathology (Nieto-Sampredo and Mora, 1994). Elevated ammonia concentrations have been shown to affect phagocytosis and cytokine production by a murine microglia cell line (Atanassov *et al.,* 1994). Atanassov *et al,* (1994; 1995) reported that ammonium acetate

(AA) affects the endocytosis and cytokine production by astroglia and microglia cell lines. It is known that ammonia is endogenously generated in brain in Alzheimer's disease (Hoyer *et al.,* 1990), the pathological concentrations of ammonia in brain may alter the functions of glia in Alzheimer's pathogenesis (Atanassov *et al.,* 1995).

As ammonia is known to act either as sole neurotoxin or in conjunction with other toxins in all these disorders, many animal models have been developed to study the neurotoxic effects of ammonia. Most of the models were based on administration of hepatotoxins like galactosamine, CCl_4, and thioacetamide and portacaval anastomosis and animals with specific gene mutations were intended to reproduce the effects of acute or chronic liver failure. Other models include administration of ammonium salts. Much work has been done in these models and in disorders of hepatic function. A brief review of literature would give an idea about the work so far done in this field.

Production and Detoxification of Ammonia in the Body

Ammonia is chief metabolite of amino acid and nucleotide metabolism Most of the ammonia formed in the body is derived from 1. metabolism of glutamine by intestinal mucosa (Hanson and Parsons, 1988; Souba, 1991; Souba *et al.,* 1985; Windmueller, 1984) and 2. hydrolysis of urea and contents of colon by the action of bacteria harbored by intestine (Jones *et al.,* 1969; Summerskill and Wolpert, 1970; Wolpert *et al.,* 1971). This ammonia enters portal circulation and consequently the hepatic portal blood ammonia concentration raises to 300-600 μM. This is essential for effective urea synthesis but would be toxic if this concentration of ammonia exists in systemic circulation. This blood is delivered to ascini of liver, where the ammonia is converted to urea in periportal hepatocytes (lacking glutamine synthetase) and to glutamine in perivenous hepatocytes

(lacking carbamyl phosphate synthetase, Haussinger, 1983; 1990; Jungerman and Katz, 1982). Carbamyl phosphate synthetase (rate limiting enzyme in urea synthesis) has a high K_m for ammonia, owing to which there is a requirement of high concentrations of ammonia for urea synthesis. This is made more efficient by hydrolysis of glutamine at the site of carbamyl phosphate synthesis to provide even high concentrations of ammonia. The hydrolysis of glutamine is catalyzed by phosphate dependent glutaminase present in periportal hepatocytes and is stimulated by ammonia. Thus, it acts in positive feed back manner to provide high concentrations of ammonia for urea synthesis. Ammonia diffused from periportal hepatocytes is scavenged to perivenous hepatocytes where it is converted to glutamine. About 90% of ammonia that enters liver is converted to urea and two thirds of urea nitrogen originates from portal ammonia and most of the remainder from glutamine (Hawkins *et al.*, 1994). Thus, the efficient functioning of liver maintains the ammonia concentration at low levels in peripheral circulation.

In case of hepatic inadequacy or hepatic dysfunction, ammonia escapes the detoxification mechanism and enters systemic circulation and floods all the tissues. Small amounts of ammonia is also released into blood from extrasplanchnic tissues like kidney, muscle and brain in the reactions mediated by glutamate dehydrogenase (GDH), glutaminase, AMP deaminase and to a lesser extent by other enzymes (Good and Knepper, 1985; Lockwood *et al.,* 1979; Souba *et al.,* 1985). Out put from kidney may be responsible for the hyperammonemia observed in some individuals on valproate therapy (Wartor *et al.,* 1983).

Uptake of ammonia may not be same for all the tissues and is dependent on the type of tissue. Ammonia enters brain through blood-brain-barrier (BBB) by diffusion (Cooper *et al.*, 1979). The brain uptake index (BUI) for ammonia increases with increase in blood pH (Raichle

and Larson, 1981) and strong positive correlation has been observed between the pH of the test bolus and BUI (Lockwood *et al.,* 1980). Ammonia exists in two forms a non-diffusible ionic form (NH_4^+) and a diffusible gaseous form (NH_3). At physiological pH, 98% of ammonia exists in ionic form. Since the gaseous form is lipid soluble and diffusible through BBB, the BUI for ammonia is expected to increase as the fraction of ammonia in gaseous form increases. This in turn increases the partial pressure of ammonia in blood and facilitates the forward flux of ammonia into the brain. Hence the blood-brain pH gradient plays a major role in determining the forward flux of ammonia into brain (Cooper *et al.,* 1981; Lockwood *et al.,* 1980). In addition to blood derived ammonia, endogenous metabolism of amino acids, nucleotides and monoamines also contribute to cerebral pool of ammonia (Benjamin and Quastel, 1974). Most of the endogenous ammonia produced in brain is derived from glutamine and glutamate by the action of glutaminase and GDH, respectively. Immunocytochemical and biochemical studies revealed that glutaminase is predominantly localized in neurons (Hogstad *et al.,* 1988; Rao, 1991; Wenthold *et al.,* 1986). The neuronal localization of glutaminase suggests its role in maintaining neurotransmitter pool of glutamate. The neuronal glutaminase is inhibited by its products glutamate and ammonia (Benjamin, 1981; Kvamme and Lenda, 1981; 1982). Decrease in glutaminase activity has been reported under hyperammonemic states (Bradford *et al.,* 1978; Kvamme and Lenda, 1982; Subbalakshmi and Murthy, 1983). This could be because of the feed back inhibition of glutaminase by elevated brain ammonia levels under hyperammonemic conditions. Formation of ammonia and α-ketoglutarate (α-KG) from glutamate is catalyzed by GDH. Ammonia formed by oxidative deamination of glutamate is very less compared to ammonia

derived from glutamine, as the reaction equilibrium is towards the formation of glutamate under physiological conditions (Engel and Dalziel, 1967). The direction of the reaction in which the enzyme operates depends on the intracellular concentrations of substrates and products (Erecinska and Silver, 1990).

Detoxification of Ammonia in Brain

Major route for the detoxification of ammonia in brain is the amidation of glutamate to glutamine which is catalyzed by glutamine synthetase (Berl *et al.,* 1962; Cooper *et al.,* 1979; 1985). As glutamine synthetase is localized in astrocytes, these are considered as the cellular sites for ammonia detoxification (Cooper *et al.,* 1979; Martinez-Hernandez *et al.,* 1977; Norenberg and Martinez-Hernandez, 1979). Since the astrocytic endfeet are interposed between blood capillaries and neurons, astrocytes act as an 'enzymatic barrier' to ammonia derived from both blood and brain. This enzymatic barrier together with the diffusional properties of ammonia maintains brain to blood ammonia ratio in the range of 1.5:1 to 3:1. This ratio may exceed 3:1 under conditions in which cerebral metabolism is acutely altered. Increase in astrocytic glutamine synthetase activity has been reported in hyperammonemic states (Rao, 1991; Subbalakshmi and Murthy, 1983) but synaptosomal and mitochondrial glutamine synthetase activities have been reported to be unaltered (Rao 1991). Regional differences in ammonia detoxification has been observed. Cerebral cortex *(CC)* being less efficient compared to brain stem (BS) (Butterworth *et al.,* 1988). Cooper *et al.,* (1985) reported that glutamine synthetase operates at near maximal velocity in mammalian brain. As glutamine synthetase is operating at maximal rates, it is no longer efficient to detoxify the additional blood born ammonia entering the brain in hyperammonemic conditions unless it is induced under these

conditions. This imposes a constraint on the capacity of brain to protect itself against surges of additional ammonia, thus leading to the neurological dysfunction under such conditions.

The other mechanism for ammonia detoxification in brain which operates at very low level is the reductive animation of α-KG, catalyzed by GDH. Though it has been suggested that the reaction is favored towards the formation of glutamate under physiological conditions, it has been questioned as *in vivo* concentration of ammonia is much below the K_m of the enzyme (Dennis *et al*., 1977). The K_m of ammonia for glutamine synthetase is much less when compared to K_m of ammonia for GDH. Hence, glutamine synthetase plays a major role in ammonia detoxification in brain. Decrease in GDH activity has been reported in homogenates, neuronal pericarya, synaptosomes and mitochondria of cerebellum in ammonium acetate induced hyperammonemia (Rao, 1991). Elevation in astrocytic GDH activity has been reported in hyperammonemic states (Rao, 1991). Subbalakshmi and Murthy (1983) reported a decrease in GDH activity of astrocytes isolated from cerebral cortex of hyperammonemic rats.

It can be concluded that, the reasons for sensitivity of brain to hyperammonic states are : 1. inability of brain glutamine synthetase to remove excess ammonia fast enough to prevent accumulation, 2. lack of induction of glutamine synthetase in brain under hyperammonemic states and 3. non- availability of an alternate mechanism to remove excess ammonia.

Morphological and Physiological Changes in Brain during Hepatic Encephalopathy and Hyperammonemia

As mentioned earlier, astrocytes are considered to be the centers for ammonia detoxification in brain. Hence, astrocytes show early signs of

damage in patients with HE or hyperammonemic syndromes. These include Alzheimer's type II astrocytosis with swollen distorted watery nuclei with prominent nucleoli (Hsia, 1974). Ammonia is known to cause disaggregation of microtubules. Since these are important cytoskeletal elements particularly during mitosis, it is suggested that Alzheimer type changes are a direct cytological consequence of elevated intracellular ammonia concentrations (Flannery *et al.*, 1982). These changes have been produced in experimental animals by the concentrations of ammonia comparable to clinical conditions and are reversible when ammonia infusions are discontinued (Flannery *et al,* 1982). It has been reported that in hyperammonemic conditions induced by the administration of MSI, the main morphological change is the development of Alzheimer type II astrocytosis and increase in astrocyte cell number (Gutierrez and Norenberg, 1977; Subbalakshmi and Murthy, 1983). Brain edema and increased cerebral hypertension are fatal complications and represent a major cause of death in patients with HE (O'Grady *et al.,* 1989). Increased brain water content in brain edema could be due to the changes in the permeability of BBB (vasogenic edema) or due to intracellular uptake of water (cytotoxic edema) which leads to brain swelling (Klatzo, 1967). Swelling of astrocytes has been observed in conditions of portacaval anastomosis (Laursen and Diemer, 1980; Zamora *et al,* 1973). Traber *et al,* (1987) suggested that the marked swelling of astrocytes in gray matter may contribute to the pathogenesis of brain edema in fulminant hepatic failure. Ammonia, a major neurotoxin implicated in the mechanism of HE is known to be associated with pathogenesis of brain edema. Elevation of glutamine content (Butterworth *et al.,* 1987; Hawkins *et al.*, 1987) was proposed to be a cause of brain edema in hyperammonemic states (Hilgier and Olson, 1994; Takahashi *et al.,* 1991; Watson *et al,* 1985). Neary *et al,* (1994) showed that ammonium chloride reduces glial fibrillary acidic

protein (GFAP) mRNA at 2, 5 and 10mM concentrations in cultured astrocytes without inhibiting total mRNA synthesis. With nuclear runoff experiments and using amanitin, they identified that the decreased GFAP mRNA was due to decreased stability of mRNA and not due to the inhibition of transcription. The addition of extracellular ATP prevents the loss of GFAP mRNA and the removal of ammonium chloride restores GFAP to normal levels. They suggested that ATP prevention of loss of GFAP mRNA may be due to the activation of purinergic receptors by ATP.

Mechanisms of Pathogenesis of Hepatic Encephalopathy and Hyperammonemia

Several mechanisms have been proposed to explain the pathogenesis of hepatic encephalopathy (Bessman and Bessman, 1955; Fischer and Baldessarini, 1971; James *et al*., 1979; Schafer and Jones, 1982; Zieve *et al*., 1974).

The energy depletion hypothesis, proposed by Bessman and Bessman (1955), centers around the involvement of GDH and glutamine synthetase in the detoxification of ammonia and in the pathogenesis of hyperammonemia. Due to the operation of these two processes, a-ketoglutarate was supposed to be drained out of the citric acid cycle, thus affecting the production of ATP. Further, oxidation of NADH to NAD in GDH reaction bypasses the electron transport chain. Utilization of ATP for the synthesis of glutamine may also deplete cerebral energy stores.. However, experimental results both in favor (Bessman and Pal, 1976; McCandeless and Schenker, 1981) and contradictory (Hindfelt and Siesjo, 1971; Ratnakumari and Murthy, 1989) to the above hypothesis have been reported. Murthy and his group and Hindfelt and Siesjo supported the energy depletion theory but not the proposed mechanism. They suggested

that in presence of excess of ammonia cytosolic glutamate is utilized for the synthesis of glutamine, thus making it unavailable for operation of malate aspartate shuttle which in turn affects the cytosolic lactate/pyruvate ratio (Fitzpatrick *et al,* 1983) and NADH/NAD ratio. The NADH generated in cytosol may not reach the electron transport chain for production of ATP. This may affect the carbon flow through glycolysis (Hertz *et al,* 1987; Murthy and Hertz, 1988; Ratnakumari, 1990).

James *et al,* (1979) proposed that deranged plasma amino acid patterns (low blood branched-chain amino acids (BCAA)/aromatic amino acids (AAA) ratio) and increased glutamine in brain act in concert to raise the brain neutral amino acid concentrations and disturb the normal neurotransmitter metabolism by producing metabolites like 5-hydroxyindole acetic acid, β-phenylethanolamine, and octopamine which act as false neurotransmitters. The hypothesis was based on the following observations

1. Deranged plasma amino acid pattern i.e. low blood BCAA/AAA ratio. (James *et al.,* 1976; Mans *et al.,* 1979; Schenker, 1970; Smith *et al.,* 1978; Soeters *et al.,* 1977; Strombeck *et al.,* 1978).
2. Increased brain glutamine levels in hyperammonemic states (Hawkins *et al.,* 1973; Kosenko *et al.,* 1993; Lin and Raabe, 1985; Williams *et al.,* 1972).
3. High circulating glucagon in cirrhotic patients and in dogs after portacaval anastomosis (Sherwin *et al.,* 1974; Soeters *et al.,* 1977).
4. Sustained hyperinsulinemia secondary to decreased hepatic catabolism of insulin.
5. Increase in activity of blood -brain amino acid transport (James *et al.,* 1978)

Based on these observations they hypothesized that, high blood ammonia stimulates glucagon secretion which promotes gluconeogenesis from amino acids and hence increases the production of ammonia Thus, high blood ammonia levels tend to become self sustaining. Increased level of insulin maintains glucose concentration within normal limits but favors the uptake and utilization of BCAA by muscle. Plasma phenylalanine, tyrosine and methionine concentrations are elevated as protein breakdown is favored, because of decreased glucose production and utilization of BCAA. Thus the establishment of a characteristic plasma amino acid pattern low BCAA and high AAA favors the entry of AAA into brain as both BCAA and AAA are transported into brain by the same carrier system (James *et al.*, 1979).

Ammonia is detoxified in brain by formation of glutamine. Glutamine, so formed, is transported into blood by neutral amino acid carrier system. The carrier system which mediates efflux of glutamine also mediates influx of neutral amino acids in proportions determined by their relative luminal and antiluminal concentrations and affinities for the transport system. This favors influx of AAA into brain as their concentration is higher when compared to BCAA under hyperammonemic conditions. Increased brain glutamine concentration, probably impair the efflux of other neutral amino acids from brain because of competition for available transport sites. Accumulated AAA undergo direct decarboxylation and form false neurotransmitters. The false neurotransmitters might interfere with synthesis, release and action of normal neurotransmitters in brain. They might even interfere with brain oxidative functions and absorption of precursors for normal neurotransmitters (James *et al.*, 1979).

Schafer and Jones (1982) hypothesized that, during liver failure, gut derived γ-amino butyric acid (GABA) escapes the hepatic detoxification mechanism, and enters brain and induces its own receptors. The GABA receptor is a supramolecular complex which has sites for the binding of synergestic ligands GABA, barbiturates and benzodiazepines. Binding of each of these ligands promote conductance of chloride ion across the membrane with resultant hyperpolarisation of the membrane and generation of an inhibitory post-synaptic potential. Thus, the neuroinhibitory actions of benzodiazepine and barbiturates are mediated by the GABA neurotransmitter system (Paul *et al.,* 1981; Skolnick *et al.,* 1981). Increased number of GABA binding sites in diseases associated with liver dysfunction seem to mediate the increased sensitivity to barbiturates and benzodiazepines (Fessel and Conn, 1972; Fowler and Schafer, 1981; Krnjevic, 1974; Schafer *et al.,* 1981; Schenker, 1970).

Apart from this, much work has been done in hyperammonemic states on ionic balance across the cerebral membranes. Na^+, K -ATPase plays a pivotal role in maintenance of transmembrane ion gradient. Hyperammonemia is known to stimulate Na , K^+-ATPase activity. Because of the similarities in ionic radii ammonium ions may replace K^+ ions, thus increase the enzyme activity (Sadasivudu *et al.,* 1977; Subbalakshmi and Murthy, 1981). However, recently Kosenko *et al.,* (1994) reported that increased Na^+, K^+-ATPase activity in hyperammonemic states is due to decreased phosphorylation of the enzyme mediated by activation of N-methyl D-aspartate (NMDA) receptors. They also reported that ammonia induced ATP depletion is a consequence of increased Na^+, K^+-ATPase activity. Both the depletion of ATP and increased ATPase activity could be prevented by the administration of MK-801, an antagonist of NMDA receptors. They also

reported that Na , K -ATPase activity in samples from ammonia injected rats could be normalized by *in vitro* incubation with phorbol 12-myristate 13-acetate, an activator of protein kinase C. Based on these results, they suggested that ammonia induced ATP depletion might be mediated by activation of the NMDA receptor, which results in decrease in the protein kinase C mediated phosphorylation of ATPase (Kosenko *et al,* 1994). Ammonium ions are also known to inhibit outwardly directed chloride pump resulting in a shift in inhibitory post-synaptic potential (IPSP) and thus alter the post-synaptic inhibitory neurotransmission (Llinas *et al,* 1974; Lux *et al,* 1970; Raabe and Gunmit, 1975; Raabe and Lin, 1985). Raabe (1989; 1991) reported that this effect was maximum at 1mM and at high concentrations (> 2mM) NH_4^+ can depolarize neurons and interfere with synaptic transmission.

Studies on the metabolism of neurotransmitters in conditions of liver cirrhosis, surgically constructed portacaval shunting and administration of ammonium salts in experimental animals have shown that liver impairment and hyperammonemic conditions are associated with altered glutamatergic function (Butterworth *et al,* 1987; 1991; Peterson *et al.,* 1990; Rao *et al.,* 1992). Increase in Ca^2 dependent glutamate release has been reported in hyperammonemic states (Butterworth *et al.,* 1991; Erecinska *et al,* 1987; Moroni *et al.*, 1983; Nicholls and Attwell, 1990; Rao and Murthy, 1991). An increase in GABA (γ-amino butyric acid) binding sites have been reported by Ferenci *et al,* (1984) in fulminant hepatic failure. Rao *et al,* (1991) reported an increase in muscimol binding and decrease in glutamate binding in synaptic membranes isolated from CE of acute AA administered rats. Regional differences in glutamate binding has been reported in galactosamine induced HE (Reeba, 1995). Peterson *et al,* (1990) reported a decrease in NMDA subclass of glutamate

receptors in rats with portacaval anastomosis Alterations in glutamate uptake has also been reported in hyperammonemic states (Norenberg *et al,* 1985; Rao and Murthy, 1991).

Alterations in other neurotransmitter systems have also been reported in HE and hyperammonemia. Increased serotonin levels observed in hyperammonemic states appears to be due to high concentrations of its precursor tryptophan (Baldessarini and Fischer, 1973; Cummings *et al.,* 1976; Curzon *et al,* 1975) and high concentrations of phenylalanine and tyrosine promote the synthesis of false neurotransmitters which may interfere with catecholaminergic function (James *et al.*, 1976; Smith *et al.*, 1978). Decrease in neurotransmitter acetylcholine and decreased activities of choline acetyltransferase have been reported in hyperammonemic states (Braganca *et al,* 1953; Ratnakumari *et al.*, 1994). Ammonium ions are known to elevate cholinesterase activities (Sadasivudu *et al,* 1983; Rukmini and Murthy, 1993) and interfere with packing of acetylcholine into Torpedo synaptic vesicles (Anderson *et al,* 1982; Van Der Kloot, 1987).

The available information in HE and hyperammonemic states shows that many neurotransmitter systems might be involved in pathogenesis of these syndromes. Knowledge about the functional status of neurotransmitter systems except glutamatergic system is very meagre in hyperammonemia. The little information reported on cholinergic system fails to give details about metabolism and neurotransmitter functions of acetylcholine such as 1. availability of precursors for acetylcholine synthesis; 2. release and post-synaptic action of acetylcholine; and 3. inactivation of acetylcholine catalyzed by acetylcholinesterase (AChE). Present study is concentrated on few of these aspects 1. Synthesis of acetylcholine using glucose as precursor for acetyl moiety of acetylcholine 2. choline (one of the precursors for acetylcholine) uptake in

synaptosomes 3. Nicotinic and muscarinic receptor ligand binding 4. choline acetyltransferase (CAT) and cholinesterase activities in AA and MSI induced hyperamrnonemia.

It is very clear that ammonia at pathophysiological concentrations is neurotoxic and interferes with many metabolic and neurotransmitter functions of brain. Inspite of much work, still an efficient therapeutic treatment has not been developed which is evident from the deaths due to liver dysfunction and those who survived suffer with neurological disorders like mental retardation (Conn and Lieberthal 1979, Cooper and Plum 1987). Despite improvements in intensive care therapy, the mortality of acute liver failure still remains around 80%. This could be because of the complex neurotransmitter systems involved in brain management. Hence, the better understanding of molecular mechanism of ammonia toxicity may help in adopting efficient therapeutic measures. Present study on acetylcholine metabolism and neurotransmitter functions might give an insight into the status of cholinergic function in hyperammonemic states.

Diagnosis and Therapy of Hepatic Encephalopathy and Hyperammonemia

The diagnosis of HE is mainly based on detection of various degrees of mental impairment along with biochemical and neuromuscular abnormalities. Three components of mental state may be impaired in HE: the level of consciousness, personality and the upper intellectual functions. The most common neuromuscular abnormality is asterixis (flappy tremor) in lower degrees of HE and in advanced stages muscular rigidity and hyper flexia are common. Based on the severity of these parameters, HE has been classified into four grades. To assess the severity of HE more accurately, a numerical index (Porto Systemic encephalopathy index), described by Conn and Lieberthal (1979), is being used.

As ammonia is suspected to be an important factor in the cerebral dysfunction of hepatic failure and in other disorders in which pronounced hyeprammonemia occurs, reducing the degree of hyperammonemia was taken as one of the primary objectives of therapy. Based on this and many other factors, therapeutic measures have been developed for these disorders (Conn and Lieberthal, 1979; Cooper and Plum, 1987; Ferenci *et al.*, 1989; Gerlach, 1994; James *et ah*, 1979; Mas *et al.*, 1994; Morsy and Caskey, 1994; Schenker *et al.*, 1974)

1. Administration of lactulose, lactulose + antibiotics like neomycin and dietary restriction aid in decreased production of ammonia in intestine

2. Infusion of BCAA and BCKA to compete with AAA at the BBB

3. Infusion of amino acids which stimulate the urea cycle (L-arginine, L-ornithine, L-citrulline, L-ornithine+L-aspartate, N-acetylglutamate, N-carbamyl glutamate, etc.) gives protection against ammonia intoxication in patients with functional liver.

4. To intervene at the level of neurotransmitter metabolism with agents like levodopa.

5. Oral flumazenil administration has been used exceptionally in the management of chronic HE.

6. Attempts have been made in developing a model for gene therapy for urea cycle disorders

7. Recently hybrid artificial liver systems have been used as extracorporel transient liver support therapy

8. In advanced stages, dialysis and finally liver transplantation are suggested.

Aim and Scope of the Present Investigation

Hyperammonemia is associated with many pathophysiological and neurological disorders and ammonia is known to act as a neurotoxin in all these disorders. Despite much work, still the exact molecular mechanism of ammonia toxicity remains unclear due to several controversies. This could be because of the multiple neurotransmitter systems involved in brain function and heterogeneity of the brain tissue. For better understanding of the mechanism of ammonia toxicity, there is a need to study the neurotransmitter systems involved in ammonia toxicity. Though much work has been done in glutamatergic system, not much information is available on the functional status of other neurotransmitter systems in hyperammonemic conditions.

Present study is concentrated on metabolism and neurotransmitter functions of acetylcholine in hyperammonemic states induced by administration of either AA or MSI in a rat model. The rationale in selecting acetylcholine is that, its synthesis from acetyl CoA was shown to be dependent on pyruvate generated from glycolysis. Earlier studies in hyperammonemic states demonstrated marked changes in the cerebral activities of glycolysis and citric acid cycle enzymes (Murthy and Hertz, 1988; Ratnakumari *et al*., 1985; 1986; Ratnakumari and Murthy, 1989). Since acetyl CoA synthesis is intimately associated with metabolism of glucose, it was felt worthwhile to study the cholinergic function in hyperammonemic states. Such a study would help in understanding the mechanisms involved in neural dysfunction associated with hyperammonemia. The knowledge thus gained would help in the design of new therapeutic approaches for diseases associated with hyperammonemia.

PART II

EXPERIMENTAL

Chemicals

Acetylcholine chloride, acetylcholinesterase, acetyl-CoA, acetylthiocholine iodide, adenosine 5`-triphosphate, ammonium reineckate, bovine serum albumin, l,5-bis(4-allyldimethyl-ammoniumphenyl)pentan-3-one dibromide (BW 284 C51), l,4-bis(5-phenyl oxazole)benzene (POPOP), choline chloride, 2,5-diphenyloxazole (PPO), 5,5`-dithiobis-(2-nitrobenzoic acid) (DTNB), eserine sulfate, hyamine hydroxide, nicotine, polyethylenimine and tetraphenylboron were purchased from Sigma Chemical Co., USA. Ammonium acetate, and tetraethylammonium chloride were purchased from Merck, Germany. Ficoll-400 was purchased from Pharmacia Fine Chemicals, Sweden. Sucrose was purchased from BDH, England. Pirenzepine hydrochloride was purchased from Research Biochemicals Inc., Natick, USA. [^{14}C]Acetyl CoA (200 mCi/mmole) and [^{3}H]L-nicotine (82 Ci/mmole) were purchased from Radiolabelled Chemicals Inc., USA. [^{14}C]Choline (45.5 mCi/mmole) and [U-^{14}C]glucose (53 mCi/mmole) were purchased from Bhabha Atomic Research Center, India. [^{3}H]Quinuclidinyl benzilate (43.5 Ci/mmole) was purchased from Dupont, NEN products, Boston, USA.

Animals

Adult albino Wistar rats weighing 200-240 g were used in the present study. Animals were maintained in cages with free access to food and water under natural light (12 hr.) and dark (12 hr.) cycles at 25°C in air conditioned rooms. Balanced pellet diet (Hindustan Lever Ltd., India) and water was provided *ad libitum.*

Induction of Hyperammonemia

Hyperammonemia was induced by intraperitoneal administration of either AA or MSI. AA is a direct source of ammonia, and MSI increases blood and brain ammonia levels by inhibiting glutamine synthetase.

Induction of Hyperammonemia with AA

Hyperammonemia was induced as per the method described by Ratnakumari and Murthy (1989).

Acute (convulsive) Group: Animals were rendered hyperammonemic by intraperitoneal administration of single dose (25 mmoles/Kg body wt.) of AA dissolved in saline and pH adjusted to 7.4. Animals were decapitated either during convulsive phase or at different time intervals after administration of the drug.

Subacute (non convulsive) Group: Hyperammonemia was induced in this group of animals by intraperitoneal administration of 2.5 mmoles of AA/Kg body weight. Animals were decapitated 20 min. after administration of the drug to keep parity with the acute group animals.

Induction of Hyperammonemia with MSI

Hyperammonemia was induced with MSI, following the method described by Subbalakshmi and Murthy (1983). A single dose of MSI, dissolved in saline and pH adjusted to 7.4 (300 mg/kg body wt.), was administered intraperitoneally to the animals. Rats in this group were killed 3.0 hr. after administration of the drug (just before entering into the convulsive phase).

***In vitro* Experiments**

To test the direct effects of AA, all the experiments were performed with the preparations of normal rats in presence of 1, 2, 5, or 10 mM AA.

Preparation of Brain/Serum Extracts for Ammonia Estimation

Animals were decapitated and the head was allowed to fall directly into liquid nitrogen and was allowed to freeze for 15 min. at this temperature. Brains were chiseled out of the cranial vault and powdered in stainless steel mortar under liquid nitrogen. Powdered tissue was

immediately transferred to pre-weighed tubes containing 3 ml of 5% ice-cold PCA, the tubes were re-weighed and was homogenized at 4°C in Potter-Elvehjem homogenizer with serrated teflon pestle. Homogenate was centrifuged at 5000 rpm at 4°C and the supernatant was collected. The supernatant was neutralized with solid potassium carbonate in an ice bath and the precipitated potassium perchlorate was removed by centrifugation at 10,000 rpm for 15 min at 4°C. The neutralized extract was used for ammonia estimation.

Blood from neck wound of the animal was collected into centrifuge tubes, allowed to clot at room temperature. This was centrifuged at 4°C and 5000 rpm and serum was collected. To 0.5 ml of serum, 1 ml ice-cold PCA was added and kept in ice for 15 min. Supernatant was collected by centrifugation and was neutralized with solid potassium carbonate. The precipitated perchlorate was removed by centrifugation and the neutralized supernatant was used for ammonia estimation.

Preparation of Synaptosomes using Sucrose Density Gradients

Synaptosomes were prepared followed by the method of Whittaker and Barker (1972). Immediately after decapitation, brains were removed from cranial vaults of normal and hyperammonemic rats and washed with ice-cold 0.32 M sucrose. Brains were pressed between two sheets of Whatman No. 1 filter paper to remove excess fluid, surface capillaries and meninges. CC, CE and BS were separated from the brains and homogenates were prepared (5% W/V) using motor driven Potter-Elvehjem homogenizer with a serrated teflon pestle at 800 rpm with 8-10 up and down strokes. Homogenates were centrifuged at 1000g for 5 min. and the resultant pellet (P_1) was discarded. The supernatant (S_1) was centrifuged at 15,000g for 15 min. to obtain crude mitochondrial pellet (P_2) and post-mitochondrial supernatant (S_2). The P_2 pellet was suspended in

0.32 M sucrose and was layered on to a pre-formed discontinuous sucrose density gradient (15 ml each of 0.8 M and 1.2 M sucrose). Tubes were centrifuged at 53,000g for 2 hrs. Synaptosomes, present at the interface of 0.8 M and 1.2 M sucrose layers were aspirated, diluted with 0.32 M sucrose and centrifuged at 15,000g for 20 min. The synaptosomal pellet, thus obtained, was suspended in 0.32 M sucrose and used for enzyme assays. All the centrifugations were done at 4°C.

Preparation of Synaptosomes using Ficoll-400 Gradients

Synaptosomes were prepared following the method of Cotman (1974). The P_2 pellet obtained as described above was suspended in 0.32 M sucrose. This was layered on pre-formed discontinuous Ficoll-400 density gradient (10 ml each of 4%, 6% and 13% Ficoll-400 in 0.32 M sucrose). The tubes were centrifuged at 63,000g for 45 min. Synaptosomes present at the interface of 6% and 13% Ficoll layers, were aspirated, diluted with 0.32 M sucrose and centrifuged at 15,000g for 20 min. Final pellet was suspended in appropriate medium and used for further studies.

Preparation of Membranes

Membranes were prepared from CC, CE, and BS of normal and hyperammonemic rats according to the method of Rao *et al.*, (1991). Homogenate (5%) was prepared in 0.32 M sucrose containing 5 mM Tris-HCI buffer pH 7.4. The homogenate was centrifuged at 1000g for 5 min. at 4°C. The supernatant was diluted with 3 volumes of ice-cold double distilled water and centrifuged at 36,400g for 15 min. at 4°C. The pellet was homogenized in 5 mM Tris buffer and centrifuged at 36,400g for 15 min. at 4°C. The pellet was homogenized in 40 mM Tris-HCI buffer pH 7.4 and centrifuged at 36,400g for 15 min. This step was repeated twice. The final pellet containing membranes was suspended in 40 mM Tris-HCI buffer and used for binding studies.

Estimation of Serum and Brain Ammonia

Ammonia content of brain and serum was determined by using phenol-hypochlorite reagent as described by Rao (1991). To 1 ml of supernatant (obtained after neutralization with potassium carbonate), 1.5 ml of solution A (50g of phenol and 250 mg sodium nitroprusside in 3.75 1 water) and 2 ml of solution B (8.4g NaOH, 89.2g disodium hydrogen phosphate and 10 ml of 5% sodium hypochlorite/1) were added. After 20 min at room temperature, absorbance of the sample was read at 630 nm. Ammonium chloride (0.1-1.0 μmoles) was used as standard.

Protein Estimation

Protein content was estimated by the method of Lowry *et al*., (1951) using bovine serum albumin as standard.

Cholinesterases

Both acetyl (AChE) and pseudocholinesterases (PChE) were assayed by the method of Ellman *et al*., (1961) as described by Whittaker and Barker (1972). Thiocholine formed by the action of AChE on acetylthiocholine, reacts with DTNB to give a yellow anion 5-thio-2-nitrobenzoate and 2-nitrobenzoate-5-mercaptothiocholine. The formation of yellow anion was continuously monitored colorimetrically for five minutes at 420 nm. The absorption coefficient of yellow anion 1.36 x m mol^{-1} x mm^{-1} was used for the calculation of enzyme activity.

Reaction mixture for total cholinesterase consisted of 100 mM phosphate buffer pH (7.9), 0.5 mM acetylthiocholine iodide, 0.3 mM DTNB and 10 μg synaptosomal protein in a final volume of 1 ml. The reaction was initiated with the addition of substrate and change in absorbance was measured at 420 nm for 5 min.

For the assay of PChE, protein was preincubated for 10 min with 0.2 mM 1,5 bis(4-allyldimethylammonium-phenyl)pentan-3-one dibromide

(BW 284 C51) (AChE inhibitor) and reaction was initiated with the addition of acetylthiocholine iodide. Difference in the activities of cholinesterases measured in absence (total cholinesterase) and in presence (PChE) of inhibitor was taken as AChE activity. For the assay of cholinesterases, synaptosomes were kept frozen at -20°C, and the assay was done, a day after the isolation.

Choline Acetyltransferase

Choline acetyltransferase was assayed according to the method of Fonnum (1975). Reaction mixture consists of 0.2 mM [^{3}H]acetyl CoA (4800 dpm/nmole), 50 mM phosphate buffer, 300 mM sodium chloride, 8 mM choline chloride, 20 mM EDTA, 0.2 mM BW 284 C51, 0.02% Triton X-100 and 30 µg of synaptosomal protein in a final volume of 50 µl. In blanks, BW 284 C51 was excluded. The protein was preincubated with BW 284 C51 for 20 min. except for blanks. Reaction was initiated with the addition of [^{3}H]acetyl CoA and incubated at 37°C for 15 min. The reaction was stopped by transferring the Eppendorf tubes into a scintillation vial containing 3 ml of 10 mM phosphate buffer. Acetylcholine was extracted into 10 ml toluene scintillation fluid (0.05% PPO and 0.02% POPOP in toluene) with 2 ml acetonitrile containing 10 mg tetraphenylboron. Radioactivity was determined with Beckman LS-1800 liquid scintillation spectrometer for 5 min. Prior to these experiments, optimum protein and optimum time of incubation were determined.

Choline Uptake into Synaptosomes

Incubation medium for choline uptake consists of well oxygenated Krebs Ringer-phosphate-glucose-HEPES medium pH 7.4 (HEPES 10 mM, $NaHPO_4$ 10 mM, NaCl 110 mM, KC1 5 mM, $CaCl_2$ 1 mM, Mg SO_4 1.3 mM, glucose 5 mM), eserine sulphate 0.2 mM, and [^{14}C]choline (0.2 µCi, 0.5 µM to 100 µM) in a final volume of 500 µl. The reaction mixture was

incubated at 37° C for 5 min. and uptake was started by the addition of 300 μg of synaptosomal protein and incubation was continued at 37° C for 5 min. with constant shaking. Uptake was terminated by rapid centrifugation at 10,000 rpm for 1 min. and the supernatant was discarded. Pellet was washed twice with one ml of buffer containing the same concentration of non-radioactive choline. The protein pellet was dissolved in 100 μl of 0.1 N sodium hydroxide and transferred to a scintillation vial containing 5 ml Bray's mixture (PPO 4 g, POPOP 200 mg, naphthalene 60 g, methanol 100 ml, and ethylene glycol 20 ml made to 1 litre with 1,4-Dioxan). After twelve hours of storage at room temperature (to reduce the chemiluminiscence), radioactivity was determined in Beckman LS-1800 liquid scintillation spectrometer. Optimum amount of protein and optimum time was determined as a part of preliminary standardization. In the former, 100 to 1000 μg of synaptosomal protein was incubated for 15 min. in reaction mixture containing 100 |iM choline. For the latter, 300 μg protein was incubated in reaction mixture containing 100 μM choline for 2 to 60 min. In each experiment, non-specific uptake was determined by carrying out incubations at 0° C and due corrections were made to obtain the specific uptake.

$^{14}CO_2$ Production from [U-^{14}C]Glucose

$^{14}CO_2$ produced from [U-^{14}C]glucose was measured by the method of Ratnakumari (1990). Reaction mixture contained NaCl 110 mM, KC1 5 mM, $CaCl_2$ 1 mM, Mg SO_4 1.3 mM, glucose 2.5 mM, and 1 mg synaptosomal protein in a final volume of 1 ml. The reaction was performed in vials closed with air tight rubber stoppers from which center wells containing a strip of Whatman No.1 paper, were suspended. Reaction was started by the injection of 1 mg protein into the vial and incubated at 37°C for 45 min. in a shaker water bath. The reaction was

terminated by injecting 200 µl of hyamine hydroxide into a center well and 200 µl of 3 M PC A into a reaction vial. To controls, PC A was added before commencing the reaction. Incubations were continued for another 1 hr. at 37°C, then the center wells were removed and transferred to scintillation vials containing 10 ml Bray's scintillation fluid. Radioactivity was determined in LS-1800 liquid scintillation spectrometer for 5 min.

Synthesis of Acetylcholine from [U-^{14}C]Glucose

Synthesis of acetylcholine from [U-^{14}C]glucose was studied by the method of Gibson *et a!.,* (1975) with few modifications. Reaction mixture contains sodium phosphate 10 mM, $CaCl_2$ 1 mM, $MgSO_4$ 1.3 mM, KC1 5 mM, Choline chloride 2 mM, glucose 2.5 mM and eserine sulfate 0.5 mM adjusted to pH 7.4 in a final volume of 1 ml. Synaptosomes were pre-incubated with 0.5 mM eserine sulfate for 20 min. and the reaction was initiated by the addition of labeled glucose (2.5 mM) and incubated for 45 min. at 37° C with constant shaking. In blanks, eserine sulfate was omitted. The reaction was terminated with the addition of 0.5 ml of 1.4 M PCA containing 35 mM tetraethylammonium chloride (TEAC). Flasks were rinsed twice with 0.5 ml of 0.2 M PCA containing 5 mM TEAC and transferred to centrifuge tubes, homogenized, centrifuged and the supernatant was used for acetylcholine estimation.

Estimation of Acetylcholine

Acetylcholine present in the supernatant was precipitated with 3.5 ml of ice-cold ammonium reineckate, adjusted to pH 2.0 with concentrated PCA. The precipitation was allowed to proceed for 1 hı in ice. Tubes were centrifuged at 25,000g for 20 min. and the precipitate was washed twice with 5 ml of 0.2 M PCA. The final pellet was suspended in 2 ml methanol adjusted to pH 4.5 with concentrated acetic acid. The tubes were centrifuged and 0.5 ml supernatant was added to scintillation vials

containing 10 ml Bray's scintillation fluid. Radioactivity was determined with LS-1800 liquid scintillation spectrometer.

Binding Studies

Nicotinic and muscarinic receptor binding was studied with specific ligands [^{3}H]nicotine and [^{3}H]quinuclidinyl benzilate (QNB) in synaptic membranes isolated from CC, CE and BS of normal and AA treated rats.

[^{3}H]Nicotine Binding

Synaptic membrane protein (200 μg) was incubated with 10 nM [^{3}H]nicotine in 500 μl of 40 raM Tris-HCl buffer pH 7.4 for 15 min. at 37° C. Binding was terminated by diluting the incubation mixture with 6 ml ice-cold Tris-HCl buffer and rapid filtration under negative pressure through GF/C glass microfiber filters followed by two washes with 6 ml ice-cold buffer. Non-specific binding was determined by parallel assays performed in presence of 100 μM unlabelled nicotine. Filters presoaked in 0.1% polyethylenimine were used, to reduce the non-specific binding of ligand to filters. Filters were transferred to scintillation vial containing 5 ml of Bray's mixture and radioactivity was determined with liquid scintillation spectrometer. Specific binding was taken as the difference between total binding and non specific binding.

Reversibility of nicotine binding was studied by incubating 200 μg of membrane protein with 20 nM [^{3}H]nicotine in 40 mM Tris-HCl buffer for 30 min. at 37°C, and various concentration of unlabelled nicotine was added. Binding was stopped 10 min. after the addition of unlabelled nicotine. Amount of nicotine bound was determined as described above.

[^{3}H]QNB Binding

Synaptic membrane protein (200 ug) was incubated with 1 nM [^{3}H]QNB in 500 μl of 40 mM Tris-HCl buffer pH 7.4 for 15 min. at 37° C. Non-specific binding was determined in parallel assays performed in

presence of 100 μM atropine. In order to study the subtypes of muscarinic receptors, 50 nM pirenzepine was used to block the M_1 receptors and [H]QNB bound under these conditions was considered as M_2 binding. M_1 binding was taken as the difference between the binding in absence and in presence of pirenzepine. The binding was stopped by diluting the assay mixture with 6 ml ice-cold Tris-HCl buffer and rapid filtration under vacuum through GF/C glass microfiber filters followed by two washes with 6 ml Tris-HCl buffer. To reduce the non specific binding of the ligand to filters, filters presoaked in 0.1% (W/V) polyethylenimine were used. Filters were transferred to scintillation vial containing 5 ml Bray's mixture and radioactivity was determined with Beckman LS-1800 liquid scintillation spectrometer at 55% efficiency. Specific binding was taken as difference between binding performed in absence and in presence of atropine. Prior to this optimum protein concentration and optimum time of incubation were standardized.

Reversibility of QNB binding was studied by incubating 200 μg of membrane protein with 1 nM [^{3}H]QNB in 40 mM Tris-HCl buffer for 30 min at 37°C, and 100 μM atropine was added. Binding was stopped either immediately or at various time periods by dilution and filtration as described above. To determine the K_d and B_{max} values, assay was performed with various QNB concentrations (0.005-5.0 nM)

Calculation of Kinetic Constants

Kinetic constants for enzyme assays, uptake studies and binding experiments were calculated by Scatchard analysis of the data using Sigma Plot program and plot statistics option.

Statistical Methods

Neuman-Keul's multiple range test was used to calculate the statistical significance between more than two groups and Student's t-test was used to calculate the statistical significance between two groups.

PART III

RESULTS AND DISCUSSION

CHAPTER 1
PRELIMINARY STUDIES

Patency of Hyperammonemia

Hyperammonemia is a consistent finding in conditions of liver dysfunction such as liver cirrhosis, necrosis, fulminant hepatic failure and other abnormalities like defects in urea cycle enzymes. As ammonia is considered to play a main role in pathogenesis of all these disorders, increased blood ammonia levels is used as one of the criteria in diagnosing the severity of the disease. Ammonia diffuses through BBB and rapidly equilibrates with brain ammonia (Cooper *et al.,* 1979). Hence, alterations in blood ammonia levels are rapidly reflected in brain. In the present study, animals were injected with ammonium salts or with hyperammonemia inducing drug, MSI. Patency of these two models has been assessed by estimating brain and blood ammonia levels.

In rats administered with acute dose of AA, blood and brain ammonia concentrations were estimated 5, 10 and 20 min. after administration of the drug. In acute MSI and subacute dose of AA administered rats, ammonia levels were estimated at the end of 3 hr. and 20 min. respectively after administration.

In control animals, brain ammonia content (280 μM) was about four times to the serum ammonia content (70 |iM) and the values were within the range of values reported earlier (Cooper *et al.,* 1985; Ehrlich *et al.,* 1980; Hindfelt *et al.,* 1977; Rao and Murthy, 1991; Ratnakumari and Murthy, 1989). Administration of AA or MSI elevated the levels of ammonia both in blood and brain (Fig. 1-3). In animals administered with acute dose of AA, rise in blood and brain ammonia levels was similar up to 10 min., indicating a rapid equilibration of these two pools of ammonia.

But at the end of 20 min. brain ammonia content increased only two fold while four fold increase was observed in serum ammonia levels, when compared to earlier time period. This suggested that brain ammonia levels might be saturated at 2 mM and subsequent changes in blood ammonia levels had very little effect on brain ammonia levels. This value represents a seven fold increase in the cerebral ammonia content of a normal animal (Fig. la and b) Manifestation of convulsions was seen as brain ammonia reaches this value. In animals administered with subacute dose of AA, rise in blood and brain ammonia levels (190 and 90%, respectively) was of lesser magnitude than in acute group of animals (Fig. 1.2). There was no significant difference in the ammonia content of synaptosomes isolated from normal (8.63 ± 1.2 nmoles/mg protein, n = 4) and hyperammonemic rats (10.1 ± 0.7 nmoles/mg, n = 4, p > 0.1). Lack of changes in ammonia content of synaptosomes isolated from normal and experimental animals might be due to the diffusion and dilution of ammonia into the medium during homogenization, centrifugation.

Changes observed in blood and brain ammonia levels in the present study are in agreement with values reported earlier (Rao, 1991; Ratnakumari and Murthy, 1989). Similar changes have been reported in cerebral ammonia content in other animal models for hyperammonemia and also in human subjects with hyperammonemic disorders (Butterworth *et al*., 1988; Hindfelt *et ai,* 1977; Rao *et ai,* 1992). Results of the present study indicate that the brain levels of ammonia are at least 3-4 fold higher than that of serum ammonia levels. Similar reports were also made by other investigators (Rao and Murthy, 1991; Ratnakumari and Murthy, 1989). It has been suggested that the brain pH (6.5-6.8) might be lower than that of blood and cerebrospinal fluid. As a result ammonia entering

the cell quickly forms an ammonium ion thus facilitating the accumulation of ammonia in brain (Cooper *et al.,* 1979; Hindfelt, 1975; Hsia, 1974).

Regional variations have been reported in the efficiency of ammonia detoxification and *CC* was found to be less efficient in removing the incoming ammonia (Butterworth *et al.,* 1988). Interestingly very few studies were conducted on the regional variation in cerebral ammonia content. Though such a study is desirable, methodological limitations precludes that study. For example, in the present study brains were frozen in liquid nitrogen to arrest post-mortem changes. Under such conditions, it becomes very difficult to dissect different regions of brain. Hence, in the present study, ammonia content was estimated in whole brain extracts.

Administration of MSI elevated the blood and brain ammonia levels (170 ± 19 μM, 1.07 ± 0.2 mM) by 2.5 fold and 3.8 fold respectively (Fig. 1.3). These results are in agreement with the results reported by the earlier investigators (Folbergroa *et al.,* 1969; Gutierrez and Norenberg, 1977). Rise in blood and brain ammonia content in MSI treated animals is known to be due to the irreversible inhibition of glutamine synthetase.

Behavioral Changes

A sequence of behavioral changes were observed following the administration of AA. In these rats the onset of changes were observed immediately after administration of the drug. After 5 min. of AA administration animals became dull and exhibited splayed leggedness. The hind limbs were affected first as a result the animals were unable to move forward and exhibited rotational behavior. This stage was followed by the loss of equilibrium and if the posture was altered manually, the animal was unable to correct its posture. At the end of 15 min. animals became unconscious and the eyes were protruding. At the end of 20 min., animals exhibited severe convulsions. These animals very rarely entered into

coma. Mortality rate was very high at this stage, hence, were killed just when they entered into convulsive phase. No such changes were observed in rats administered with subacute dose of AA. Behavioral changes observed in MSI administered rats were similar as reported earlier (Jyothi, 1994; Ratnakumari *et al*., 1985; Subbalakshmi and Murthy, 1981). At the end of 1 hr, there was a progressive decrease in physical activity and at the end of 2 hr. animals exhibited splayed leggedness, wobbly gait and also involuntary movements. Three hours after the drug treatment animals lost their sense of equilibrium, this was followed by tonic and clonic convulsions. Mortality rate was very high at this time period, hence animals were killed at the end of 3 hr.

Purity of Synaptosomes

Initially, synaptosomes were isolated by the method of Whittaker and Barker (1972). These synaptosomes were used for determining the activities of cholinesterases and CAT. Wherein the synaptosomes were disrupted either by freeze thawing or using detergents such as Triton X-100. There was no loss in cholinesterase activity after freeze thawing compared to freshly isolated synaptosomes.

It has been observed that the functional properties of membranes were altered in synaptosomes isolated in sucrose gradients. This was evinced by very high rates of choline uptake when choline uptake was performed in these synaptosomes. Moreover in these experiments very little difference was observed between specific and non specific uptake of choline. Hence, to carryout uptake and metabolic studies synaptosomes were isolated by the method of Cotman (1974) using discontinuous density gradients of Ficoll-400. Earlier studies from this laboratory indicated that the synaptosomes isolated by this method are 80-90% pure

even in hyperammonemic states (Ratnakumari and Murthy, 1989; Rao, 1991).

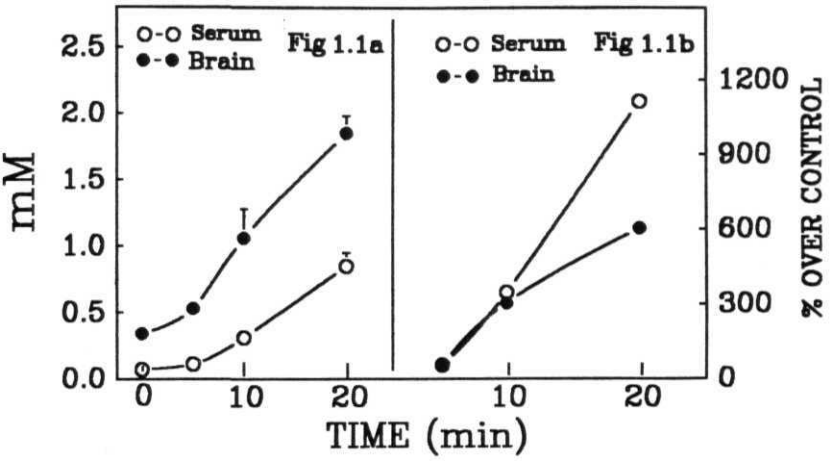
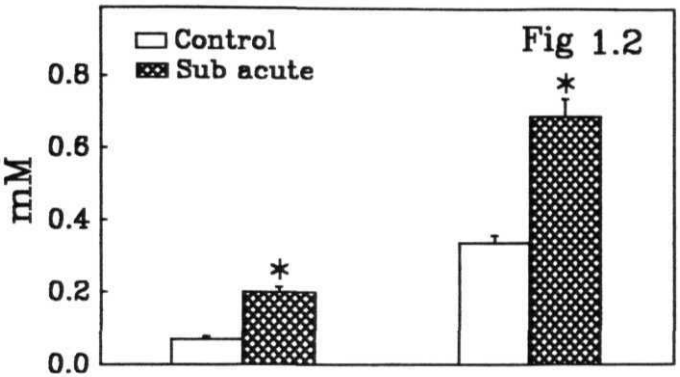


Fig 1.1: Ammonia content in serum and brain in control rats and rats administered with acute dose of AA. (a) ammonia content and (b) percentage change over control Values are Mean ± S.D of four experiments done in duplicates All values are significantly different compared to controls ($p < 0\ 05$). Brain water content was assumed to be 80% and values obtained in μ moles were converted accordingly to mM Values at t = 0 indicate the ammonia content in control animals



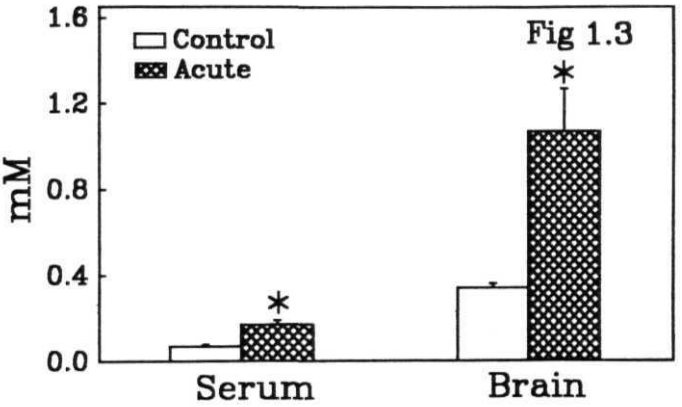


Fig. 1.2: Ammonia content in serum and brain in control rats and rats administered with subacute dose of AA

Fig 1.3: Ammonia content in serum and brain in control rats and rats administered with acute dose of MSI Values are Mean ± S D of three experiments done in duplicates. *. Statistically significant with controls ($p<0.05$)

CHAPTER 2
ACETYLCHOLINE SYNTHESIS

Acetylcholine and Cholinergic System

Cells in CNS communicate with each other either electrically or chemically. The neurons communicate with each other through specialized zones called "synapses". The communication in a great majority of synapses in mammalian CNS is mainly mediated by chemicals called "neurotransmitters" released by the presynaptic neuron. This synaptic transmission provides a basis for intercellular communication in nervous system. Nearly 50 putative neurotransmitters have been identified in brain, among which glutamate, acetylcholine, GABA and glycine play a major role in central synaptic transmission.

The term "Cholinergic System" is used to describe pathways of central and peripheral nervous systems that use acetylcholine as neurotransmitter. Cholinergic neurons in CNS are important components of neural circuitry of learning, memory and cognition. The cholinergic cells, located in the basal fore brain are involved in memory and arousal (Bartus *et **ai,*** 1982; Collerton, 1986; Smith, 1988; Stewart *et ai,* 1984;). Pontomesencephalic cholinergic neurons play a role in sleep wakefulness locomotor behavior and memory (Garcia-Rill *et ai,* 1987; Kessler *et ai,* 1986; Webster and Jones, 1988). Cholinergic neurons localized in the striatum play a role in a variety of voluntary locomotor behaviors (Costall *et **ai,*** 1972; Kelly *et ai,* 1989; Vrijmoed-DeVries and Cools, 1986).

Acetylcholine acts as neurotransmitter, neuromodulator and some times as local hormone and trophic factor. Acetylcholine plays a role in cellular differentiation (Lauder, 1993), regulates neurite outgrowth during early development through both nicotinic and muscarinic receptors (Lipton

et al, 1988; Small *et al,* 1995; Zheng *et al,* 1994). It also inhibits the differentiation of haemopoietic stem cells into megakaryocytes (Patinkin *et al,* 1990). Acetylcholine in placenta might play a role in transport mechanisms. Acetylcholine is present in highest concentrations in corneal epithelium, its role in this tissue is not yet known.

Acetylcholine is synthesized in neuronal perikarya and nerve terminals. In nerve terminals, acetylcholine is stored in synaptic vesicles and upon receiving Ca^{2+} signal (Dunlap *et al,* 1995) generated by incoming action potential, it is released into synaptic cleft by exocytosis mediated by several proteins, such as Ca^{2} binding proteins, rab 3a, synapsin 1, synaptotagmin, synaptobrevin, syntaxin and SNAP-25 (Bennett and Scheller, 1994; Geppert *et al.,* 1994; Lledo *et al.,* 1994; Rasahl *et al,* 1993). Exocytosis in neurons followed by endocytotic membrane retrieval which is triggered by an increase in cytosolic Ca^{2+} concentration (Burgoyne and Morgan, 1995). It has been suggested that modulation of neurotransmitter release, which involves cascade of steps might play a role in synaptic plasticity, the molecular basis of learning (Catsicas *et al,* 1994). Once the transmitter is released, it binds to either pre-synaptic (autoreceptors: regulates the release of transmitter) or post-synaptic receptors (transmits or evokes the same response in post-synaptic neuron). After transmitting the signal, the neurotransmitter function of acetylcholine is terminated by the action of AChE which is present on out side of the synaptic membrane and the product choline is taken up by nerve terminals for reacetylation. Hence, the neurotransmitter function of acetylcholine involves

1. Synthesis of acetylcholine
2. Release of acetylcholine upon stimulation
3. Binding of the transmitter to receptors
4. Termination of neurotransmitter function by cholinesterase

Choline Acetyltransferase

Acetylcholine is synthesized from acetyl CoA and choline by the action of choline-o-acetyltransferase (EC 2.3.1.6). The reaction involves reversible transfer of an acetyl group from acetyl CoA to choline (Nachmanson and Machado, 1943). In the nervous system, (CAT) is expressed selectively in cholinergic neurons (Cheney *et al,* 1976; Rossier, 1977a). The gene for CAT was reported to encode two proteins, CAT and vesicular acetylcholine transporter (Grosman *et al,* 1995). Bejanin *et al,* (1994) reported that the gene encoding putative rat vesicular acetylcholine transporter (VAT) is located on the sense strand of the first intron of CAT gene. They have also reported that the two mRNAs would be produced by alternative splicing. The CAT and VAT genes have been reported to be unique, since both coding sequences lie in the same orientation and both their products are required to express the cholinergic phenotype (Bejanin *et al,* 1994). This linkage was conserved from caenorhabditis (Rand, 1989) to mammals and this conservation between two evolutionarly separated species indicates its functional significance. In mammalian brain, CAT is expressed specifically in cholinergic neurons and hence is used as a marker for these neurons.

CAT has been purified from variety of sources (Bruce *et al,* 1985; Dietz and Salvaterra, 1980; Ryan and McClure, 1979) and used to produce both polyclonal and monoclonal antibodies (Bruce *et al,* 1985; Crawford *et al,* 1982; Eckenstein *et al,* 1981; Levey *et al,* 1981). Specific antibodies raised against this enzyme were used in immunohistochemical mapping of cholinergic pathways (Berrard *et al,* 1987). It has been reported that CAT is synthesized in the cell body of neuron and transported to neuronal processes by slow axonal transport (Dahlstrom, 1983; Heiwall *et al,* 1979; Saunders *et al,* 1973, Tucek, 1975). In the

experiments with subcellular fractionation of brain, the highest proportion of CAT is recovered in nerve terminal fraction, the major site of acetylcholine synthesis (Tucek, 1967; Whittaker, 1965).

Exact localization of this enzyme within the nerve terminal has been the subject of controversy. Though it is established that CAT is a cytosolic enzyme (Fonnum, 1968; Rossier, 1977a) several reports indicated a association of significant amounts of this enzyme with nerve terminal membranes (Benishin and Carroll, 1983; Peng *et al.*, 1986; Smith and Carroll, 1993). It has been reported that, soluble forms of CAT can bind reversibly to membranes (Fonnum, 1968) and a membrane bound form of CAT has been described which is not dissociated from synaptosomal membranes with salt washes (Smith and Carroll, 1993). They suggested that binding of CAT to plasma membranes could represent a regulatory mechanism in acetylcholine synthesis. It has been suggested that the isozymes of CAT differ in their outer charge and thus in their affinities for the membrane (Fonnum and Mathe-Sorenssen, 1973). It is yet to be resolved which CAT of the two forms is physiologically relevant (Cooper, 1994). Schmidt and Rylett (1993a) reported that chloride channel blockers or low chloride medium reduced the membrane bound CAT to 10%, without impairing the basal acetylcholine synthesis. They suggested that membrane bound CAT may not play a significant role in cerebral acetylcholine synthesis. The K_m of choline for brain CAT is in the range of 0.4-1.0 mM and for acetyl CoA 7-45 μM. The activity of the isolated enzyme in the presence of optimal concentrations of cofactors was reported to be far greater than that reflected at *in vivo* concentrations of choline and acetyl CoA, 50 μM and 5-7 μM respectively (Tucek, 1985). Hence, it was suggested that in *in vivo* conditions CAT activity would depend on the availability of precursors.

Under *in vivo* conditions, CAT activity might be influenced by factors like intraterminal ionic environment (Hersh and Peet, 1978; Rossier, 1977b) and possibly by calcium dependent kinase mediated phosphorylation (Bruce and Hersh, 1989; Schmidt and Rylett, 1993b). Rossiet (1977b) reported that the kinetic properties of CAT were affected by the chloride ion. They suggested that the chloride ions entering presynaptic nerve terminals during the action potential activate CAT and increase its V_{max}. They also suggested that the affinity of the enzyme for both of its substrates was decreased, rendering the catalytic activity of the enzyme to depend more on intraneuronal choline and acetyl CoA concentrations and at the same time the K_i of the enzyme for acetylcholine was increased which accelerates the acetylcholine synthesis. Phosphorylation of the enzyme was reported to enhance its activity. Phosphorylated CAT was purified from freshly prepared rat brain synaptosomes by Bruce and Hersh (1989). They suggested that the phosphorylation of enzyme might regulate the function in ways other than directly altering the enzyme activity, probably by protecting against the action of proteases. The phosphorylation could affect subcellular localization and was suggested that phosphorylation lowered the affinity of the enzyme protein to synaptic membranes when compared to the native protein (Bruce and Hersh, 1989).

CAT activity is modulated by several hormones and growth factors viz. thyroid hormone (Hefti *et al,* 1986), estrogen (Luine *et al,* 1986), interleukin-3, basic fibroblast growth factor (Knusel *et al,* 1990), brain derived neurotrophic factor (Knusel *et al,* 1991) and nerve growth factor (Gnahn *et al,* 1983; Honegger and Lenoir, 1982).

Abnormalities in the structure and function of cholinergic system were observed in neurodegenerative diseases like Alzheimer's disease and Parkinson's disease (Aubert *et al,* 1992; Lange *et al,* 1993; Perry *et al.*,

1985). In Alzheimer's disease, loss of cholinergic neurons and decrease in CAT activity have been reported. Reasons for the selective loss of cholinergic neurons in this disease are yet to be elucidated. However, it has been shown that the cause of this disease has a genetic basis rather than of physiological origin. But the toxins generated during degeneration are expected to have secondary role in aggravating the disease and one of these toxins was reported to be ammonia (Seiler, 1993). In many clinical cases, hyperammonemic conditions were observed to be associated with this disease. This was strengthened by observation of Ratnakumari *et ah,* (1994). In sparse fur mutant mice (animal model for chronic hyperammonemia), they observed a decrease in CAT activity and they proposed this as an evidence for cholinergic neuronal loss in hyperammonemic states. However, no detailed studies were conducted on the effects of ammonia on cholinergic neuronal function. Hence, this aspect was studied in the present investigation.

Presently, CAT was assayed in synaptosomes prepared from CC, CE and BS of control, acute AA and acute MSI treated rats. Preliminary standardization of the enzyme was done with synaptosomes isolated from the CC of control rats. The reaction was linear upto 60 μg of synaptosomal protein (Fig. 2.1). Incubation time was standardized using 30 μg of protein. The acetylcholine synthesis was linear upto 40 min. of incubation (Fig. 2.2). Hence, 30 μg synaptosomal protein and 15 min. of incubation time were chosen for further studies.

Regional differences were observed in the activity of CAT. Highest activity was observed in synaptosomes isolated from BS and CC followed by CE. The CAT activity in BS was 6.5 fold higher than in CE and 1.2 fold to that of CC (Fig. 2.3). Such differences might be due to the extent of

cholinergic innervation and expression of genes involved in the synthesis of CAT.

There was no significant change in synaptosomal CAT activity in all the three brain regions studied in rats administered with acute dose of AA. CAT activity in CC was suppressed by 15% and in BS, CAT activity was elevated by 9%, with marginal decrease in CE (Fig. 2.4). *In vitro* addition of AA suppressed CAT activity by 28% , 17%, and 13% at 5, 2 and 1 mM concentrations respectively in synaptosomes isolated from CC of control rats. Significant change was observed only with 5 mM AA (Fig. 2.5). MSI had no significant effect on CAT activity in both *in vivo* and *in vitro* conditions (Fig. 2.6).

In the present study, though decrease in CAT activity was observed in acute hyperammonemic conditions, the change was not significant. Ratnakumari *et al.*, (1994) reported significant decrease in CAT activity during hyperammonemic states. This discrepancy in the results could be because of the difference in animal models and methods applied to study the CAT activity. They have done it in sparse fur (sfs) mutant mice with congenital ornithine transcarbomylase deficiency, an animal model for chronic hyperammonemia, but in the present study hyperammonemia was induced by administering acute dose of either AA or MSI. Significant loss in choline acetyltransferase activity was also reported in Alzheimer's disease associated with hyperammonemia. These two models were chronic hyperammonemic models, hence, CAT activity might be altered on prolonged exposure to ammonia.

Provision of Choline for Acetylcholine Synthesis

Choline required for the synthesis of acetylcholine is derived from more than one source, a). hydrolysis of acetylcholine and recycling of choline, b). hydrolysis of choline containing phospholipids present in

neuronal membranes, c). endogenous synthesis of choline and d) choline supplied through the blood.

Following the depolarization of presynaptic cholinergic nerve terminal, acetylcholine is released into the synaptic cleft. This is hydrolyzed to choline and acetic acid by cholinesterases present on exoplasmic face of the synaptic membranes. Choline, thus formed, might be transported into presynaptic nerve terminal and reutlized for synthesis of acetylcholine. It has been suggested that 50-70% of choline required for the synthesis of acetylcholine is derived by this mechanism (Tucek, 1985). Rest of the choline required for the synthesis of acetylcholine in brain is derived from other sources.

Wurtman and his group reported that choline derived from hydrolysis of phosphotidylcholine present in the neuronal membranes by the action of phospholipase C might also serve as precursor for acetylcholine synthesis. They have also suggested that this might be the reason for selective vulnerability of cholinergic neurons in neurodegenerative disorders of CNS, such as Alzheimer's disease (Ulus *et al.,* 1989; Wurtman, 1992). However, precise contribution of this pathway in providing choline for acetylcholine biosynthesis was not quantified by these investigators.

It was believed for a long time that brain is incapable of synthesizing choline. Even as late as 1993, Klein *et al.,* proposed that blood borne choline is the only source for choline in brain. However, several investigators reported that brain can synthesize choline by sequential methylation of phosphotidylethanolamine (Blusztajn *et al.,* 1979; 1987; Blusztajn and Wurtman, 1983; Crews *et al.,* 1980; Mozzi and Porcellati, 1979). It is however, not known whether choline formed in this pathway would serve as a precursor for the biosynthesis of acetylcholine and precise contribution of this pathway was also not evaluated.

Choline derived from blood also serves as precursor for the cerebral pool of choline. Choline is taken up from blood by carrier mediated process present in BBB (Cornford *et al.,* 1978). Dietary intake of choline rich food increases plasma choline levels (Hirsch *et al.,* 1978; Jope, 1982; Zeisel *et al.,* 1980). However, increased plasma choline levels has very little effect on brain choline and acetylcholine concentrations under normal physiological conditions (Klein *et al.,* 1992).

Choline is transported into cholinergic neurons by a sodium dependent, hemicholinium-3 sensitive, high affinity transport system (HAUS) and sodium independent low affinity (less sensitive to hemicholinium-3) transport system (LAUS) (Jope, 1979; Murrin, 1980; Tucek, 1978; 1984). It has also been suggested that HAUS plays a regulatory role in acetylcholine synthesis (Simon *et al.,* 1976; Sterling *et al.,* 1986). It has been reported that incorporation of choline into acetylcholine, but not into lipids is susceptible to inhibition by hemicholinium, an inhibitor of sodium dependent HAUS for choline (Bhatnagar and Macintosh, 1967; Guyenet *et al.*, 1973). Hence, it is believed that choline transported by HAUS is used as a precursor for acetylcholine synthesis while the choline transported by LAUS is used for lipid biosynthesis (Jope, 1979; Tucek, 1985; Yamamura and Snyder, 1973). As HAUS is associated with acetylcholine synthesis, it is used as a marker for cholinergic neurons (Kuhar *et al.,* 1973). Hemicholinium-3, a potent and selective inhibitor of HAUS, has been used as a quantitative marker for high affinity choline uptake sites present in cholinergic neurons (Happe and Murrin, 1993).

HAUS for choline has been isolated from locust head ganglion and reconstituted into liposomes (Knipper *et al.*, 1989). Protein purified with antibodies that block HAUS was found to be capable of accumulating choline into liposomes and ionic requirements for choline transport in this

system were similar to the naturally occurring HAUS (Knipper *et ai,* 1989; 1991). High affinity choline transporter from rat spinal cord was reported to have been cloned (Mayser *et ai,* 1992). They found it as hemicholiniun-3 insensitive and suggested to be due to the existence of different subtypes of choline transporter with distinct pharmacologies. However, it was later found that the transporter protein cloned by Mayser *et ai,* was a creatine transporter but not the choline transporter (Cooper, 1994).

Regional heterogeneity has been reported for the distribution of HAUS for choline in rat brain with a rank order of striatum > hippocampus > cortex > cerebellum. This distribution was correlated well with the other cholinergic markers such as acetylcholine and CAT (Happe and Murrin, 1993).

The driving force for choline transport was reported to be the electric potential across the neuronal membrane (Beach *et ai,* 1980; Vaca and Pilar, 1979). Sodium ions were known to alter kinetic parameters for choline uptake (Carroll and Buterbaugh, 1975; Simon and Kuhar, 1976; Wheeler, 1979). It was proposed that sodium ions and choline are transported together, but no direct evidence is available for the cotransport of choline and sodium ions (Vaca and Pilar, 1979; Wheeler, 1979).

Synaptic activity or prolonged depolarization enhances choline uptake in nerve terminals. This stimulation of uptake could be a consequence of acetylcholine release from nerve terminals. This is supported by an inverse correlation observed between the rate of post-depolarization choline uptake and the level of acetylcholine in synaptosomes (Weiler *et al.,* 1978). It was further supported by other investigators, they reported that the uptake of choline was decreased in synaptosomes prepared from brain of animals which were treated with drugs that decrease the release of acetylcholine (Atweh *et ai,* 1975;

Jenden *et al,* 1976; Richter *et al,* 1982; Simon and Kuhar, 1975). It has been suggested that the depolarization induced increase in choline uptake may involve activation of pospholipase A_2. The release of calcium from internal stores activates membrane phospholipase A_2, which catalyzes the break down of membrane phospholipids releasing unsaturated fatty acids such as arachidonic acid which might unmask the previously hidden trasporter sites (Yamada *et al,* 1988). Regulation of choline transport may also occur by a conformational change in transport site, by changes in local membrane properties or by phosphorylation of transporter protein (Saltarelli *et al,* 1987). It has been reported that sodium flux enhancers increase the high affinity choline transport without altering the affinity for choline (Antonelli *et al,* 1981; Murrin and Kuhar 1976) and metabolic inhibitors such as ouabain and 2,4-dinitrophenol decrease HAUS activity for choline (Simon and Kuhar, 1976). Hence, it is suggested that under physiological conditions, choline transport into nerve terminals depends on 1. nerve terminal activity, 2. content of acetylcholine in the nerve terminal, and 3. membrane potential. As nerve terminal activity and membrane potential are altered in hyperammonemic states (Raabe, 1991), it was planned to study choline transport in hyperammonemic states.

Presently, studies were performed on choline uptake in synaptosomes isolated from the cerebral cortex of control and hyperammonemic rats. Choline uptake was linear up to 500 μg of synaptosomal protein and 10 min. of incubation at 37°C (Fig. 2.7 and 2.8). To determine the kinetic constants of the transport process, choline concentrations ranging from 10-100 μM were used. Throughout this study, non-specific uptake was found to be 10-20% of the total uptake (Fig. 2.9). It was observed that all the data points could not be fitted into a single rectangular hyperbola (Fig. 2.10a). Scatchard analysis of the data also

revealed the presence of two different uptake systems for choline, one with high affinity (low capacity) and the other with low affinity (high capacity) for choline (Fig. 2.10b and 2.10c). It was further confirmed by performing the uptake in presence of hemicholinium-3, a potent and specific inhibitor for high affinity choline transport (K_i = 25-100 nM) and a much weaker inhibitor of low affinity (Kj= 50 μM) transport system (Happe and Murrin, 1993). In presence of hemicholinium-3, choline uptake was suppressed by 80% when the choline concentration was between 0.1 to 2.5 μM. However, at choline concentrations 5 and 10 μM only 40% suppression was observed in choline uptake while no suppression was observed at 20-100 μM (Fig. 2.11a and 2.11b). This suggested that the transition point between HAUS and LAUS was around 5 μM, where the high affinity transport system would be fully saturated and low affinity system starts.

K_m and V_{max} values obtained for high and low affinity systems, in the present study, were 1.74 μM, 1.9 nmoles/mg/hr and 15.4 μM, 5.7 n moles/mg/hr respectively. Reported K_m values are in range of 1.6-5 μM for high affinity transport and 30-100 μM for low affinity system. The reported V_{max} values are in the range of 0.7-2.0 nmoles/mg/hr for high affinity system and 3.5-6.0 n moles/mg/hr for low affinity uptake system (Ferguson *et al.*, 1991; Hrdina and Elson, 1979; Sterling *et al.*, 1986). K_m and V_{max} values obtained in the present study were within the range of reported values except the K_m value for LAUS (Table 2.1). This could be because of differences in the preparations used. In earlier studies crude synaptosomes (P_2 pellet, which contains mitochondria) were used by the authors whereas in the present study purified synaptosomes were used.

Comparison of apparent Michaelis kinetic constants derived from Scatchard plots (Fig 2.12a and 2.12b) revealed that, following the intraperitoneal injection of an acute dose of AA, there was significant

suppression in the V_{max} i.e., by 38% and 33% for HAUS and LAUS respectively. K_m for HAUS was increased by 18%, which was statistically not significant, while there was no effect on the K_m for LAUS under these conditions (Table 2.1). Administration of subacute dose of AA had no effect on choline transport (Fig. 2.13a and 2.13b). When the uptake was performed in synaptosomes (isolated from normal rats) in presence of 5 mM AA (Fig. 2.14a and 2.14b), V_{max} was suppressed by 23% and 18% for HAUS and LAUS respectively, with marginal decrease in K_m values for both the systems. Administration of acute dose of MSI (Fig. 2.15a and 2.15b) increased K_m value for HAUS 26% with marginal decrease in V_{max} values for both the systems (Table 2.1).

Significant finding in the present study was the decrease in rate of choline uptake without significant alterations in K_m. As K_m is the measure of affinity of transporter for choline, it was suggested that the affinity of receptor for choline was not affected in hyperammonemic states induced with AA. The change in V_{max} could be either due to decreased capacity of transporter site in transporting choline or due to decrease in number of transporter sites. Change in the number of transporter sites might be due to changes in rate of synthesis (in cell body), axonal transport and incorporation of the transporter protein into the membrane or rate of degradation of transporter proteins. However, both these possibilities might be ruled out, as the time lapse between administration of AA and killing of the animal was very short ($\cong$ 20 min.) and might not be adequate to bring such significant changes in the above said processes. As transport proteins are integral proteins of the membrane, the observed change in choline uptake may be attributed to alterations in membrane architecture such as fluidity and viscosity and changes in local environment of the

transporter proteins. Similar suggestions were made earlier, for changes observed with membrane bound enzymes, transport systems and receptor proteins in hyperammonemic conditions (O'Conner *et al,* 1984; Rao *et al,* 1991).

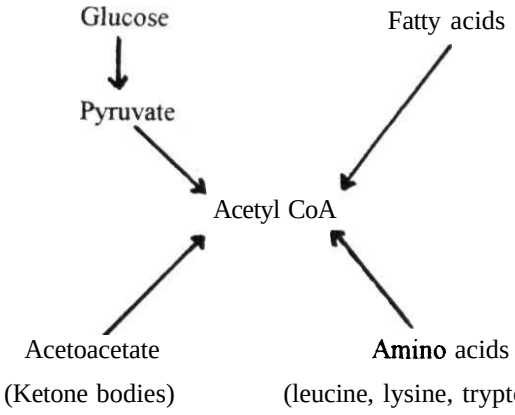
It was suggested that ammonia exists in two forms NH_3 and NH_4^+ which are in equilibrium with each other. NH_3 is readily diffusible through biological membranes while NH_4 is impermeable. If the change was due to NH_3, it might be similar to those of gaseous anesthetics. Ammonium ion might interact with hydrophilic moiety of the membrane, disrupting the molecular forces that govern lipid-lipid and or lipid-protein interactions, there by creating disorder in membrane architecture. As membranes are rich in acidic groups, their neutralization by ammonium ions might also induce notable alterations in membranes (O'Conner *et al,* 1984).

Significant increase in K_m for high affinity system with marginal decrease in V_{max} value suggested that, the affinity of transporter was affected in MSI rats. This suggests that the effects of hyperammonemia induced with MSI was different from the ammonia toxicity induced by administration of AA.

As high affinity uptake of choline is known to play a regulatory role in acetylcholine synthesis, conditions that influence the HAUS might affect the synthesis of acetylcholine in brain. As choline uptake was suppressed in hyperammonemic states, there might be an impaired synthesis of acetylcholine under these conditions.

Provision of Acetyl CoA for Acetylcholine Synthesis

As mentioned earlier acetyl CoA is another substrate required for the synthesis of acetylcholine. There are several pathways for acetyl CoA production.



However several studies have revealed that cerebral pool of acetyl CoA is derived exclusively from glucose (Browning and Schulman, 1968; Lefresne *et ai*, 1973; Tucek, 1983; Tucek and Cheng, 1970; 1974). It has been shown earlier that the operation rates of β-oxidation of fatty acids are very low and do not contribute significantly to cerebral pool of acetyl CoA. Though brain can metabolize ketogenic amino acids such as leucine, lysine and tryptophan, contribution to cerebral acetyl CoA pool by these pathways is very less compared to acetyl CoA derived from glucose. Metabolism of ketone bodies (especially β-hydroxybutyrate and acetoacetate) is high in neonatal stages and in conditions of prolonged starvation (Krebs *et ai*, 1971; Owen, *et ai*, 1976). The rate of metabolism of ketone bodies, however, is very low in normal adult brain (Klee and Sokoloff, 1967). All these studies thus, conclusively indicate that cellular pool of acetyl CoA originates exclusively from glucose.

Bulk of acetyl CoA produced in the brain is oxidized in citric acid cycle and very small amount serves as precursor for the production of acetylcholine. Evidence for incorporation of glucose carbons into

acetylcholine was provided by Browning and Schulman (1968) and Rincy and Tucek (1981).

Acetyl CoA is produced by oxidative decarboxylation of pyruvate mediated by the enzyme pyruvate dehydrogenase, which is localized in mitochondria. Rincy and Tucek (1981) demonstrated that inhibition of pyruvate dehydrogenase by bromopynivate adversely affects the levels of both acetyl CoA and acetylcholine, thus establishing the relationship between acetyl CoA production from pyruvate and the synthesis of acetylcholine. The most interesting and controversial issue with regard to acetylcholine synthesis in brain is the mode of transport of acetyl CoA from mitochondria to cytosol. As pyruvate dehydrogenase is present in mitochondrial matrix, it is implicited that acetyl CoA is produced in the same compartment. However, the enzyme CAT is localized in cytosol. Hence, acetyl CoA has to be transported from mitochondria to cytosol to be utilized for synthesis of acetylcholine. This problem is further accentuated by the impermeability of inner membrane of mitochondria to acetyl CoA. Several mechanisms have been proposed for the transfer of acetyl groups across the inner mitochondrial membrane.

One of the mechanisms proposed suggests the involvement of citrate in this process. Citrate is synthesized in mitochondria from acetyl CoA and oxaloacetate in the presence of citrate synthase. Tricarboxylic acid carrier present in the inner mitochondrial membrane transports citrate into cytosol. In the cellular compartment, citrate is converted back to acetyl CoA and oxaloacetate by the action of cytosolic ATP-cirrate lyase. This process is similar to that described for the provision of acetyl CoA for fatty acid synthesis in liver. It has been shown that when ATP-citrate lyase was inhibited with hydroxycitrate, synthesis of acetylcholine from glucose and pyruvate was reduced by 30% (Gibson and Shimada, 1980; Sterling and O'Neill, 1978; Sterling *et al,* 1981; Szutowicz *et al.,* 1977; 1981;

Tucek *et at.,* 1981). Hence, it was suggested that about one third of acetyl groups for acetylcholine synthesis is supplied through the ATP-citrate lyase.

Direct passage of acetyl CoA through the mitochondrial membrane was another mechanism proposed in this respect. It has been shown that the passage of acetyl CoA through mitochondrial membrane is increased *in vitro* in presence of Ca^{2+} (Benjamin and Quastel, 1981; Benjamin *et at.,* 1983). They reported that Ca^{2} (10 μM) enhances the permeability of inner mitochondrial membrane to acetyl CoA and suggested that conditions that increase cytoplasmic calcium concentration might increase the rate of transfer of acetyl CoA from mitochondria to cytosol. Rincy and Tucek (1983) also reported an increase in leakage of acetyl CoA from mitochondria when Ca^{2+} concentration was raised from 10^{-8} to 10^{-7} M

Acetylcarnirine is also known to act as a precursor for acetylcholine synthesis, but it is not considered as a major source of acetyl CoA for acetylcholine synthesis (Dolezal and Tucek, 1981; White and Scates, 1990). However, it was suggested that carnitine and its acyl derivatives were similar to choline and acetylcholine, may take part in neurotransmission (Falcheto *et at.,* 1971). They were proven to be useful pharmacological agents for treatment of chronic degenerative diseases in aging human subjects (Rebouche, 1992). Acetylcarnirine has been shown to be effective in slowing down the mental deterioration in Alzheimer's disease (Forloni *et al.,* 1994; Spagnoli *et at.,* 1991) and the mechanism of observed effect has been suggested to be associated with cholinergic nerve transmission (Rebouche, 1992). Recently Wawrzenczyk *et at.,* (1994; 1995) proposed that carnitine can stimulate acetylcholine synthesis only when P-oxidation is low as in adult brain and in transformed cells. Ketone bodies were also suggested to contribute for acetylcholine synthesis when

their concentrations were abnormally high or when the glucose metabolism was reduced (Sterling *et al,* 1981).

It was proposed that acetyl CoA generated by extramitochondrial pyruvate dehydrogenase could be utilized for acetylcholine synthesis (Lefresne *et al*., 1978). But it was contradicted by the experiments with α-cyanocinnamate, which blocks pynivate transport into mitochondria (Jope and Jenden, 1980). Gibson *et a.,l* (1975) showed that in experiments with brain minces the rate of acetylcholine synthesis was directly proportional to the rate of intramitochondrial acetyl CoA production

It may be concluded that the major sources of acetyl CoA for acetylcholine synthesis would be pynivate and glucose and the intramitochondrial acetyl CoA might reach cytosol by more than one mechanism.

Regulation of Acetylcholine Synthesis

Various factors are involved in control of acetylcholine synthesis in brain (Cooper *et al,* 1991; Jope, 1979; Tucek, 1988). These include

1. The availability of acetyl CoA and choline
2. Acetylcholine concentration in the nerve terminal
3. High affinity uptake of choline
4. The activity of choline acetyltransferase

Barker and Mittag (1975), based on the comparison of transport and acetylation of choline and its analogues, hypothesized that the transport and acetylation were not independent processes but are coupled processes. However, it has been shown that acetylcholine is synthesized both from choline supplied by the carriers and also derived from the intracellular pool (Marchbanks and Kessler, 1982). Acetylation can be altered without change in the transport (Jope *et al,* 1978) or acetylation can be postponed (Jope and Jenden, 1980). This was in support of the tenet that choline

uptake and acetylation of choline were not coupled but independent processes.

It is evident from experiments done under sustained synaptic activity that the availability of both choline (Jope, 1982; Trommer *et al.*, 1982; Wecker and Schmidt, 1980) and acetyl CoA (Dolezal and Tucek, 1982) act as regulatory factors in synthesis of acetylcholine. It was reported that preloading of both choline and glucose helped to maintain acetylcholine concentrations under sustained synaptic activity. Thus, the pretreatment with precursors would act by improving the availability of acetyl CoA and choline when the release of acetylcholine is high.

Metabolism would be adversely affected in hyperammonemic states of various etiologies. Different groups of investigators proposed different mechanisms to explain the reduction in glucose oxidation in hyperammonemic states. Available reports indicated an increased production of lactate and a decrease in the operation rates of citric acid cycle (Fitzpatrick *et al.,* 1983). Such a condition would result in a decrease in the conversion of pyruvate to acetyl CoA. It has been suggested that in hyperammonemia, operation of malate aspartate shuttle (MAS) would be suppressed due to the channeling of cytoplasmic pool of glutamate for glutamine formation (Duffy and Plum, 1982; Fitzpatrick *et al,* 1983; Hindfelt *et al*., 1977; Ratnakumari *et al*., 1985; 1986). It was shown earlier that in such conditions acetyl CoA will accumulate and exert a feed back inhibition on pyruvate dehydrogenase and suppress its own production (Murthy and Hertz, 1988).

Irrespective of the mechanism by which ammonia suppresses the glucose oxidation, it could lower the production of acetyl CoA and this might affect the availability of acetyl CoA for the synthesis of acetylcholine. This tenet was verified by studying the incorporation of ^{14}C-carbon from [U-^{14}C]glucose into acetylcholine, in the synaptosomes

isolated from normal and experimental animals. $^{14}CO_2$ production from [U-^{14}C]glucose was studied in synaptosomes isolated from CC of control rats and rats administered with acute dose of AA and acute dose of MSI, at three time intervals viz. 15, 30 and 60 min. of incubation. CO_2 production was significantly reduced at 60 min. (18%), while no effect was observed at 15 and 30 min., in MSI administered rats. In AA administered rats, CO_2 production was suppressed by 15%, 11%, and 20% at 15, 30 and 60 min. respectively (Table 2.2). CO_2 production was significantly reduced in cortical synaptosomes only when the time of incubation was prolonged for 1 hr and this effect was observed only in rats administered with AA or MSI but not in control rats. However, when CO_2 production was tested *in vitro*, it was suppressed by 11.5% and 17% at 5 and 10 mM AA respectively while no effect was observed with 1 mM AA (Table 2.3).

In the present study, wherein no significant effect was observed in CO_2 production either at early time periods or at lower concentrations of AA (1 to 5 mM) were in agreement with the results of Hertz *et ah,* (1987) in primary cultures of astrocytes and of neurons.

It was reported earlier that ammonia at pathophysiological concentrations would affect cerebral glucose metabolism by depleting a-KG from citric acid cycle and /or by inhibiting the α-ketoglutarate dehydrogenase activity. These two processes would have an adverse effect on the operation of rates citric acid cycle and this might result in reduced production of acetyl CoA (Bessman and Bessman, 1955).

Decreased CO_2 production from [U-^{14}C]glucose at 60 min. but not at the earlier time periods in synaptosomes isolated from rats administered with acute dose of AA may be explained on the basis of reports of earlier investigators. It is well known that brain depends upon the MAS for the transport of reducing equivalents generated in cytosol during glycolysis. It

has been reported that activity of aspartate amino transferase, an enzyme that participates in the operation of MAS is inhibited in hyperammonemic states (Lai *et al.,* 1989; Ratnakumari and Murthy, 1989). It was also reported that the oxaloacetic acid required for the operation of TCA cycle is provided by this pathway. Moreover, it has been demonstrated that conversion of pyruvate to acetyl CoA is suppressed under these conditions in astrocytes but not in the neurons (Hertz *et al.,* 1987; Murthy and Hertz, 1988). Under such conditions CO_2 production from glucose should be suppressed due to the non-availability of NAD for glycolysis and oxaloacetate for TCA cycle. Such effect, indeed, was reported in astrocytes but not in neurons. This is understandable as neurons are the sites for the large pool of glutamate while astrocytes harbor small pool of glutamate. As large pool of glutamate has slow turn over rate, it might be anapleurotically replenishing α-ketoglutarate, thus oxaloacetate for the operation of TCA cycle atleast during the early time periods. However, under conditions of prolonged incubation even the larger pool of glutamate might be exhausted thus lowering CO_2 production from glucose. This might explain the suppression of CO_2 production at 60 min. of incubation but not in the earlier time periods. In astrocytes, which contain the small and rapidly turning over pool of glutamate, CO_2 production from glucose would be affected much earlier than neurons due to the early depletion of endogenous glutamate pool.

Results of the present study on *in vitro* effects of different concentrations of AA on CO_2 production from glucose indicated a reduction in the presence of 10 mM AA while no changes were observed at lower concentrations. The interpretation given above might also explain this effect. At higher concentrations AA might be inhibiting the operation of MAS more efficiently than at lower concentrations.

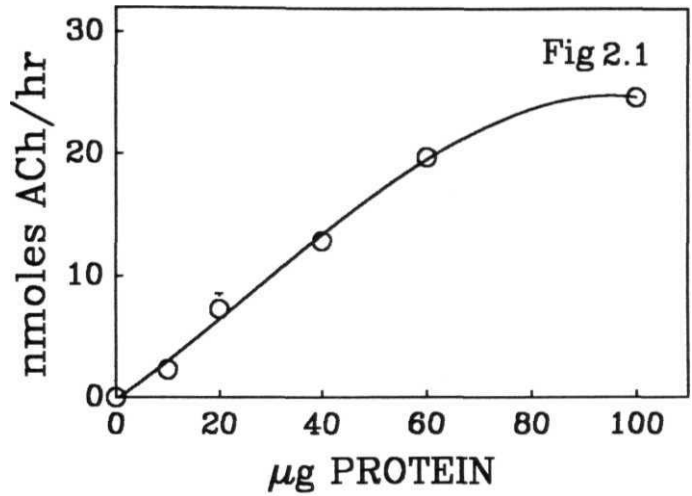
. *In vivo* administration of AA or MSI had no effect on acetylcholine synthesis from [U-^{14}C]glucose. But when acetylcholine synthesis was studied in synaptosomes isolated from control rats in presence of ammonium acetate, significant decrease was observed at 10 mM while there was no effect at 1 and 5 mM concentrations (Table 2.4). Results of the present study indicated that hyperammonemic conditions have no adverse effects on acetylcholine synthesis except at abnormally high concentrations.

If the acetylcholine synthesis is related to CO_2 production from [U-^{14}C]glucose and choline uptake, the results are contradictory. In the present study high affinity uptake of choline was suppressed by 38%, while no change was observed in acetylcholine synthesis from [U-^{14}C]glucose in acute hyperammonemic states. As high affinity uptake of choline was proved to be rate limiting step in acetylcholine synthesis, one would expect acetylcholine synthesis to decrease with a decrease in choline uptake.

However, it must be mentioned that the experiments on acetylcholine synthesis and choline uptake were performed under different conditions. The rationale for using such experimental conditions was to know the effect of impaired energy metabolism (known to exist under hyperammonemic conditions) on acetylcholine synthesis. For choline uptake studies, choline concentration was between 0.5-100 μM, where as for the synthesis of acetylcholine 2 mM choline was used. Such high concentration of choline was used to prevent choline uptake from acting as a rate limiting factor. At this concentration diffusion of choline into synaptosomes would be higher than the combined rates of both HAUS and LAUS. This was evident from the observation that, when the choline concentration was increased beyond 200 μM, the accumulated label inside

the synaptosomes was very high (Fig. 2.16). Earlier Cornford *et al.,* (1978) reported such simple diffusion of choline through BBB when choline concentrations were increased beyond 200 μM. Moreover, it has been reported that the choline derived from intracellular pool also serves as a precursor for acetylcholine synthesis (Marchbanks and Kessler, 1982).

Acetylcholine synthesis has been reported to be dependent on energy metabolism. Though CO_2 production was reduced by 20% in acute hyperammonemic states, acetylcholine synthesis was found to be normal. This suggests that acetyl CoA would be spared for acetylcholine synthesis even under impaired energy metabolism. It can be suggested that unless glucose metabolism is severely affected, as in hypoxic conditions or in severe hypoglycemia (Dolivo 1974; Ghajar *et al.,* 1985, Gibson and Blass 1976), acetylcholine synthesis might occur at normal rate. Hence, it can be suggested that hyperammonemia may not interfere with the supply of acetyl CoA from glucose for acetylcholine synthesis.



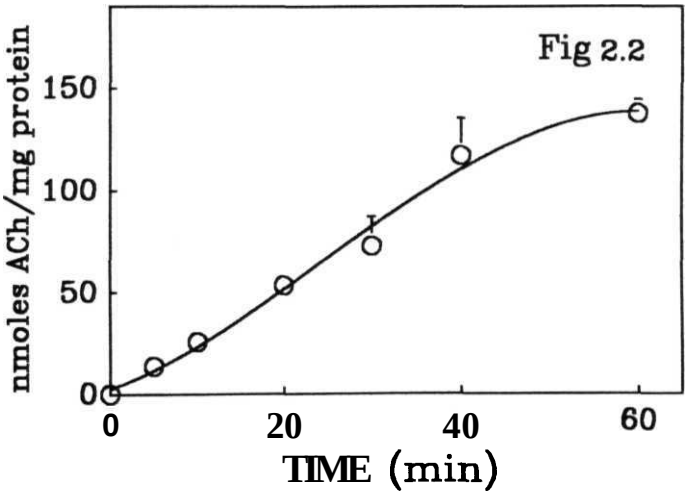


Fig. 2.1: CAT activity as a function of added synaptosomal protein

Fig 2.2: CAT activity in cortical synaptosomes as a function of incubation time Values are Mean ± S.D of three experiments in duplicates

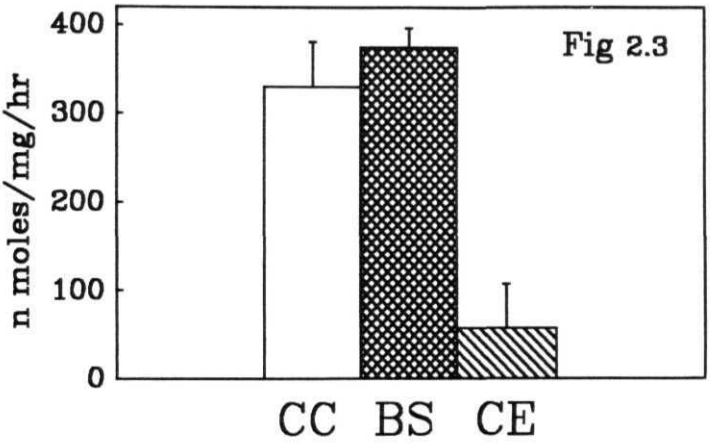
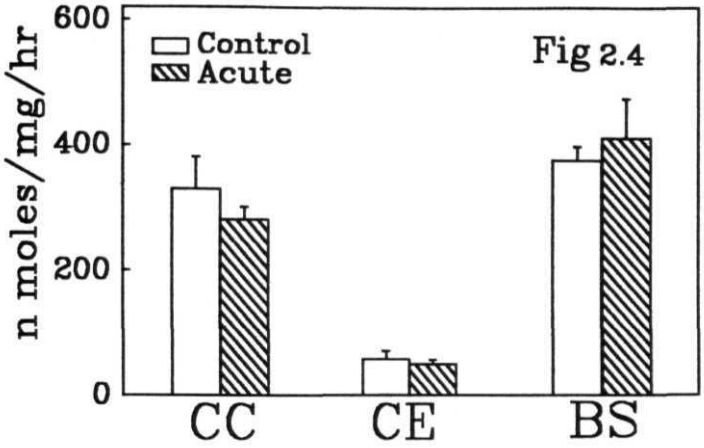


Fig. 2.3: CAT activity in synaptosomes isolated from CC, CE and BS of control rats indicating the regional distribution of CAT. Values are Mean + S.D of three experiments done in duplicates.



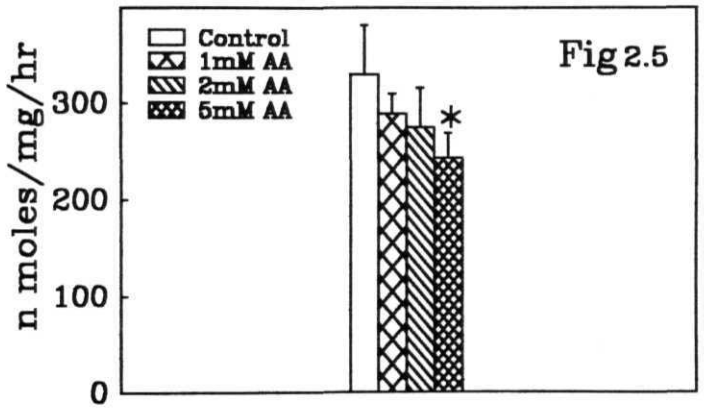


Fig. 2.4: Synaptosomal CAT activity in CC, CE and BS of control rats and rats administered with acute dose of AA

Fig 2.5: CAT activity, in synaptosomes isolated from CC of control rats in presence of 1, 2 and 5 mM AA Values are Mean ± S.D, of three experiments done in duplicates * : Statistically significant compared to controls ($p < 0.05$)

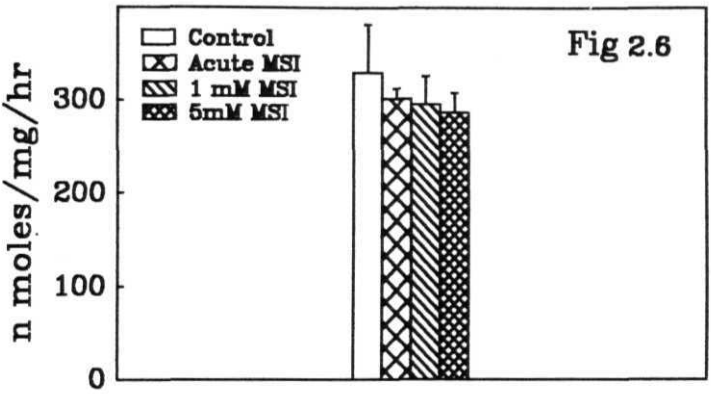
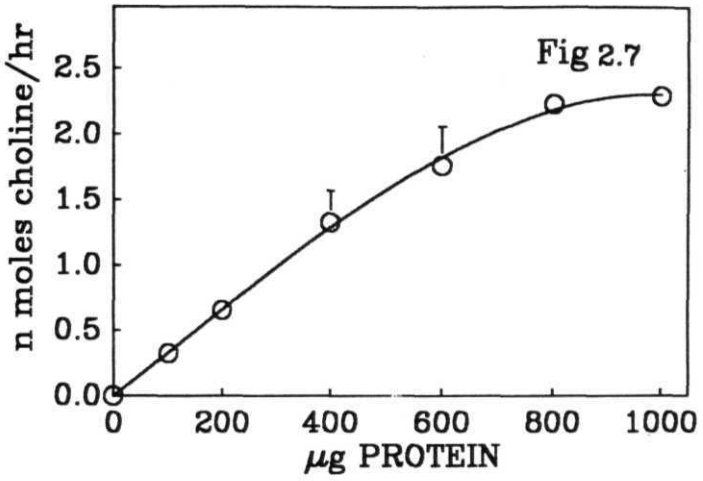


Fig 2 6: Synaptosomal CAT activity in CC of control rats and rats administered with acute dose of MSI (acute MSI) 1 and 5 mM MSI represents the CAT activity in cortical synaptosomes of control rats studied in presence of 1 and 5 mM MSI Values are Mean + S.D of three experiments done in duplicates.



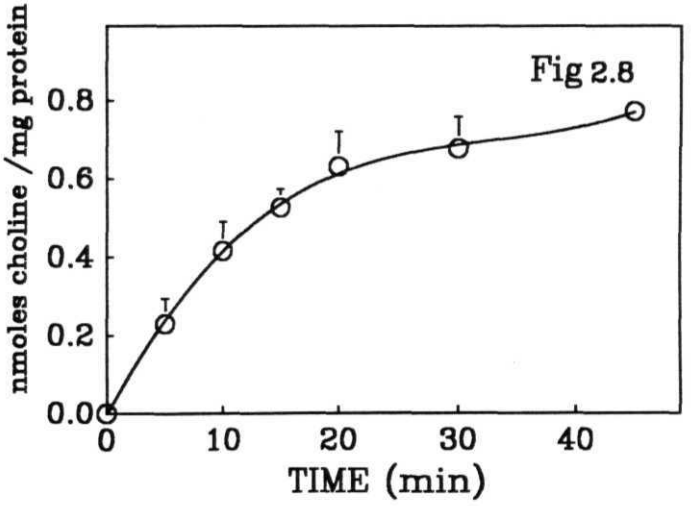


Fig 2.7: Choline uptake as a function of added synaptosomal protein (from CC)

Fig. 2 8: Choline uptake in cortical synaptosomes as a function of incubation time Values are Mean ± S D of three experiments in duplicates

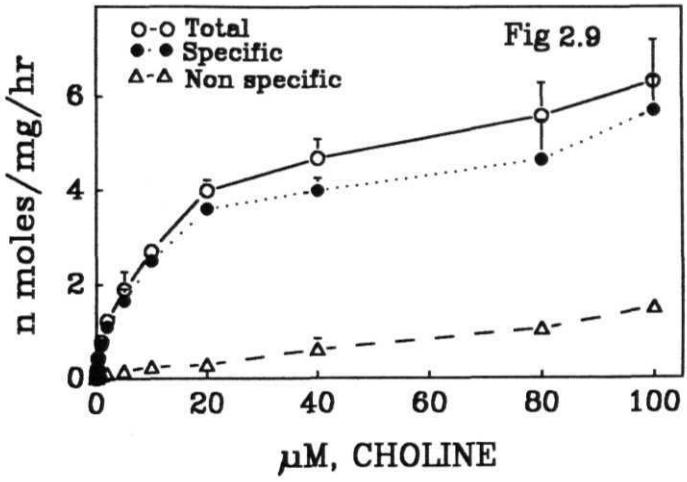
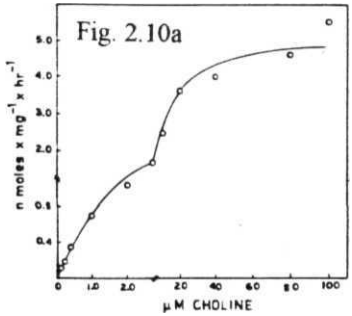


Fig 2.9: Total, specific and non specific choline uptake into cortical synaptosomes Values are Mean ± S D of four experiments done in duplicates.



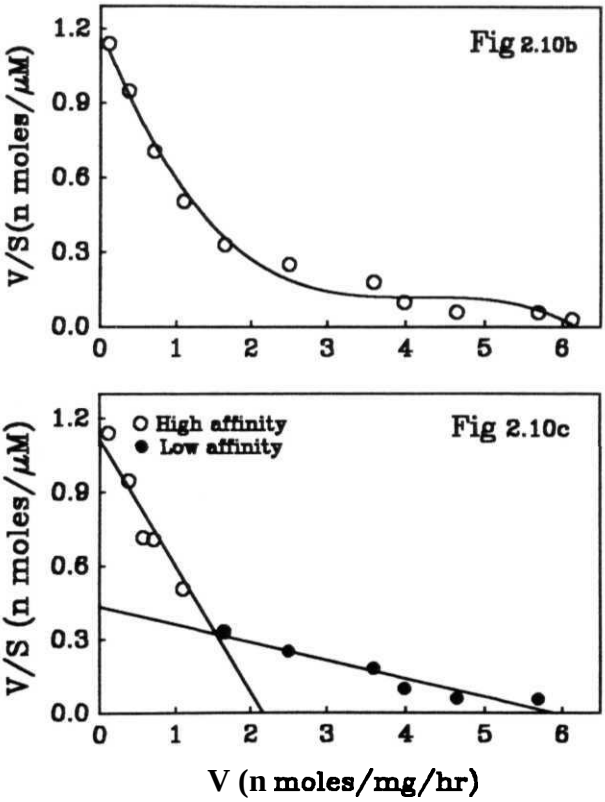


Fig 2.10: Choline uptake into cortical synaptosomes (a) Saturation isotherm showing biphasic nature of uptake (b) Curvilinear nature of the Scatchard plot (data of saturation isotherms) (c) Scatchard plots constructed with first order linear regression, showing two affinity systems for choline uptake Values are Mean ± S.D of four experiments done in duplicates.

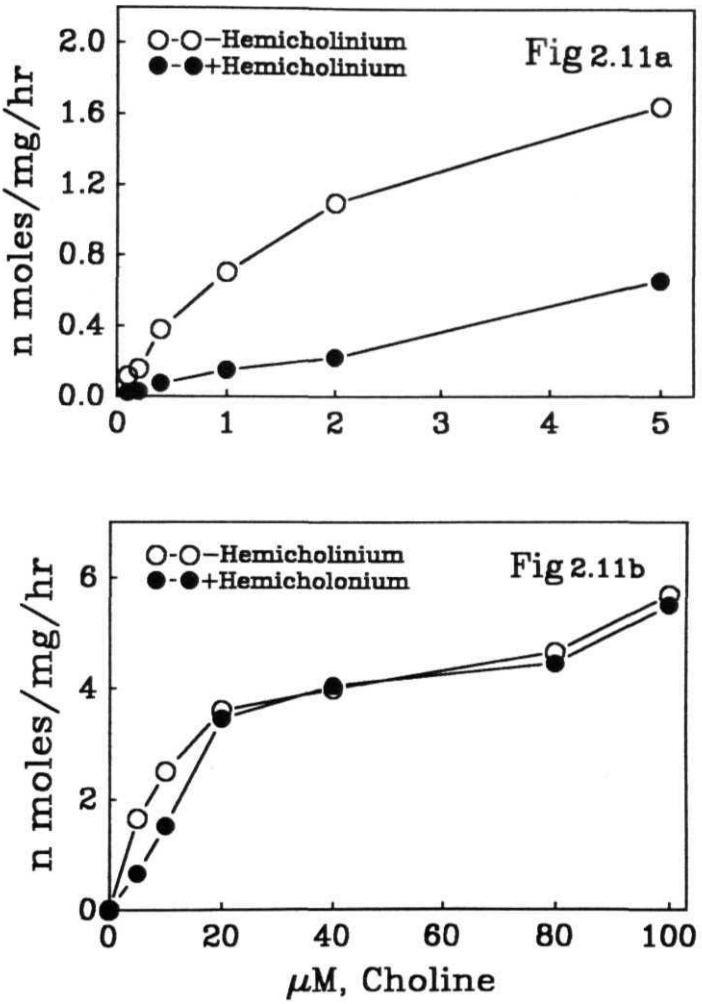


Fig 2.11: Choline uptake into synaptosomes in absence (-) and in presence (+) of hemichonium-3 as a function of choline concentration (a) high affinity (b) low affinity **uptake**

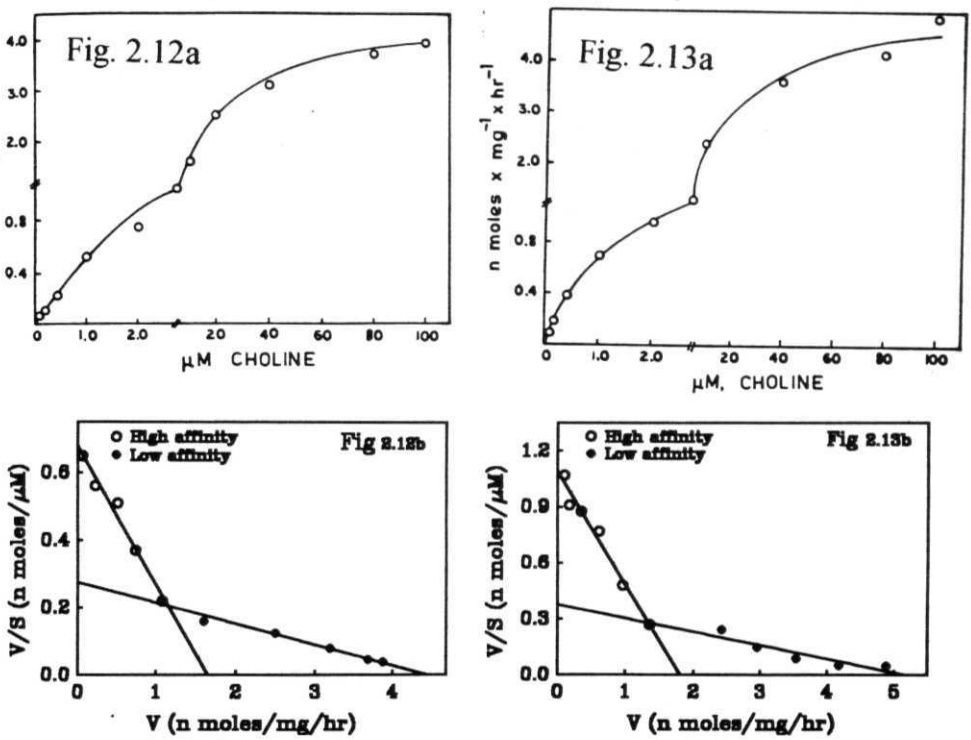


Fig. 2.12: Choline uptake into synaptosomes isolated from CC of rats administered with acute dose of AA (a) and (b) Scatchard plot of the uptake process. Values are Mean of four experiments done in duplicates

Fig. 2.13: Choline uptake in synaptosomes isolated from CC of rats administered with subacute dose of AA. Rest of the legend same as in Fig. 2.12.

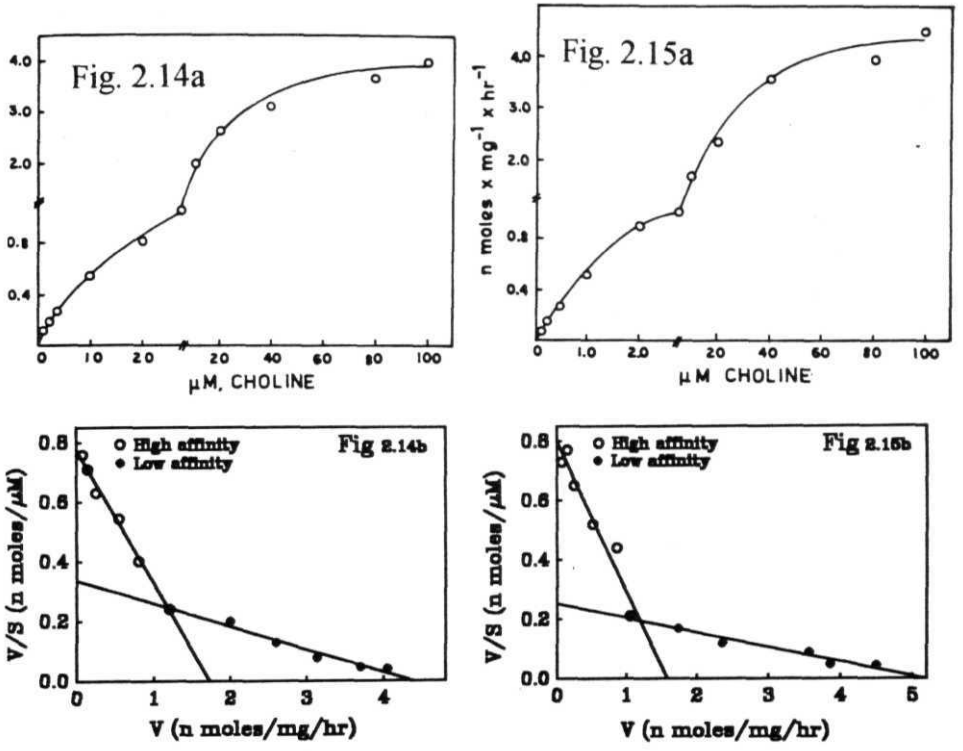


Fig. 2.14: Choline uptake in synaptosomes isolated from CC of rats administered with acute dose of MSI. Rest of the legend same as in Fig. 2.12.

Fig. 2.15: Choline uptake in synaptosomes isolated from CC of control rats, studied in presence of 5 mM AA. Rest of the legend same as in Fig 2.12

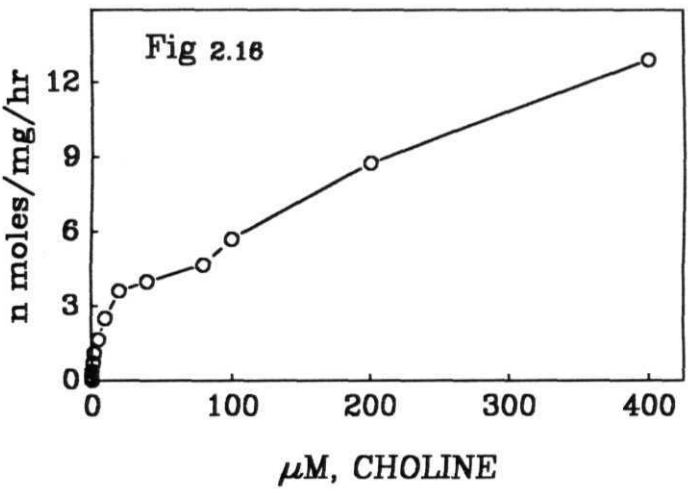


Fig. 2.16 Choline uptake in synaptosomes showing enormous increase in uptake when choline concentration was increased beyond 200

Table: 2.1 Kinetic constants for choline uptake in control and hyperammonemic rats

	HIGH AFFINITY		LOW AFFINITY	
	K_m	V_{max}	K_m	V_{max}
CONTROL	1.74 ±0.23	1.9 ±0.2	15.4 ±2.2	5.7± 1.3
ACUTE AA	2.07 ±0.33	1.3 ± 0.14**	14.3 ±2.2	3.9 ± 0.57*
SUBACUTE AA	1.67 ±0.17	2.0 ±0.42	15.6 ±4.2	5.7 ±0.50
5mM AA (in vitro)	1.56 ±0.24	1.5 ±0.24	13.5 ±1.8	4.7 ±1.2
ACUTE MSI	2.2 ±0.4*	1.8 ±0.23	16.7 ±4.0	5.3 ±0.73

K_m values are in μM and V_{max} values are expressed as n moles/mg/hr. *In vitro* indicates the uptake of choline in presence of AA in synaptosomes isolated from control rats. Values are Mean ± S.D. (n = 4). Statistically significant compared to controls **: $p < 0.001$, *: $p < 0.02$

Table: 2.2 $^{14}CO_2$ production from [U-^{14}C]glucose in synaptosomes prepared from CC of control and hyperammonemic rats

TIME (min.)	CONTROL	ACUTE AA	ACUTE MSI
15	21.06 ±4.7	17.7 ±1.2	21.8 ± 1.1
30	41.34 ±1.1	37.0 ±2.6	39.4 ±4.1
60	74.64 ± 2.7	59.8 ±3.6*	61.5+1.2*

$^{14}CO_2$ production from [U-^{14}C]glucose was studied in synaptosomes isolated from CC of control rats and rats administered with acute dose of AA (acute AA)/MSI (acute MSI). All the values are Mean ± S.D. of three experiments done in duplicates.CO_2 produced was calculated taking one sixth of the radioactivity present in reaction mixture and expressed as n moles of CO_2 produced per mg protein. *: Statistically significant compared to controls $p < 0.05$.

Table: 2.3 I*n vitro* effects of AA on $^{14}CO_2$ production from [U-^{14}C]glucose in cortical synaptosomes

AA(mM)	n moles CO_2/mg/hr
0	74.9 ±2.1
1	73.3 ±4.6
5	66.3 ±1.6
10	62.1 ±1.2*

In vitro effects of AA on CO_2 production from [U-^{14}C]glucose was studied in synaptosomes isolated from CC of control rats in presence of 1, 5 and 10 mM AA. All the values are Mean ± S.D. of three experiments done in duplicates. *: Statistically significant compared to controls $p < 0.05$.

Table: 2.4 Synthesis of acetylcholine with [U-^{14}C]glucose as precursor for acetyl moiety of acetylcholine

GROUP	ACETYLCHOLINE n moles/mg/hr
CONTROL	1.36 ±0.05
ACUTE AA	1.44 ±0.29
ACUTE MSI	1.41 ±0.12
IN VITRO	
1mM AA	1.46 ±0.03
5mM AA	1.39 ±0.07
10mM AA	1.04 ±0.03*

Acetylcholine synthesis was studied in synaptosomes isolated from CC of control rats and rats administered with acute dose of A A/MSI. *In vitro* indicates the experiments done with synaptosomes isolated from control rats in presence of AA at specified concentrations. Specific activity was corrected taking one third of the radioactivity present in the reaction mixture. All the values are Mean ± S.D. of three experiments done in duplicates. * statistically significant compared to controls $p < 0.05$.

CHAPTER 3
RECEPTORS

Neurotransmitter released from the nerve terminal binds either to pre-synaptic (auto receptors) or post-synaptic receptors to mediate its designated function. Neurotransmitter by binding to the pre-synaptic receptor modulates its own release while binding to post-synaptic receptor results in propagation of the signal in post-synaptic neuron.

Neurotransmitter receptors have been classified into ionotropic and metabotropic receptors. The former type of receptors span the entire bilayer and are associated with an ionic channel. Binding of neurotransmitter to this class of receptors results in opening of the channel and influx of ions into post-synaptic neurons. This may result either in depolarization (excitation) or hyperpolarization (inhibition) of post-synaptic neuron. In contrast to this, binding of the neurotransmitter to the metabotropic receptor generates second messengers which mediate a cascade of metabolic reactions. Post-synaptic response mediated by ionotropic receptors are usually fast and transitory while metabotropic receptors mediate delayed and prolonged response.

Receptors are specific in their interaction with neurotransmitters. Despite this, several related compounds also interact with the receptors. Some of these compounds mimic the action of neurotransmitters (agonists) and others antagonize the effects of neurotransmitters (antagonists). Based on the specificity of the interaction of agonists and antagonists, several subtypes of neurotransmitter receptors have been identified.

Accordingly, acetylcholine receptors have been classified into nicotinc and muscarinic receptors based on their pharmacological

specificity for nicotine and muscarine respectively. Brief survey of literature on these two types of receptors is presented.

Nicotinic Acetylcholine Receptors

The presence of these receptors has been identified in many neurons of CNS, pre ganglionic neurons of autonomic nervous system, and also in adrenal chromaffin cells. Wide spread presence of these receptors in neuromuscular junctions, especially of skeletal muscles, has been reported in several vertebrates. Among the invertebrates, electric organ (electroplax) of cartilaginous fishes, especially, electric ray is cited as a classical example for the presence of nicotinic receptors.

Nicotinic acetylcholine receptors (nAChR) are ligand gated ionotropic receptors and are known to be associated with cationic channels (Fieber and Adams, 1991; Mulle and Changeux, 1990; Nutter and Adams, 1991). The nAChR has special place in the history of ionic channels as it has several 'firsts' to its credit. It was the first channel to be purified, first to be sequenced, first to be functionally reconstituted in a synthetic bilayer, first for which electric signals from a single channel were recorded, first ionic channel for which a gene has been cloned and sequenced (Alberts *et al.,* 1994). Most of the nicotinic receptors are associated with sodium channel. Binding of acetylcholine results in opening of an ionic channel, which leads to an increased influx of Na ions into a cell and shifting of membrane potential close to threshold value. This depolarizes the post-synaptic neuron and generates action potential. In recent years it has been shown that these channels may even permit the entry of potassium and calcium ions, which may activate second messenger systems (Mulle *et al.,* 1992; Vernino *et al.,* 1992)

The *Torpedo* nAChR has been well characterized and can be taken as a model, for nAChRs in other systems. The receptor is a pentameric

protein consisting of four different types of subunits. They are two a, and one each of 3, y and 8 subunits. The two a subunits are located opposite to each other and are separated by (3 subunit on one side and γ, 8 subunits on the other side. The five subunits together form a cylindrical structure which projects over the exoplasmic face of the membrane and also span the entire bilayer. Each subunit consists of a large hydrophilic region which projects out and four membrane spanning hydrophobic domains (M1-M4). Acetylcholine binds to the site contributed by a subunits while the ionic channel is made of membrane spannig segment M_2 from each subunit. The clusters of negatively charged amino acids at either end of the channel confer cation selectivity to the channel. An uncharged ring at the center, where M_2 segments bend, participates in closing the ion channel when the receptor becomes desensitized to acetylcholine (Changeux, 1993). When fully opened, this channel allows ions with a diameter 0.65 run to pass through it. Thus, the channel is not specific for any cation and allows not only sodium ions but also potassium and calcium ions.

However, the influx of sodium ions through the open channel dominates over the other two ions due to the high driving force on sodium ions (110 mV) when compared to that of potassium ion (15 mV), and also the relatively high extracellular concentration of sodium ions (120 mM) when compared to Ca^2 ions (1 mM). When the neurotransmitter binds to a-subunit, conformation change is induced and the channel opens. This opening of the channel is a very short lived phenomena and losts for only a millisecond. Acetylcholine dissociates from the receptor and the receptor returns to its original closed conformation of low energy state. During this brief period when the channel opened, about 30,000 sodium ions are supposed to pass through the channel and this induces depolarization (Alberts *et al.*, 1994).

nAChR has been purified from various sources. Results of these studies indicated the diversity in structure and composition of these receptors, though all of them perform the same function. Three distinct types of nAChRs have been characterized, from the electric organ of electric fish *{Torpedo californica)*, skeletal muscle and nervous system.

The muscle nicotinic receptor also contains α_2, β, *y* and 8 subunits in fetal type but in adult γ is replaced by e (Takai *et al.*, 1985). The neuronal nicotinic receptors are made of only two types of subunits a and {3. These two subunits in different combination form diverse types of receptors. Different isoforms ($\alpha_{2\text{-}8}$, $\beta_{2\text{-}4}$) of these subunits have been cloned from both avian and rodent nervous system and they have a distinct pharmacological profiles and localizations (Cooper *et al.,* 1990; Couturier *et al.,* 1990; Deneris *et al.,* 1988; 1989; 1991; Duvoisin *et al.,* 1989; Luetje *et al.,* 1990). Only few human neuronal nAChR subunits have been characterized viz. (X5, α_7, β_2 and β_4 (Anand and Lindstrom, 1990; Chini *et al.*, 1992; Tarroni *et al.,* 1992). These genes encode peptides with four hydrophobic transmembrane domains (M1-M4). The overall amino acid homology between the products of these genes and muscle genes from same species is 40-55%. This homology approaches 100% in putative transmembrane regions (M_1, M_3) and in selected stretches of N-terminal extracellular domain. Amino acid sequence in the cytoplasmic segment between M_3 and M_4 was also reported to be divergent (Changeux, 1990; Karlin, 1991).

Neuronal nicotinic receptors differ from muscle type nicotinic receptor with respect to their sensitivity to a-bungarotoxin. Most of the neuronal receptors are sensitive to K-neurotoxin present in the venom of the same elapid snakes, few are sensitive to a-neurotoxin and the other type are insensitive to both a and K-neurotoxins (Hucho, 1993).

Nicotinic receptors in CNS, are involved in neuronal excitability, memory, control of temperature and motor function (Deneris *et al.*, 1991; Luetje *et al.,* 1990) and are affected in human degenerative disorders like Alzheimer's and Parkinson's disease (Aubert *et al.,* 1992; Giacobini *et al.,* 1988; Perry *et al.,* 1987; Xuereb *et al.,* 1990).

Muscarinic Acetylcholine Receptors

Muscarinic acetylcholine receptors (mAChR) are abundant not only in CNS but also in heart, gastrointestinal tract, smooth muscle, secretary organs and also in circulating cells such as erythrocytes and T lymphocytes (Jarv and Bartfai, 1988). In CNS, most of the cholinergic innervation is of muscarinic type and is known to play a key role in neuronal mechanisms underlying learning and memory (Drachman, 1977; Gitelman and Prohovnik, 1992), control of movement (Klawans, 1975), neuroendocrine regulation in hypothalamus (Ganong, 1975), thought processing in CC (Itill and Fink, 1968), in normal aging (Vannucchi and Goldman-Rakic, 1991) and are affected in neuropsychiatric diseases like Alzheimer's, Parkinson's and also in depression (Dilsaver, 1986; Lange *et al.,* 1993).

mAChRs were defined by Sir Henry Dale as receptors which are stimulated by acetylcholine and muscarine and blocked by atropine. Later, the concept of muscarinic receptor subtypes emerged because of the finding of tissue selective muscarinic antagonists. Among which pirenzepine provided the major impetus for the definition of receptor subclasses. Based on pharmacological specificity to pirenzepine, the mAChRs are classified into two groups, one in the brain which has high affinity for pirenzepine and the other in peripheral organs with low affinity for pirenzepine(Jarv and Rinken, 1993). The M_1 is characterized by having high affinity for pirenzepine. However, the M_1 receptors are not found to

be a homogeneous population of receptors. They are further classified into hippocampal ($M_{1\alpha}$) and ganglionic ($M_{1\beta}$), based on their response to an agonist MCN-A-343 (4-(m-chlorophenyl-carbamyl-oxy)-2-butyryltrimethylammonium chloride). MCN-A-343 has weak stimulating activity at $M_{1\beta}$ receptors (Gmelin, 1985; Ladinsky *et al.,* 1990). The muscarinic receptors with low affinity for pirenzepine are subclassified into three types- a cardiac M_2 type which has a high affinity for AF-DX 116 (11 -[2-(diethylaminomethyl-1 -piperidinyl)-5,11 -dihyro-6H-pyrido(2,3-6)(l,4)benzodiazepine-6-one), while glandular (M_3) is characterized by having low affinity for AF-DX 116, and a third type M_4 is characterized by having intermediate affinity for AF-DX 116 when compared to cardiac and glandular types of mAChR. Methoctramine was found to be a selective antagonist for M_2 receptors. These receptors are present in cardiac tissue and certain regions of brain (Mei, *et al.,* 1989). M_3 subtype of mAChR include the receptors in rat pancreas and *CC* and 4-DAMP was claimed to be selective for these receptors (Louie and Owyang, 1986). One more subtype of muscarinic receptor M_5 has been found to be expressed in brain. All these receptors are supposed to be separate gene products and m_1 gene has been cloned from human (Allard *et al.,* 1987; Bonner *et al.,* 1988; Peralta *et al.*, 1987), rat (Bonner, *et al.,* 1987), and pig (Kubo, *et al.,* 1986), m2 and m3 from human, rat and pig (Bonner *et al.,* 1987; Kubo, *et al.,* 1986; Liao *et al.,* 1989), the m4 and m5 from human and rat (Bonner *et al.*, 1988, Liao *et al.,* 1989).

M_1 receptors are post-synaptic in localization and are mainly involved in the transmission of signal whereas M_2 receptors are believed to be located in pre-synaptic and dendritic portions of neurons (Vogt, 1988). They might be involved in the regulation of the release of acetylcholine from the nerve terminal. It has also been shown that, M_2 receptors are

reliable markers for cholinergic cell groups and projection areas (Spencer *et al.*, 1986).

Muscarinic receptor is a glycoprotein and resembles the receptors for catecholamines, serotonin and for certain peptides. Muscarinic receptors show greatest homology with α_2 adrenergic receptors and least homology with peptide receptors (Hulme *et al.,* 1990). There are seven transmembrane domains for muscarinic receptor which are connected by four extracellular and four intracellular loops with the amino terminus at the exoplasmic face and carboxyl terminus inside the cell. Purified muscarinic receptors from brain and heart have homology of about 38% mostly in transmembrane domains (Kubo *et al.,* 1986; Liao *et al.,* 1989). It is believed that the aspartic acid residues at positions 77, 99, 105 and 122 are highly conserved and located in or near the second and third transmembrane domains. These residues along with the tyrosine residue in seventh transmembrane domain have been proposed to be possible candidates for the ligand binding. Comparison of the amino acid sequences of different muscarinic receptor subtypes indicated greatest sequence homology in the membrane spanning regions while the third intracellular loop varies greatly among receptor subtypes (Hulme *et al.,* 1990).

Muscarinic receptors belong to the super family of the receptors which are associated with G proteins. It has been reported that different subtypes of muscarinic receptors are associated with different types of G proteins. As a consequence, the post-synaptic action of muscarinic receptors varies from tissue to tissue and even within the tissue (in different regions of brain). Depending on the type of the cell, activation of muscarinic receptors can lead to an increase/decrease in levels of cAMP, release of arachidonic acid, Ca^{2} , activation of K^{+} or Cl^{-} channels and an inhibition of M-current (Berridge, 1993; Braun *et al,* 1987; Conklin *et al.*,

1988). M_1, M_3 and M_5 interact with G_{q11} type of G protein which contains three subunits α_{q11}, β and y (Rhee and Choi, 1992). Binding of acetylcholine to these receptors brings about conformational change in G protein, as a result the bound GDP is exchanged for GTP leading to the dissociation of a subunit from β y complex. The a subunit activates membrane bound phosphoinositide specific phospholipase C-β resulting in the hydrolysis of membrane bound inositol containing phospholipids. This results in the production of IP_3 and diacylglycerol (Berridge, 1993; Cockcroft and Thomas, 1992; Nishizuka, 1992). IP_3 binds to the IP_3 receptor in the membranes of endoplasmic reticulum, resulting in the efflux of Ca^{24} from endoplasmic reticulum into cytoplasm. Ca^{2} stimulates specific protein kinases, leading to the phosphorylation of several proteins including ionic channel proteins. Protein phosphorylation is known to initiate a cascade of metabolic events. Phosphorylation of ionic channel proteins modifies kinetic behaviour of the channels. Ca^{2} stimulates phospholipase A_2 which may hydrolyze arachidonic acid from diacylglycerol. Arachidonic acid and its metabolites such as prostaglandins, prostacyclins and leukotrienes are known to have profound effects on synaptic function. Further, protein kinase C which is a cytosolic enzyme is altered by the Ca^{2+} ions and is translocated from cytosol to plasma membrane. This enzyme is activated by a combination of Ca^{2+}, diacyglycerol and negatively charged phosphatidyl serine. As a consequence, it also phosphorylates certain membrane bound proteins (Alberts *et al*., 1994).

M_2 and M_4 type of muscarinic receptors are associated with G_i type of G proteins and receptor ligand interaction brings about an inhibition of adenylyl cyclase (Ashkenazi *et al*., 1989). The precise mechanism by which the adenylyl cyclase is inhibited is not yet clear. These subtypes of

muscarinic receptors are also known to act directly as the ionic channels especially of Ca^{2} , K^{+} and modify their ionic conductance properties.

The interaction between muscarinic receptors with G proteins also affects the properties of receptors such as agonist binding activity.

In the earlier chapter, it was indicated that ammonium ions at pathophysiological concentrations might interact directly with the membrane and alter the lipid protein interactions of the membrane. It was also suggested that this might affect the properties as well as the function of the integral proteins, such as transporters present in the membrane. As the neurotransmitter receptors are integral proteins of plasma mebrane, it is possible that their function might also be altered during hyperammonemic states. Earlier studies from this laboratory indicated that pathophysiological concentrations of ammonia exert differential effects on the subtypes of cerebral glutamate receptors such as kainate and NMDA receptors (Rao *et al.*, 1992). Hence the ligand binding properties of nAChRs and mAChRs were studied in brains of hyperammonemic rats.

Nicotinic and muscarinic acetylcholine receptors were studied using specific ligands [^{3}H]nicotine (agonist) and [^{3}H]QNB (antagonist) respectively. All the preliminary experiments were done with synaptic membranes isolated from CC of control rats.

Preliminary studies indicated that [^{3}H]nicotine binding was linear upto 10 min. of incubation and complete saturation was obtained within 30 min. of incubation (Fig. 3.1). In all the subsequent studies, samples were incubated for 15 min. at 37° C. Nonspecific binding of [^{3}H]nicotine was observed to be very high and accounted for 40% of total binding. The reason for such high nonspecific binding of nicotine is not known. However, Romano and Goldstein (1980) also reported such high nonspecific binding (60%) for nicotine (Fig. 3.3)

One of the characteristics of ligand receptor interaction is reversibility of ligand binding to the receptor. Hence, the reversibility of nicotine binding was studied with various concentrations (10-500 nM) of unlabelled nicotine. The unlabelled nicotine displaced bound [^{3}H]nicotine by 23% and 50% at 10 and 20 nM concentrations respectively. But only 54, 59, 62 and 76% displacement was observed with 50, 100, 200 and 500 nM unlabelled nicotine respectively, (Fig. 3.2) and 100% displacement was achieved with 1 mM nicotine. These results indicate the existence of more than one population of nicotinic receptors. Some of them might be low affinity receptors, hence, nicotine bound to such receptors might be displaced even with low concentrations of unlabelled nicotine. The results also suggested the existence of high affinity receptors. Bound [^{3}H]nicotine displaced at extremely high concentrations of unlabelled nicotine might represent this population of receptors. Earlier reports on nicotine binding also suggested the presence of heterogeneous population of receptor sites with low and high affinities for nicotine (Abood *et al*., 1985; Shimohama *et* or/., 1985).

Nicotine binding was studied in synaptic membranes isolated from CC, CE and BS of control and rats administered with AA. Regional differences were observed in nicotine binding, highest being in CC followed by BS and CE (Fig. 3.3). However, the difference in binding of nicotine was statistically significant only between CC and CE, while the difference between CC and BS, CE and BS were not statistically significant. Nicotine binding was elevated in all the three brain regions of the rats administered with subacute/ acute dose of AA. In acute hyperammonemic rats, nicotine binding was increased by 73%, 67% and 54% in CC, BS and CE respectively. In rats administered with subacute dose of AA [^{3}H]nicotine binding was elevated by 60%, 49% and 46% in CC, BS and CE respectively. The magnitude of change was highest in CC

followed by BS and CE. The same profile was maintained in total binding. However, there was no statistically significant change in non-specific binding in all the three regions under these conditions when compared to controls (Fig. 3.4a-3.4c).

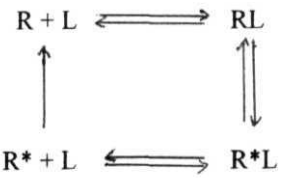
Preliminary studies indicated that [^{3}H]QNB binding was linear upto 500 μg protein and reached saturation at 1mg protein (Fig. 3.5). In further experiments, 200 μg protein was used. QNB binding was linear upto 2 min. and reached saturation levels from 5 min.onwards (Fig. 3.6). Hence, 15 min. of incubation time was chosen for further experiments. Unlike nicotine binding, non specific binding of QNB was less than 2% of the total binding (Fig. 3.7). Binding of QNB was saturable and reversible. Reversibility of QNB binding was checked by the addition of atropine (Fig. 3.8)

Regional differences were observed in saturation isotherms of QNB binding. In CE and BS, QNB binding reached saturation at 0.05 and 0.1 nM concentration of the ligand respectively (Fig. 3.9a and 3.9b). However, saturation isotherm for QNB binding in CC was observed to be biphasic in nature. The first phase of QNB binding reached saturation at 0.05 nM, and increase in QNB concentration beyond 0.05 nM showed large increase in QNB binding and the saturation was observed 0.5 nM onwards (Fig. 3.10a). Scatchard plots of the data from CE and BS gave a linear plot and the data could be fitted into a single linear curve with first order regression (Fig. 3.9a and 3.9b). In contrast to this Scatchard plot of the data from CC gave a curvilinear plot which could not be fitted into a single first order linear regression curve. However, this data fitted best into a biaffinity system (Fig. 3.10b and 3.10c). Results of the present study thus indicated the presence of biaffinity system for QNB binding in CC and single affinity system in CE and BS. Thus in CC two K_d values and two B_{max}

values were obtained The K_d and B_{max} values of low affinity QNB binding were six and three fold higher than those of high affinity system respectively (Table 3.1).

Earlier reports on QNB binding indicated the existance of single affinity binding system. This anamoly might be due to the differences in concentration range of QNB chosen to perform kinetic experiments and other conditions under which experiments were performed. Ravikumar and Sastry (1985) using 0.025-2 nM of QNB, reported a single affinity system in 1000g (10 min.) supernatant fraction of whole brain (except CE) from rats. In the present study, however, the QNB concentration ranged from 0.005 to 5 nM. More over a better membrane preparation was used as a source for receptors when compared with those of Ravikumar and Sastry (1985).

It is interesting to note that Kloog and Sokolovsky (1978) reported an apparent presence of two types of binding sites for N-methyl-4-piperidyl benzilate (4-NMPB), an analogue of benzilate series, in mouse brain membranes. They obtained similar result with another antagonist, scopolamine. They reported that the association involves a single population of binding sites but dissociation was a biphasic reaction representing the presence of two populations of ligand receptor complexes. Kloog and Sokolovsky reported that the biphasic nature of 4-NMPB binding to the receptors was temperature dependent. It was observed the presence of two types of receptors only at 25 and 37° C but not at 10° C where only single type of 4-NMPB receptors were observed. Based on these results they proposed a model



Their interpretation of results was rather interesting. Based on the results, especially of temperature dependence of the appearance of biaffinity system, they predicted the existence of single population of receptors with a single association constant and formation of a RL complex. They, however, predicted that the RL complex undergoes an isomerization resulting in the formation of R*L complex. They suggested that the dissociation constants of RL and R*L might be different and might represent two different receptor ligand complexes. They ruled out, the possible pre existance of two types of receptor sites as they observed the non identitiy of ligand receptor populations and at different temperatures with the same ligand.

Results of the present study, indicating the existance of two populations of mAChRs, were in agreement with the results of Kloog and Sokolovsky (1978). With the data available, however, it is not possible to predict whether the dual affinity systems observed in present study actually represent two isoforms of a single receptor (as suggested by KJoog and Sokolovsky) or two distinct types of muscarinic receptors. It is interesting to note that such biaffinity receptor systems have been reported for other neurotransmitters such as glutamate and GAB A. By convention, the high affinity binding system is considerd as the representation of neurotransmitter receptors.

As mentioned earlier, dual affinity systems were noticed in CC while a single affinity system was present in CE and BS. A comparison of the kinetic parameters (K_d and B_{max}) of the high affinity binding system of CC with that of CE and BS revealed an interesting pattern. Both the K_d and B_{max} values of mAChRs in CE were less than that of CC and BS. In

the latter two regions, there was no statistically significant differences between the K_d values of muscarinic receptors. This suggests that the mAChRs of CE have much higher affinity (3 fold) than present in CC and BS (Table 3.1 and 3.2). These studies do not indicate the precise reason for such regional differences in the affinity of mAChRs. Similar differences were also observed in kinetics of glutamate receptors in these regions by Reeba (1995). One of the reasons for such differences in the affinity of the receptor might be due to the differential distribution of muscarinic receptor subtypes in these three regions of the rat brain. As the receptors are integral proteins of the plasma membrane, differences in physico-chemical properties of the membrane in these three regions of brain might also influence the conformation of the receptor protein, thus its kinetic properties. Studies of Reeba (1995) indicated a higher ratio of cholesterol/phospholipid of membranes of CC and BS when compared to that of CE.

The B_{max} values obtained in the kinetic studies are usually considered as the indicators of the receptor density in the preparation. Results obtained in the present study revealed that the B_{max} values of CE were much lower than that of other two regions CC and BS. Such a differential distribution might be due to differences in the cholinergic innervation as well as the differences in the expression of genes coding for mAChRs.

It was suggested earlier that the differences in K_d values between CE and other two regions might be due to the differential distribution of receptor subtypes in these regions. This tenet was verified by determining the distribution of pirenzepine sensitive mAChRs in these three regions of the brain. It has been reported that M_1 subtype of muscarinic receptors have a greater sensitivity to pirenzipine compared to M_2 subtype. Differences in K_d values of M_1 and M_2 receptors for pirenzepine was

reported to be atleast an order of magnitude (Dadi *et al.,* 1986). In the present study 50 nM pirenzepine was used. At this concentration only M_1 receptors would be blocked while the M_2 receptors would be least affected. Hence, in these experiments QNB binding was carried out both in absence (total) and presence (M_2) of pirenzepine. The difference between the binding in absence and presence of pirenzepine would give an information about the density of M_1 receptors. In all these studies 1 nM QNB was used, which was higher than the K_d values of the receptor in three regions of the brain.

Regional differences in QNB binding (total) was observed in rat brain. Highest binding was observed in CC followed by BS and CE, indicating the order of distribution of mAChRs as COBS>CE in these regions (Fig. 3.11a). This coincides well with the distribution of mAChR derived from B_{max} values.

Regional differences were also observed in the distribution of M_1 and M_2 subtypes of mAChRs. Among the CC, CE and BS, receptor density of these receptors was much higher in CC than in CE and BS. In contrast to the above, the ratio of M_2 to M_1 receptors was much higher in CE and BS than in CC. These studies indicated the predominance of M_2 type of mAChR in CE and BS while an equal representation of both the receptor subtypes in CC (Fig. 3.11b). The results obtained in the present study were in agreement with those published earlier (Hammer *et al.*, 1980; Hulme *et al.,* 1990).

Changes in responses of muscarinic receptors in acute and subacute hyperammonemic states was studied in membranes prepared from CC, CE and BS. Regional differences were observed in response of mAChR in hyperammonemic states. Changes observed in CE mAChRs were statistically not significant under these conditions. A decrease was observed in muscarinic binding both in acute and subacute states in

membranes of CC. The magnitude of decrease observed in acute hyperammonemic state was greater than that of subacute and also statistically significant. Marginal elevation in the binding of QNB to mAChRs was observed in the membranes isolated from BS of subacute hyperammonemic rats. However, such an effect was not seen in this brain region under acute hyperammonemic states, wherein there was no statistically significant difference in QNB binding when compared to control animals (Fig. 3.12a).

To check whether the observed changes were due to direct action of AA, synaptic membranes isolated from CC of control rats were incubated in presence of 1, 2 and 5 mM AA. There was no significant change in QNB binding under these conditions compared to controls (Fig. 3.13).

Similarly, regional differences were observed in the response of M_1, M_2 subtypes of mAChRs. The binding of QNB to the M_1 receptors was observed to be suppressed in CC and BS in both acute and subacute hyperammonemic states, except that the change observed in CC in subacute state was of lesser magnitude and was statistically not significant. There was an elevation in QNB binding to M_1 receptors in the CE, though the changes observed in CE were of larger magnitude, they were not statistically significant. This may be due to lowest density of these receptors in CE, as a result of which large variation was observed in binding studies (Fig. 3.12c).

QNB binding to M_2 receptors was elevated in BS of subacute hyperammonemic rats. The changes observed in both acute and subacute states in the other two regions of brain (CC and CE) were statistically not significant (Fig. 3.12b).

Inorder to gain further insight into the effects of ammonia on muscarinic receptors, kinetic studies were carried out on QNB binding in normal and hyperammonemic rats. The results indicated a decrease in B_{max}

values of high and low affinity QNB binding systems in *CC* of hyperammonemic rats (acute), which is in agreement with the results reported above. There was no significant changes in B_{max} values of the single affinity QNB binding sites in CE and BS. Similarly there were no significant changes in K_d values for QNB binding in CC and BS. In contrast to this, there was an elevation of K_d values of cerebellar mAChRs in acute hyperammonemic states (Table 3.1 and 3.2).

Administration of acute dose of MSI had no significant effect on QNB binding to M_1 and M_2 receptors in CC, where as, a significant decrease in M_2 receptors was observed in CE. A significant decrease in QNB binding to M_1 receptors was observed in BS while there were no changes in M_2 receptors (Fig. 3.14a-3.14c).

In brief, results of the receptor binding studies indicated an increase in binding to the nicotinic rceptors and a decreased binding in muscarinic receptors in hyperammonemic states.

Observed changes in nicotine and QNB binding could be due to 1. Changes in synthesis, transport and incorporation of the receptor protein into the membrane and 2. Changes in the expression and functional properties of the receptor. As already mentioned, the first possibility is unlikely to play a role in the changes observed in acetylcholine receptors in hyperammonemic states, as the time interval between administration of the drug and killing of the animal might not be adequate to induce such changes at least in rats administered with AA. Earlier reports in acute hyperammonemic states (induced by the same method) revealed no significant changes in the electrophoretic protein profile of synaptic membranes (Reeba, 1995). Though no specific information is available on the receptor proteins in these studies, still it can be taken into consideration as membranes mainly contain receptor proteins, transport proteins and some membrane bound enzymes. Hence, such changes due to

synthesis, incorporation and degradation of the receptor might be ruled out as the responsible factors which affect the nicotinic and muscarinic receptors in hyperammonemic states.

The post-synaptic effect of neurotransmitter can be modulated by the receptor density and the properties of the receptor such as the affinity of the receptor for neurotransmitter. Receptor density is regulated by 1. its turnover, which includes synthesis, transport, incorporation into the membrane and degradation of the receptor and 2. By masking/unmasking of the receptors due to changes in the membrane architecture especially its viscosity and fluidity. As the time interval between the administration of the drug and killing of the animal might not be adequate for bringing about changes in receptor turn over. As discussed earlier, changes in the physico-chemical properties of the membrane might be responsible for the observed changes in the receptor binding studies in hyperammonemic states. It is pertinent to mention at this juncture that the response of the different types of proteins to the changes in microenvironment of membrane might be different.

Studies have been carried out in the past on the role of membrane lipids in regulation of receptor function. It has been shown that the presence of negatively charged phospholipids along with cholesterol provide an optimal environment for the incorporation and stabilization of AChRs (Criado *et al,* 1982; Dalziel *et al.,* 1980; Fong and McNamee, 1986; Jones and McNamee, 1988; Ochoa *et al.*, 1983). Experiments with acetylcholine receptor incorporated into reconstituted membranes with defined lipid composition revealed that the affinity of the receptor towards the ligand is reduced by the addition of cholesterol. However, the ion flux through the channel is enhanced by the addition of cholesterol to membrane vesicles (Fernandez-Ballester *et al,* 1994). As cholesterol modifies the membrane fluidity, it was suggested earlier that optimal

fluidity is required for the efficient functioning of receptors and other membrane proteins (Fong and McNamee, 1986). This was further supported by the direct correlation between the membrane viscosity (inversly proportional to fluidity) and ability of the membranes to support ligand binding transitions and ion flux responses of the nAChR. It was also proposed that the membrane viscosity affects protein conformation there by influencing the functional properties of the receptors. Klein *et al.*, (1995) also demonstrated that the removal of cholesterol from the myometrial membrane resulted in the transformation of high affinity oxytocin receptors (K_d=1.5 nM) to low affinity receptors (K_d=134 nM) and on increasing the cholesterol content in these membranes, the low affinity receptors reverted to high affinity receptors. These results indicate that the interaction between cholesterol and phospholipids (one of the parameters governing the membrane fluidity), affects the functioning of the membrane proteins including the nAChR.

In addition to this, cholesterol also directly binds to the AChR and other membrane proteins (for example Ca^{2+} ATPase; East *et al.*, 1984; Simmonds *et al.*, 1984). It has been suggested that cholesterol by interacting with the sites located at the transmembrane portion of the protein may play a complex role as an allosteric effector of acetylcholine receptor (Fernadez-Ballester *et al.*, 1994; Jones and McNamee, 1988).

It has been suggested earlier that, changes in receptor properties in hyperammonemic states might be due to the changes in the physico-chemical properties of the membrane such as fluidity. Cholesterol content was decreased in membranes of CC and CE, while it was increased in membranes of BS in hyperammonemic states. As a consequence the ratio of cholesterol to phospholipid (C/P) decreased both in CC, CE and registered a marginal elevation in BS. It has been shown that C/P ratio is inversely proportional to membrane fluidity. These results indicated

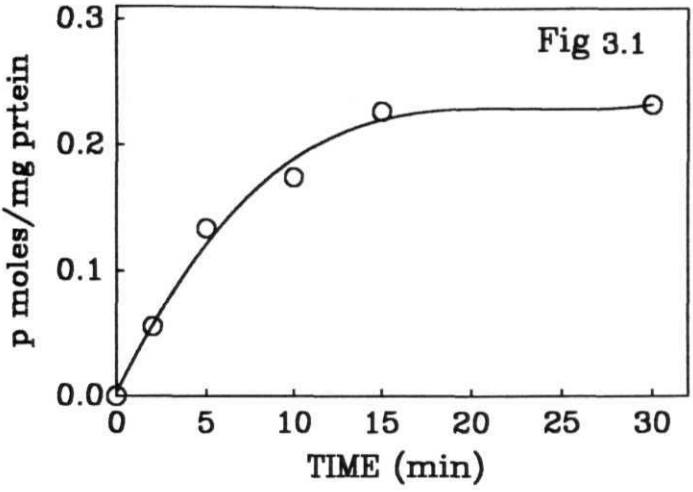
alterations in membrane fluidity in hyperammonemic states. This was further supported by fluorescent polarization studies of the membranes (Reeba, 1995). These observations are in support of the suggestion that the changes in receptor properties in hyperammonemic states may be due to the alterations in membrane architecture (Reeba, 1995). Alterations in the activities of several membrane bound enzymes such as Na^+, K^+-ATPase, y-glutamyl transpeptidase, AChE and other integral proteins of the plasma membrane such as glutamate and GABA receptors, transport proteins for glutamate, BCAA and choline are in support of this tenet (O'Conner *et al.*, 1984; Rao *et al*., 1991; 1992; Rukmini and Murthy, 1993).

The affinity of the receptor to its ligand is influenced by the architecture of the ligand binding site which inturn is regulated by a conformation of the protein molecule. Post-translational modification of proteins such as phosphorylation, carboxy methylation etc., might also alter the conformation of the ligand binding site either directly or indirectly. Protein phosphorylation is known to be involved in regulating diverse processes in nervous system, such as neurotransmitter biosynthesis, axoplasmic transport, neurotransmitter release, generation of post-synaptic potentials, ion channel conductance, neuronal shape and mobility, elaboration of dendritic and axonal processes and development and maintenance of differentiated characteristics of neurons (Nestler and Greengard, 1983). Several reports implicate protein kinase activities in the control of AChR concentration and function on the cell surface (Haga *et al.,* 1988; Kwatra *et al,* 1987; Rosenbaum *et al*., 1987). Reeba (1995) showed region specific changes in phosphorylation of membrane proteins in AA administered rats. Changes in phosphorylation during hyperammonemic states might alter the ligand-receptor interaction which leads to either increased binding or decreased binding of the ligands.

Results of the present study indicated an increase in nicotinic binding. nAChRs are ionotropic receptors and binding of neurotransmitter to these receptors depolarizes the post-synaptic neuron. Their stimulation in hyperammonemic states may or may not be directly involved in the onset of convulsions. However, as these receptors are excitatory in nature, their stimulation would atleast partially depolarize the target neurons and keeps them in a state of activation. Under such conditions a stimulus of even lesser threshold which would normally an insignificant one might also trigger convulsions.

Results of the muscarinic receptor studies indicated a decrease in activity of the receptors in CC. In CC decrease in muscarinic binding was found to be due to the changes in receptor density (as indicated by decrease in B_{max} values). In CE, K_d value for QNB binding was increased suggesting a reduced affinity of the receptor to its ligand. It is interesting to note that in CE, change in affinity was not associated with change in B_{max} value. However, the persual of saturation isotherms for QNB binding in the CE revealed an interesting pattern. Due to the decreased affinity, the saturation point of the receptor was shifted towards right hand side suggesting that the higher concentration of the ligand might be required for saturating the receptor. Moreover, at low concentrations the amount of QNB bound was less in hyperammonemic states when compared to normals (Fig. 3.15). Hence, it is suggested that the functioning of the cerebellar mAChR might be suppressed in hyperammonemic states. Though studies were not carried out on release of acetylcholine, it has been reported that ammonium ions have an adverse effect on the packing of acetylcholine into synaptic vesicles. Under such conditions, it is possible that less amount of acetylcholine may be released in hyperammonemic states. Since all the mAChRs are associated with second messengers, a decreased muscarinic function might also affcet the

production of second messengers (IP_3, cAMP) and the processes mediated by these second messenger systems. It has been suggested that the cholinergic neurons in the brain are involved in initiation and execution of motor functions and in the regulation of sleep wake cycles (Woolf, 1991). Dysfunction in cholinergic system in hyperammonemic states might be partially responsible for such behavioural changes reported in hyperammonemic states.



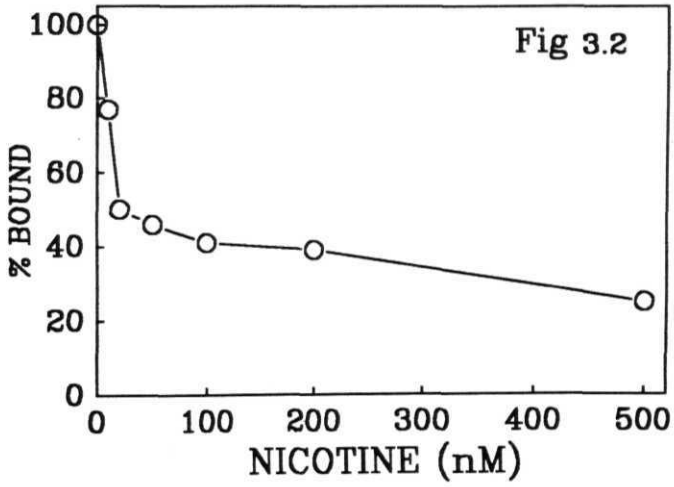


Fig 3.1: [^{3}H]Nicotine binding to cortical synaptic membranes of control rats as a function of time Values are Mean of two experiments

Fig 3.2: Displacement of bound [H]nicotine with unlabelled nicotine Membranes were pre-incubated with [H]nicotine and unlabelled nicotine was added Data plotted as concentration of unlabelled nicotine added and percent of [H]nicotine bound after the displacement Values are Mean of three experiments

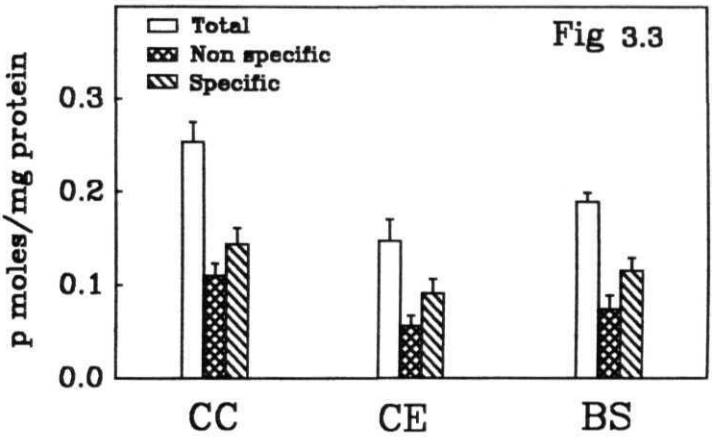


Fig 3.3: Total, non specific and specific binding of [H]nicotine to synaptic membranes isolated from CC, CE and **BS** of control rats indicating the regional distribution of nicotinic receptors in **brain** Values are Mean ± S **D** of three experiments done in **duplicates**

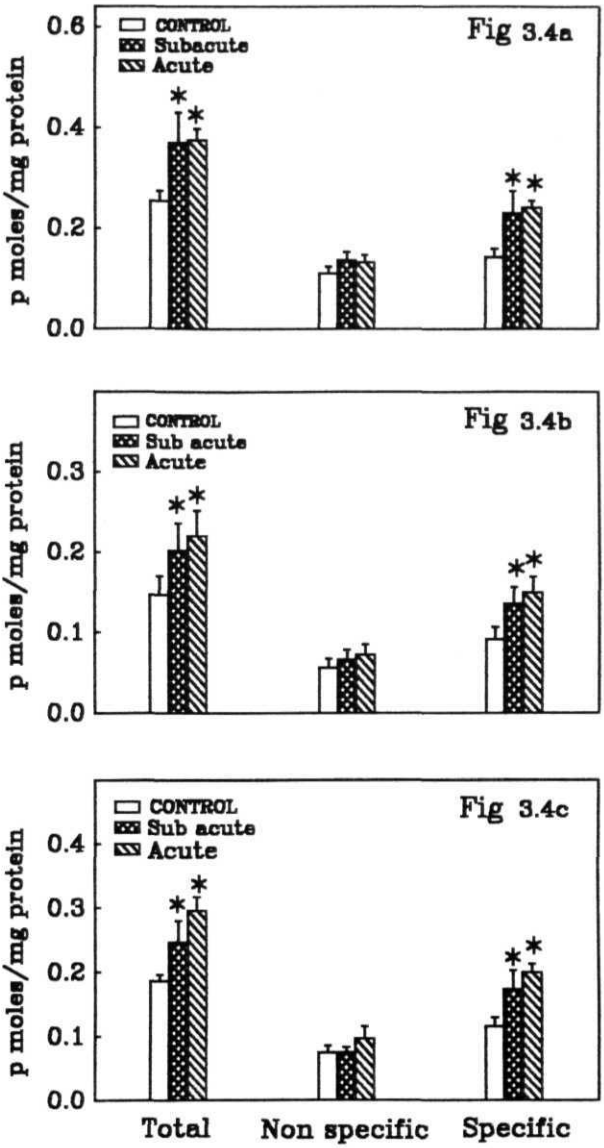
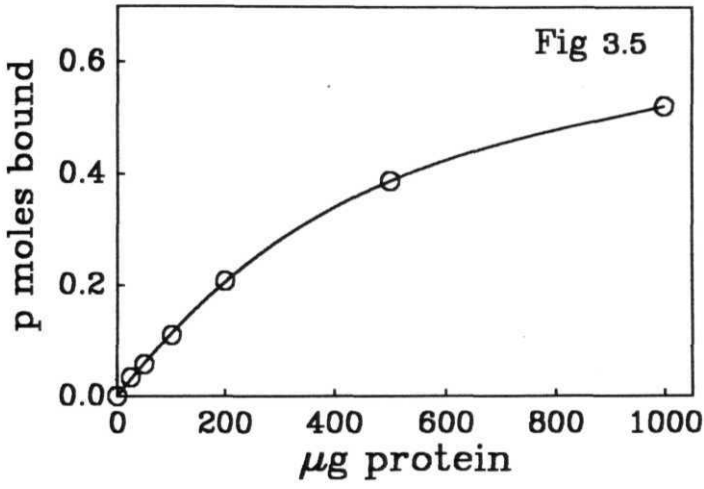


Fig 3.4: Total, non specific and specific binding of [H]nicotine to synaptic membranes isolated from (a) CC, (b) CE and (c) BS of control rats and rats administered with subacute or acute dose of AA Values are Mean ± S.D of three experiments done in duplicates * : Statistically significant compared to controls ($p < 005$).



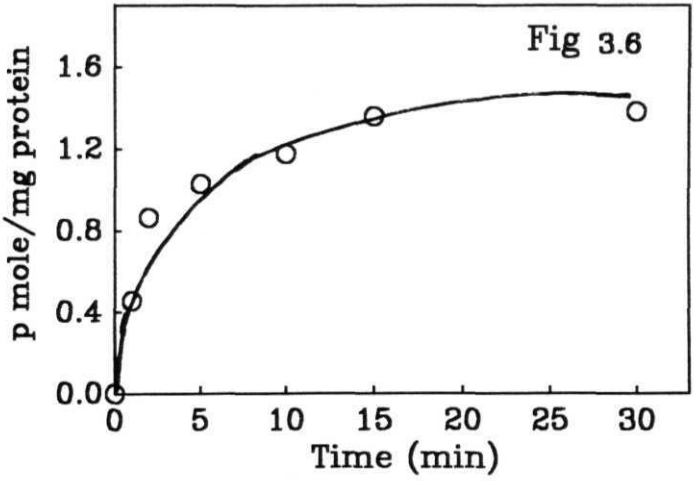


Fig 3.5: [H]QNB binding to cortical synaptic membranes as a function of protein concentration

Fig 3.6: [H]QNB binding to cortical synaptic membranes as a function of incubation time Values are Mean of two experiments.

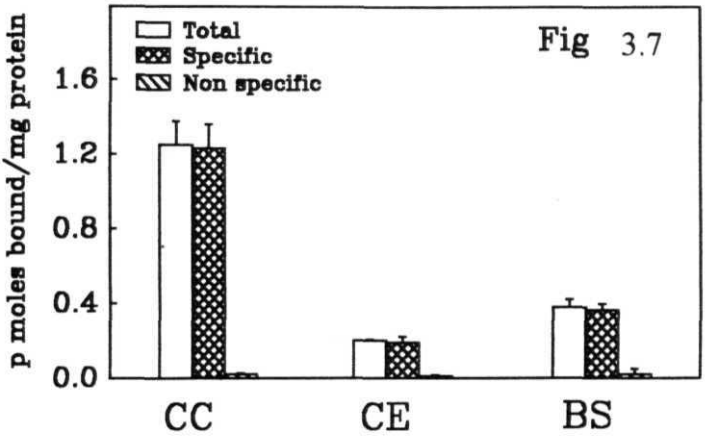


Fig 3.7: Total, non specific and specific binding of [H]QNB to synaptic membranes isolated from CC, CE and BS of control rats Values are Mean ± S.D of four experiments done in duplicates

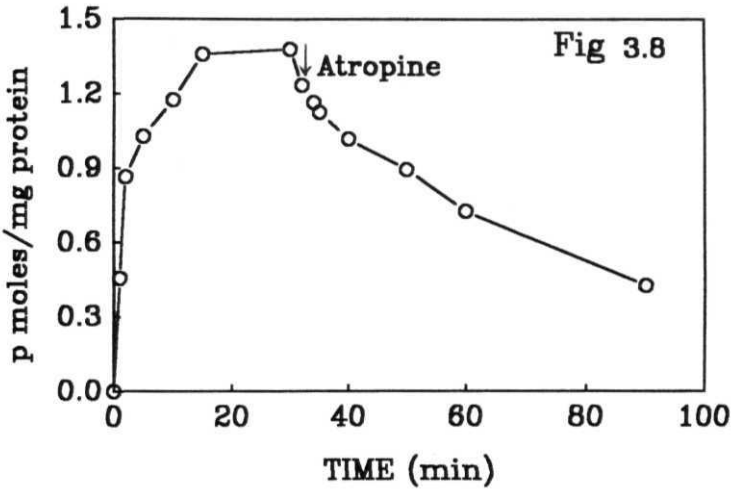
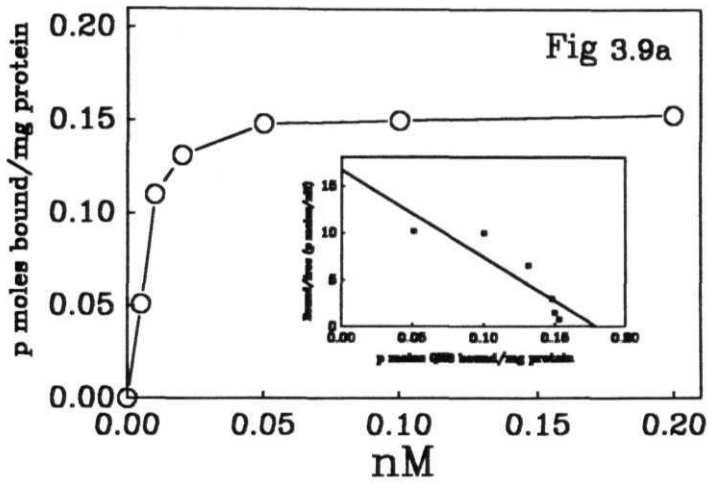


Fig 3 8: Displacement of bound [H]QNB with unlabelled atropine Membranes were pre-incubated with [H]QNB and unlabelled atropine was added Bound QNB was measured at various time periods after the addition of unlabelled atropine Values are Mean of two experiments



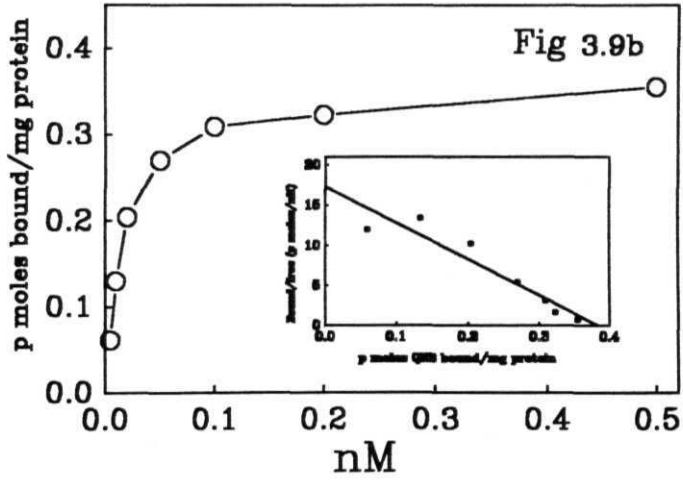


Fig 3.9: Saturation isotherms for [^{3}H]QNB binding in CE (a) and BS (b) showing single affinity system Insert: Scatchard plot of the data from saturation isotherm

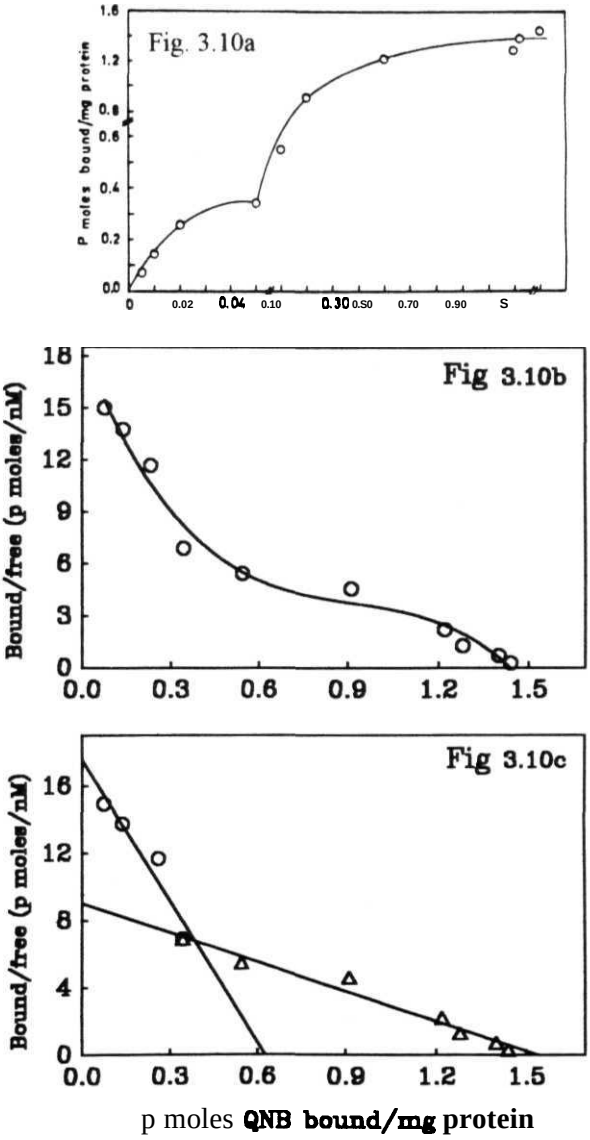
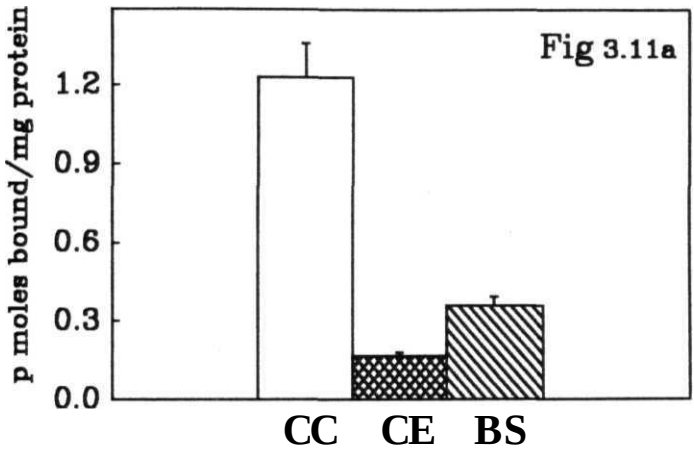


Fig. 3.10: [H]QNB binding to cortical synaptic membranes (a) Saturation isotherm showing biphasic nature (b) Curvilinear Scat chard plot of the data from saturation isotherms (c) Scatchard plots showing the possible presence of two affinity systems for [^{3}H]QNB binding in CC. Values are Mean ± S.D. of three experiments done in duplicates



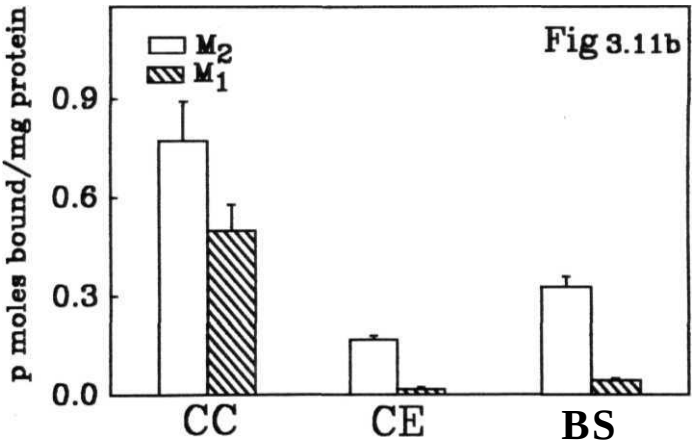


Fig 3 1 la: Specific binding of [H]QNB to mAChR of synaptic membranes isolated from CC, CE and BS of control rats, indicating the distribution of mAChR in rat brain

Fig 3.11b: [H]QNB binding to synaptic membranes in absence (total) and in presence (M_2) of pirenzepine The difference between total and M_2 was assumed as M_1 binding Regional distribution of sub-types of mAChRs (M_2 and M_1) in CC, CE and BS Values are Mean + S.D of three experiments done in duplicates

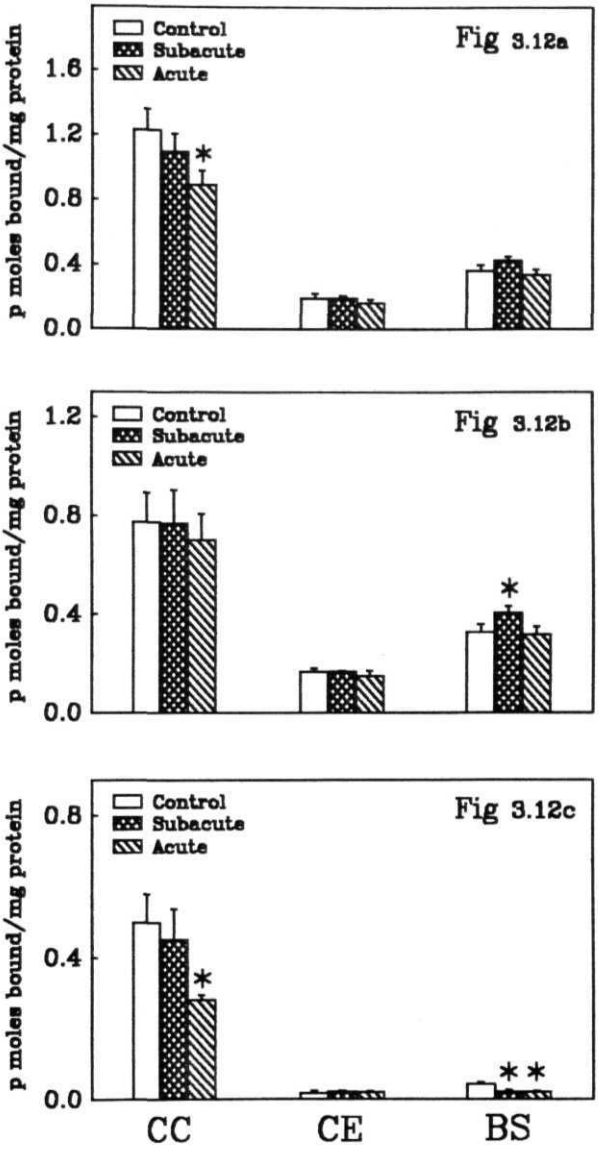


Fig 3.12: Specific [H]QNB binding to (a) total, (b) M_2 and (c) M_1 muscarinic receptors in CC, CE and BS of control rats and rats administered with subacute or acute dose of AA Values are Mean ± S.D of three experiments done in duplicates •: Statistically significant compared to controls $p < 0\ 05$

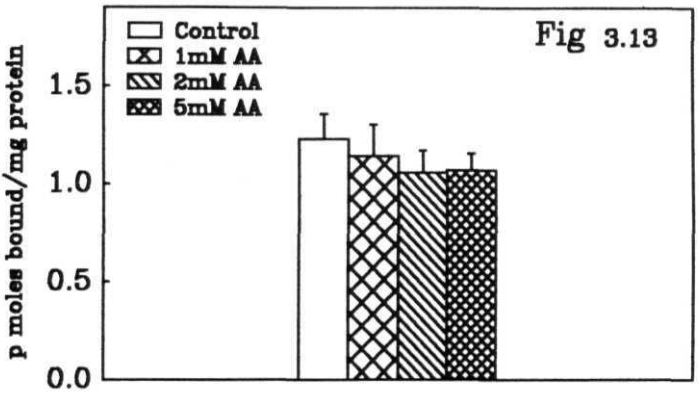


Fig 3.13: *In vitro* effects of AA on [H]QNB to synaptic membranes prepared from CC of control rats Values are Mean ± S.D of three experiments done in duplicates

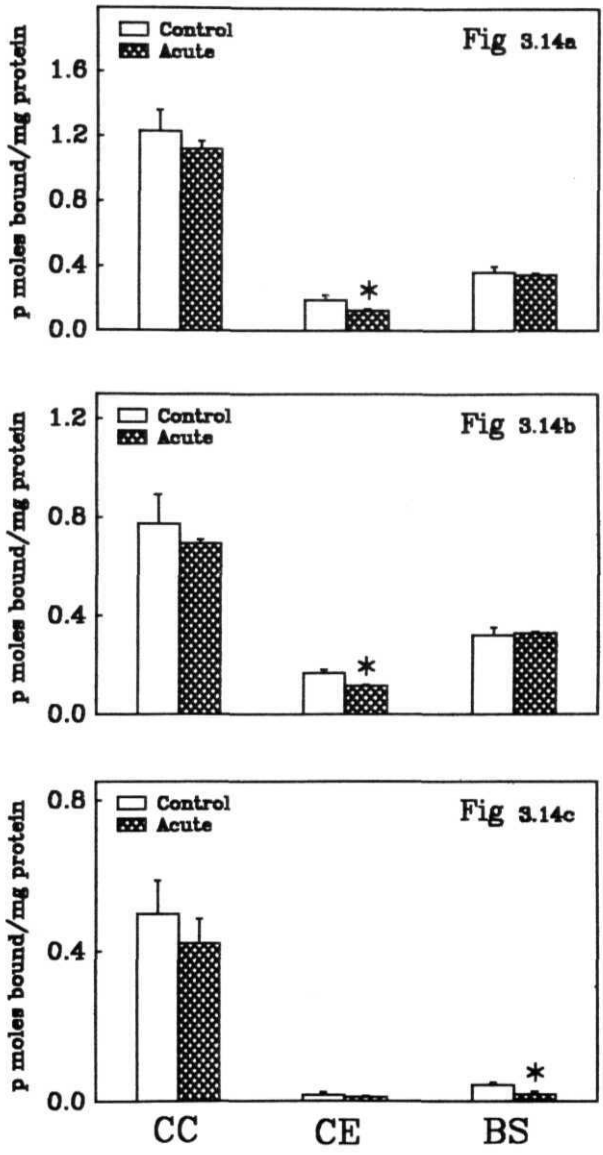


Fig. 3.14: Specific [H]QNB binding to (a) total (b) M_2 and (c) M_1 muscarinic receptors in CC, CE and BS of control rats and rats administered with acute dose of MSI Rest of the legend same as for Fig 3.12

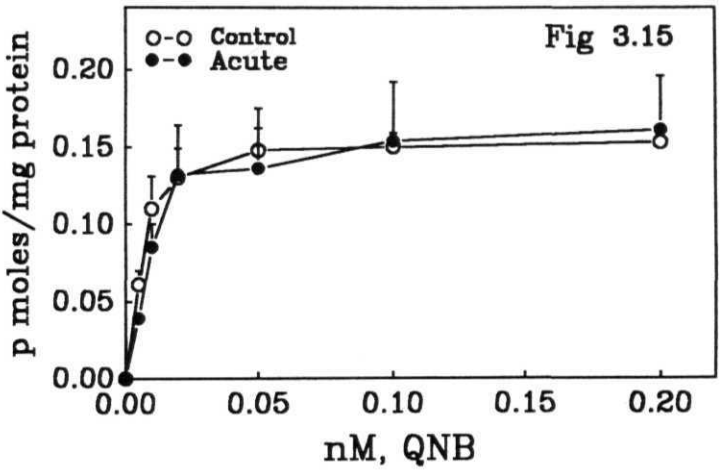


Fig 3.15: Saturation isotherms for QNB binding to synaptic membranes isolated from cerebellum of control rats and rats administered with acute dose of AA. All the values are Mean ± S.D of three experiments

Table: 3.1 Kinetic constants for [^{3}H]QNB binding in synaptic membranes isolated from CC of control rats and rats administered with acute dose of AA.

	HIGH AFFINITY		LOW AFFINITY	
	K_d	B_{max}	K_d	B_{max}
CONTROL	42 ±9	0.62 ± 0.07	189 ± 14	1.68 ±0.31
ACUTE	35 ±7	0.47 ±0.07*	213 ±21	1.01 ±0.14*

All the values are Mean ± S.D. of three experiments done in duplicates. Statistically significant *: $p < 0.01$. K_d values are in pM and B_{max} values are expressed as p moles of QNB bound/mg protein

Table: 3.2 Kinetic constants for [^{3}H]QNB binding in synaptic membranes isolated from CE and BS of control rats and rats administered with acute dose of AA.

	CONTROL		ACUTE	
	K_d	B_{max}	K_d	B_{max}
CE	9.0 + 1.6	0.17 ±0.03	12 ±1.4*	0.19 ±0.05
BS	28 ±5	0.42 ±0.03	23 ±3.5	0.37 ±0.04

K_d values are in pM and B_{max} values are expressed as p moles of ligand bound per mg protein (n=3).

CHAPTER 4
CHOLINESTERASES

Cholinesterases are hydrolytic enzymes which terminate the action of neurotransmitter acetylcholine by hydrolysis. Based on substrate selectivity, cholinesterases are divided into acetyl or true cholinesterase (AChE) and butyryl or pseudocholinesterase (PChE). The former specifically hydrolyzes acetylcholine while the latter preferentially acts on acyl esters of choline with more than two carbons in their acyl group (Bertilsson *et al.*, 1993; Taylor, 1991). AChE is an enzyme with high catalytic efficiency and hydrolyzes its substrate very rapidly. In contrast the hydrolytic action of PChE is very slow. AChE and PChE are coded by single but distinct genes. Phylogenetic analysis shows that AChE and PChE arose through gene duplication after the appearance of cholinergic system. This is also supported by the finding that *Drosophila* cholinesterase activity is intermediate in between AChE and PChE and has low substrate specificity when compared to the vertebrate cholinesterases (Toutant, 1989). PChE is primarily synthesized in liver and is secreted into the blood. Hence, this enzyme is abundant in plasma. The precise function of this enzyme either in plasma or in tissues is not yet known. But the distribution of its mutants showing resistance to naturally occurring inhibitors suggests that, its function is to hydrolyze dietary esters with potential toxicity. Still it is considered as physiologically unimportant enzyme as individuals lacking functional PChE are phenotypically normal. Though this enzyme hydrolyzes long chain acyl esters of choline, there are few naturally occurring compounds of this category. Hence, it is generally believed that this is an enzyme without natural substrate. As AChE is the major enzyme involved in the

termination of neurotransmitter activity of acetylcholine, a brief survey of literature on this enzyme is given below.

AChE is widely distributed in neural and also in non-neural tissues. It's presence has been reported both in neurons and glial cells of brain and also in neuromuscular junctions. AChE is also reported to be present in erythrocytes, platelets and lymphocytes (Appleyard, 1992). It's presence in neural tissues is understandable, while its role in non-neural tissues especially in blood cells is yet to be investigated.

Multiple molecular forms of the enzyme have been identified. These forms differ in their solubility and mode of membrane attachment but not in their catalytic activity (Massoulie and Bon, 1982; Taylor, 1991). The enzyme exists in monomeric, dimeric, and tetrameric globular forms (Muller *et al,* 1985; Rakoczay and Brimijoin, 1988; Rieger *et al.,* 1980). The globular forms of the enzyme exist both as soluble and membrane bound forms which differ in their hydrophobicity. These two species of AChE differ in the last 40 amino acid residues at the carboxy terminal end. The hydrophobic character of the enzyme is because of the presence of glycophospholipid on the carboxy terminal end (Roberts *et al.,* 1987; Silman and Futerman, 1987). The molecular weight of monomeric form is reported to be 80, 000 Daltons (Brimijoin, 1983). The multimeric form of the enzyme is characterized by the presence of a collagen like tail in which three groups of tetrameric subunits are linked to the three helical strand of the collagen tail by a disulfide bond. The collagen tail helps in attachment of the enzyme to the basal lamina of the synapse (Brandan *et al,* 1985; McMahan *et al,* 1978; Rosenberry and Richardson, 1978). This collagen tail also imparts asymmetry in the enzyme, hence, this enzyme is called as asymmetric form. This A_{12} form is enriched in basal lamina of synaptic structures and neuromuscular junctions (Couraud *et al.,* 1980). It accounts 0.02% of total AChE activity of CNS, while G_4 form contributes

to 90% of the activity in mammalian CNS (Grassi *et al.*, 1982; Rieger and Vigny, 1976; Wade and Timiras, 1980). In rat CE, both G_1 and G_4 equally contribute and in BS, G_2 form also contributes significantly (Clark and Lenz, 1983).

Membrane bound G_4 appears to be the physiologically critical form in cholinergic neurons of CNS (Muller *et al*, 1985). In human brain, high proportions of G_4 correlate with areas of high cholinergic activity (Atack *et al*, 1986; Fisman *et al*, 1986). The relative proportions of the AChE molecular forms change in relation to developmental stages of mammalian brain (Muller *et al.*, 1985; Rieger and Vigny, 1976; Zakut *et al*, 1985). Rieger and Vigny (1976) showed that the G_1 form is the first to appear in fetal rat brain and reaches adult levels by the time of birth, while the G_4 form appears later and increase slowly until maturity. The transient expression of AChE preceding the active synaptogenesis suggests that AChE aids in neurite outgrowth and extension of neurites during development (Appleyard, 1992).

The catalytic mechanism of AChE has been studied in detail. The enzyme active site has two subsites, an anionic site and an esteratic site. The positively charged trimethylammonium head group of acetylcholine binds to the anionic site and the ester bond region to the esteratic site. A key step in reaction mechanism is the acylation of serine residue of the enzyme followed by deacylation and the active site is believed to contain the catalytic triad typical of serine hydrolases with a histidine implicated as the intermediary charge relay residue. However, it is interesting to note that there is no sequence homology between AChE and other serine hydrolases such as chymotrypsin and subtilisin. The catalytic sequence in acetylcholine hydrolysis is: The active site serine (200) is rendered nucleophilic by a dicarboxylic amino acid (glutamate 327) serving as proton sink and an imidazole group of histidine 440. The serine attacks the

carbonyl carbon of acetylcholine forming a tetrahedral intermediate. The carbonyl oxygen is likely stabilized through hydrogen bonding in an oxyanion hole. Removal of the choline leaving group forms an acyl enzyme. Attack of water leaves free enzyme (Taylor and Brown, 1994). The acyl enzyme is short lived and accounts for the high catalytic efficiency of the enzyme.

Inhibitors for AChE act by different mechanisms. Inhibitors such as edrophonium bind reversibly to active site of the enzyme and prevent access of the substrate. Other reversible inhibitors such as gallamine and propidium, bind to a peripheral site on the enzyme. The carbamoylating agents, such as neostigmine and physostigmine, form a carbamoyl enzyme by reacting with the active site serine. The carbamoyl enzymes are more stable than the acetyl enzyme. The alkyl phosphates, such as diisofluorophosphate or echothiopate, act in similar manner. The alkylphosphorates and alkylphosphonates form an extremely stable bond with serine in the active site of the enzyme. The time required for their hydrolysis often exceeds that for biosynthesis and turnover of the enzyme. Thus, the acute toxicity of carbamoylesters and organic fluorophosphates is caused by the very slow hydrolysis rates of such acylenzyme intermediates and are considered as irreversible inhibitors of the enzyme (Taylor and Brown, 1994).

Functions other than hydrolysis of acetylcholine has been ascribed to AChE, 1. The reversible hyperpolarizing action via an opening of potassium channels on selective population of nigral neurons (Webb and Greenfield, 1992). 2. It has also been reported that, peptidase activity was associated with AChE. However it was found to be due to contaminating enzymes (Checler *et al.*, 1994).

AChE is synthesized in cell body of the neuron and is transported to the nerve terminal by axonal transport. It has been demonstrated that

this enzyme is rapidly transported bidirectionally and the velocities of the anterograde and retrograde axonal transport were calculated to be 400 and 260 mm/day (Lubinska and Niemierko, 1971). AChE is coded by single gene and structural diversity arises from alternative processing of mRNA and post-translational modification of subunits (Li *et al.*, 1993; Gibney *et al*, 1988; 1990; Sikorav *et al.*, 1988; Taylor, 1991). The open reading frame in mammalian AChE gene is encoded by three invariant exons (exons 2, 3 and 4) followed by three splicing alternatives. Continuation through exon 4 gives rise to a monomeric species, splicing to exon 5 gives carboxy terminal with signal sequence for addition of glycophospholipid, while splicing to exon six encodes a sequence containing a cysteine that links to other catalytic or structural subunits (Taylor and Brown, 1994).

Cholinesterases have been implicated in pathophyisiology of certain disorders associated with cholinergic dysfunction viz. general mental retardation or Down's syndrome (Price *et al.*, 1982; Propert, 1979), neural tube defects (Bonham and Atack, 1983; Smith *et al.*, 1979), Alzheimer's, Parkinson's and Huntington diseases (Arendt *et al.*, 1984; Atack *et al.*, 1985; Mesulam and Moran, 1987; Yates *et al.*, 1983) and also in tumors (Topilko and Caillou, 1988). AChE inhibitors physostigmine, heptylphysostigmine, tetrahydroamino acridine and nutrifonate have been proposed as candidates for cholinomimetic therapy of Alzheimer's disease (Becker and Giacobini, 1988; Pomponi *et al.*, 1990).

The neurotransmitter function of acetylcholine is terminated by the action of hydrolyzing enzyme, AChE. Hence, alterations in AChE activity might affect the synaptic transmission. Present study was carried out to gain an insight into AChE function in hyperammonemic states and thus the cholinergic synaptic transmission.

AChE activity was determined in synaptosomes isolated from different regions of normal and hyperammonemic rats. Regional differences were observed in the activities of synaptosomal AChE and PChE. AChE activity was the highest in BS and CC and the lowest in CE (Fig. 4. la) whereas PChE activity was highest in CE and BS and lowest in CC (Fig. 4.1b).

Administration of subacute dose of ammonium acetate resulted in an elevation in the activities of both the cholinesterases in synaptosomes isolated from all the three regions In this group of animals, magnitude of elevation in AChE was higher in CE and BS than in CC while the magnitude of elevation in PChE activity was higher in CC than in BS and CE. AChE activity was elevated by 19% (statistically not significant), 35% and 41% while the PChE activity was elevated by 66%, 18% and 45% in CC, CE and BS respectively (Fig. 4.2a and 4.2b). Administration of acute dose of AA also elevated the activities of these two enzymes and the magnitude of elevation was greater than that of subacute group (Fig. 4.3a and 4.3b). Moreover, in the acute group of animals elevation in the activities of these two enzymes was linear with respect to time. Magnitude of elevation was higher in BS and CC than in CE. At 5 min. after administration of AA, AChE activity was increased by 11%, 3% (not statistically significant) and 21% in CC, CE and BS respectively whereas PChE activity was increased by 38%, 21% and 6% (statistically not significant). After 10 min., AChE and PChE activities were elevated by 51%, 65%, 42% and 79%, 58%, 25% in *CC,* CE and BS respectively. During convulsive phase AChE activity was increased by 85%, 99.8% and 75% in CC, CE and BS respectively whereas PChE activity was increased by 114%, 93% and 38% (Fig. 4.4a and 4.4b). A similar elevation in the activities of cholinesterases was reported earlier in mice injected with a single or multiple doses of AA (Sadasivudu *et al.*, 1983). Recently

reduction in AChE activity has been reported in rats administered with AA (Kosenko *et al.*, 1994). This could be because of the difference in enzyme source, dosage of AA used for induction of hyperammonemia etc. Synaptosomal cholinesterase activitiy was also studied in hyperammonemia induced by administering acute dose of MSI. Significant elevation was observed in activity of AChE in *CC*, while no change was observed in cholinesterase activities in CE and BS (Fig. 4.5a and 4.5b).

As elevation in the activities of cerebral cholinesterases and content of ammonia in brain and blood in hyperammonemic animals were observed to be time dependent, relationship between these were elucidated. There was no correlation (r = 0.52) between rise in blood ammonia level and the activities of brain cholinesterase while a strong correlation (r = 0.98) was observed between the rise in brain ammonia content and its cholinesterase activity (in all the three regions studied; Fig. 4.6a and 4.6b).

Elevation in the activities of cholinesterases in hyperammonemic states could be due to the direct action of ammonium ion on the enzyme or an indirect process which might ultimately stimulate the enzyme activity. To elucidate the first possibility, *in vitro* effects of three different concentrations of AA (1, 5 and 10 mM; pH 7.4) were studied on the cholinesterase activity in synaptosomes isolated from normal animals and on purified AChE.

While carrying out these studies, non-enzymic hydrolysis of both acetyl and butyryl thiocholine esters was observed in presence of AA while no such effect was observed in presence of sodium acetate. This suggested that the non-enzymic hydrolysis of acetylthiocholine might be due to ammonium ion. To confirm this, non-enzymic hydrolysis was studied with different ammonium salts. Similarities in the extent of this non enzymic hydrolysis of thiocholine esters with ammonium formate,

ammonium chloride and ammonium carbonate confirmed that non-enzymic hydrolysis was due to the ammonium ion (Fig. 4.7b). Non-enzymic hydrolysis was dependent on pH, and concentration of ammonium salt (Fig. 4.7a and 4.7c). Maximum hydrolysis was observed at pH 9.0, where most of the ammonia exists as unprotonated form. This suggested that the unprotonated ammonia present in the reaction mixture might be responsible for the non-enzymic hydrolysis of thiocholine esters.

As ammonia was observed to be interfering with hydrolysis of acetylthiocholine, it is possible that the observed increase in cholinesterase activity in hyperammonemic states might be due to elevated ammonia levels in cerebral preparation rather a true rise in cholinesterase activities. Hence, ammonia content was determined in synaptosomes prepared from normal and hyperammonemic rats. There was statistically no significant difference in the ammonia content of synaptosomes isolated from normal (8.64 ± 1.16 n moles/mg protein) and hyperammonemic (10.1 ± 0.68 n moles/mg protein, n=4, $p > 0.1$) rats. However, lack of changes in ammonia content of synaptosomal preparations from normal and hyperammonemic animals indicated that the observed elevation in the activities of cholinesterases in hyperammonemic states was not due to high levels of residual ammonia in the synaptosomes of hyperammonemic rats. Apparently, these changes have taken place in *in vivo* conditions and they have persisted the rigors of isolation procedure. Suitable blanks (-enzyme + AA) for non enzymic hydrolysis were used in studies on the *in vitro* effects of ammonium ions on cholinesterase activities.

Statistically significant elevation was observed in the activity of PChE only in the presence of 5 and 10 mM AA in CC and BS while no change was observed in AChE activity (Fig. 4.8a and 4.8b) Moreover, AA

even at 10 mM, failed to elevate the activity of purified AChE (Table 4.1). This suggested that *in vivo* effects of ammonium ions on the activities of cholinesterases might be indirect.

The observed increase in cholinesterase activity might be due to changes in the affinity of the enzyme to its substrate or in the number of catalytic sites. Kinetic experiments were performed with various concentrations of acetylthiocholine (6 μM to 2000 μM) in synaptosomes isolated from CC of control and acute hyperammonemic rats. AChE was saturated at 250 uM of acetylthiocholine whereas PChE was saturated at 1000 μM of acetylthiocholine. PChE was below ihe detectable range when acetylthiocholine concentration was reduced below 100 μM while the AChE activity was detected even at 6 μM acetylthiocholine (Fig. 4.9a-4.9b and 4.10a-4.10b).

Kinetic analysis of *in vivo* activation of cholinesterases in hyperammonemic states revealed no change in K_m of both the enzymes for their substrates (Table 4.2). However, an increase was observed in the V_{max} of these enzymes in synaptosomes of hyperammonemic rats.

Cholinesterases are membrane bound enzymes and also exist as soluble cytosolic forms. It has been reported that 70-80% of total AChE activity in brain was represented by membrane bound forms (Ogane, *et al.,* 1992; Schegg *et al.,* 1992). As cholinesterases are known to be membrane bound proteins, alterations in membrane properties (such as fluidity) in hyperammonemic states might result in unmasking of more catalytic sites. In earlier reports, responses obtained with other membrane bound enzymes (Na^+, K^+-ATPase, γ-glutamyl transpeptidase), transport systems (glutamate, choline, BCAA) and neurotransmitter receptors (glutamate, GABA) in hyperammonemic states have been suggested to be due to changes in membrane properties (O'Conner *et al.,* 1984; Peterson

et al, 1990; Rao and Murthy, 1991; Rao *et al.*, 1991). The hydrophobic region of the membrane was proposed to play a regulatory role in the biochemical events catalyzed by membrane bound enzymes. Hence, change in the hydrophobic region of the membrane might alter the catalytic functions of these enzymes. It has been proposed that the change in physical state of lipids might induce a conformation change in associated proteins and thus alter their specific functions (Lenaz, 1977). The dynamic lipid-protein interactions are known to play an important role in regulating the functional activity of membrane proteins. There is selectivity in the lipid protein interactions and AChE activity is known to be modulated by different classes of lipids (Cho *et al.*, 1995). Using fluorescent probes such as DPH and TMA-DPH it has been reported that the fluidity increases at the core and decreases at the surface in CC and CE while a reverse pattern was observed in the membranes of BS during hyperammonemic conditions. In contrast, during AA induced hyperammonemia, no significant changes in membrane fluidity has been reported (Reeba, 1995). However, this fails to give any information regarding changes in lipid-protein interactions. Hence, the elevated cholinesterase activity could be a consequence of either altered membrane architecture or lipid-protein interactions. Lack of effect of ammonium ions on the purified AChE was also in favor of such a suggestion. Marginal effects observed on AChE activities *in vitro* studies with AA suggested that the perturbations in membrane architecture might be smaller under *in vitro* conditions than in *in vivo* conditions. Physiological significance of the elevated activity of AChE in hyperammonemic states is difficult to understand as this enzyme has a high catalytic efficiency and its activity under physiological conditions is usually higher than the amount of acetylcholine released. Hence, it could be a consequence of the altered

membrane architecture and might have a role to play in the neurotoxic effects of ammonia.

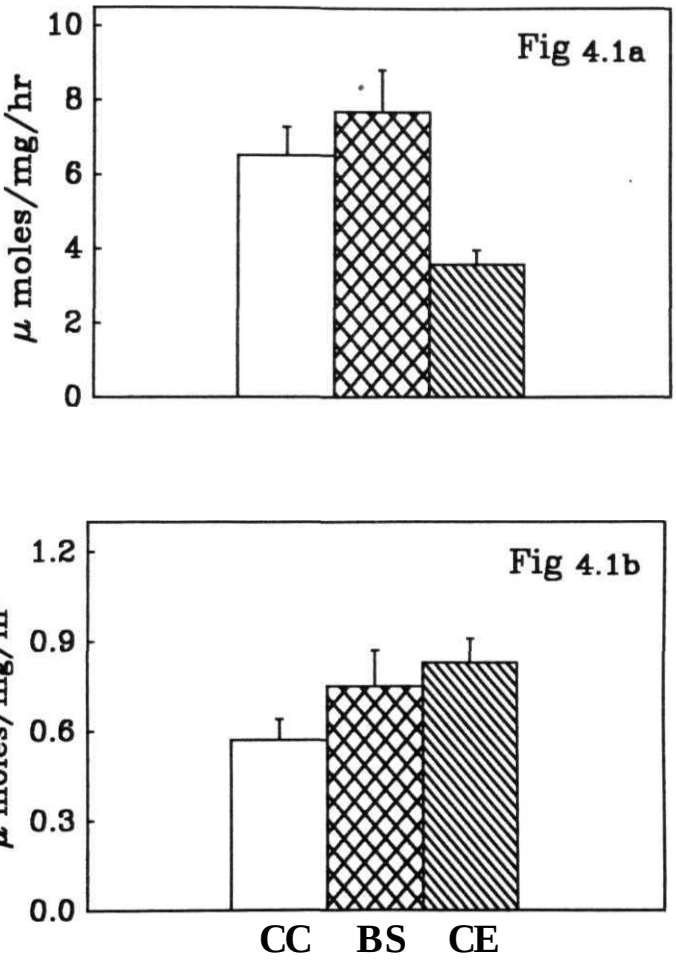


Fig 4 1: Regional distribution of synaptosomal (a) AChE and (b) PChE in CC, CE and **BS**

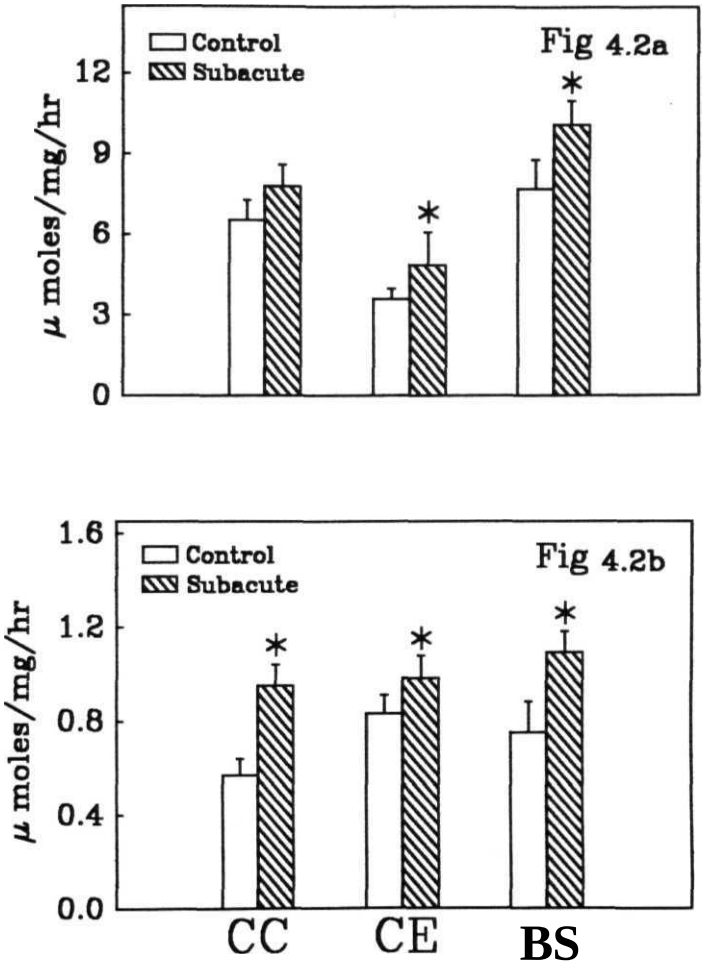


Fig 4.2 AChE (a) and PChE (b) activities in synaptosomes isolated from CC, CE and BS of control rats and rats administered with subacute dose of AA Values are Mean ± S.D. of five experiments done in duplicates *: Statistically significant compared to controls $p < 0.05$.

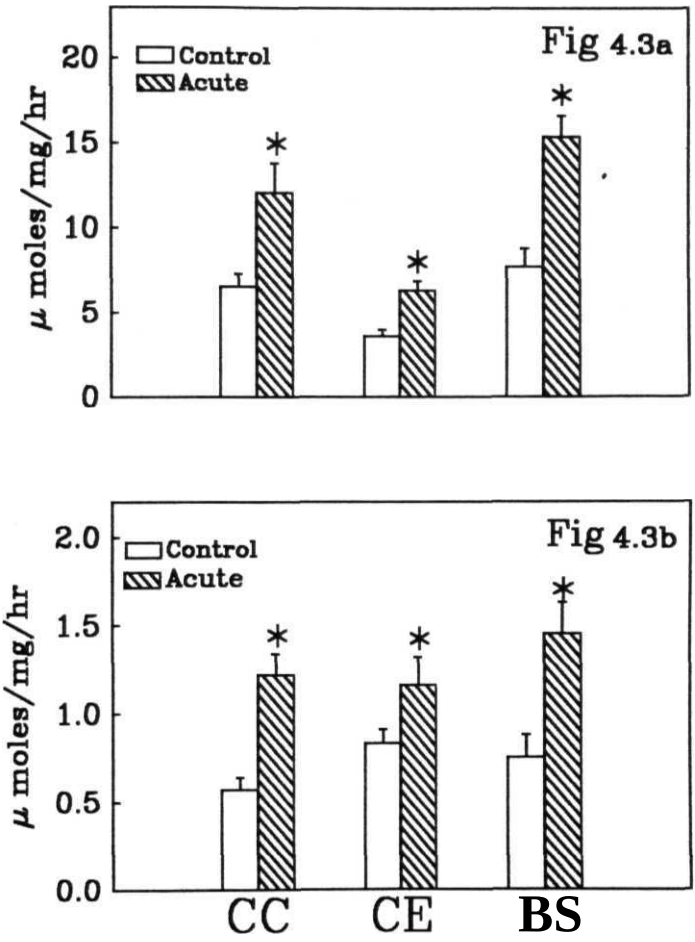


Fig. 4 3: AChE (a) and PChE (b) activities in synaptosomes isolated from CC, CE and BS of control rats and rats administered with acute dose of AA. Values are Mean ± S.D. of five experiments done in duplicates. *: Statistically significant compared to controls $p < 0.05$.

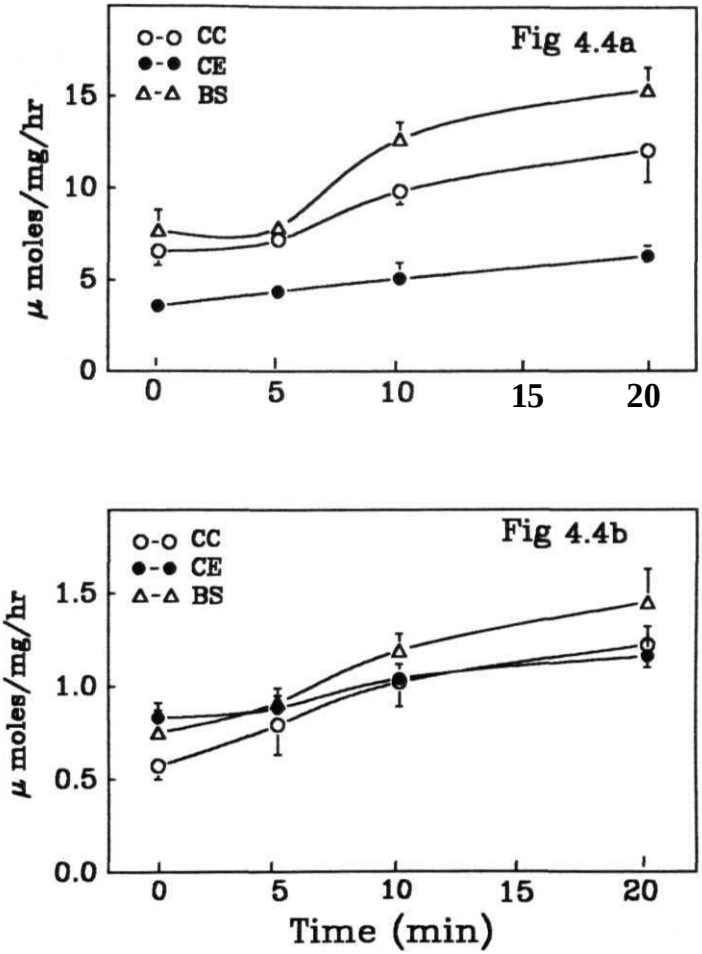


Fig 4 4 AChE (a) and PChE (b) activities in control and acute hyperammonemic states in synaptosomes isolated from CC, CE and BS at various time periods after the administration of acute dose of AA Values are Mean ± S.D. of five experiments Values at 10 and 20 min are statistically significant ($p < 0\ 05$) compared to controls and earlier time period.

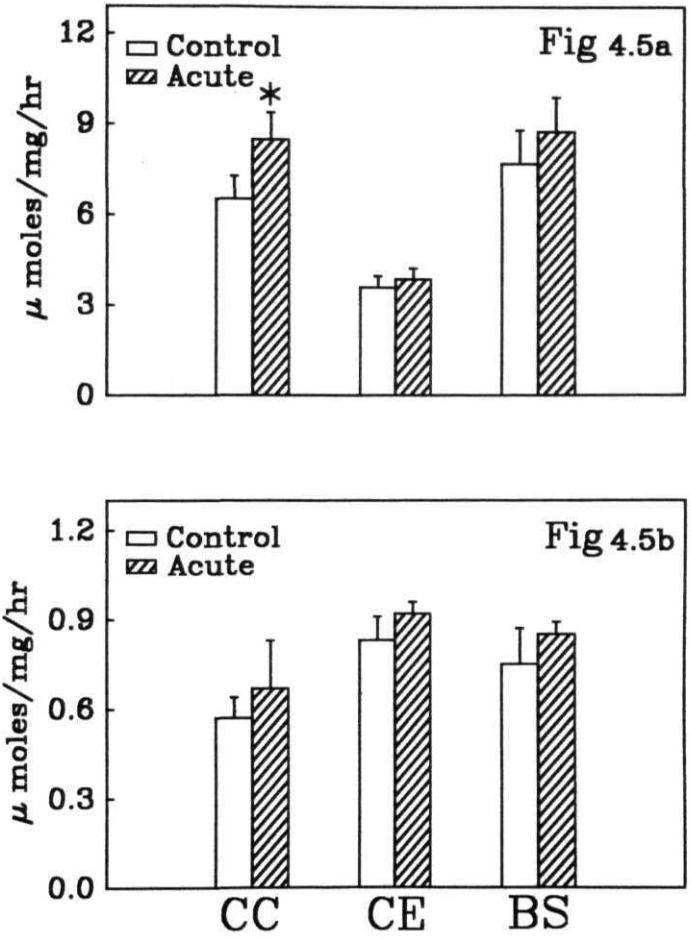
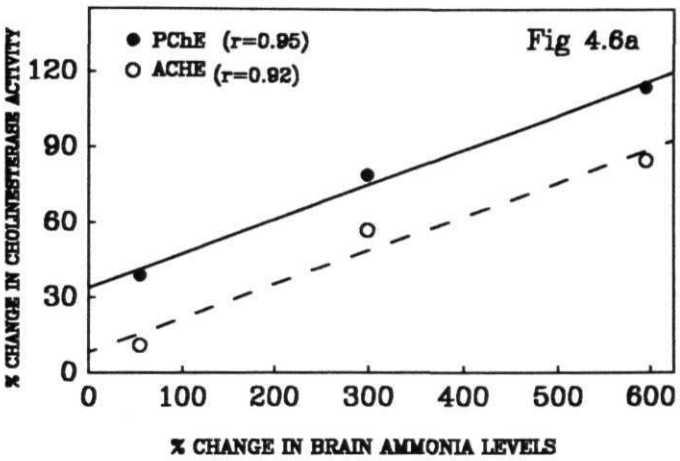


Fig: 4.5: AChE (a) and PChE (b) in synaptosomes isolated from CC, CE and BS of control and rats administered with acute dose of MSI Values are Mean ± S.D of three experiments done in duplicates •: Statistically significant compared to controls p < 0 05.



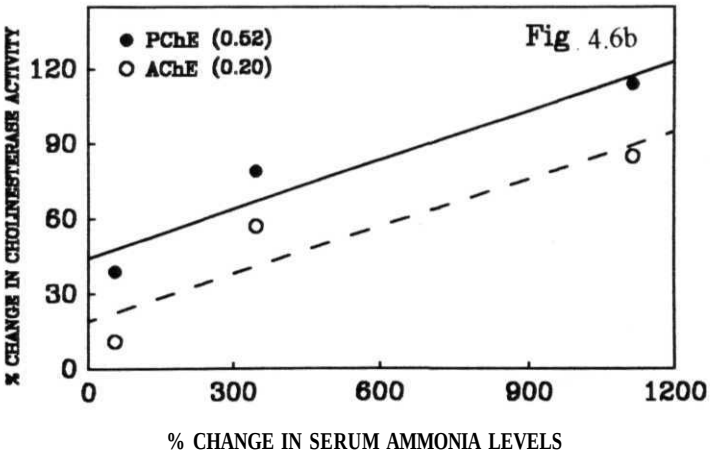


Fig 4.6: Correlation plots (a) % increase in brain ammonia levels Vs % increase in AChE and PChE activities (b) % increase in serum ammonia levels Vs % increase in AChE and PChE activities

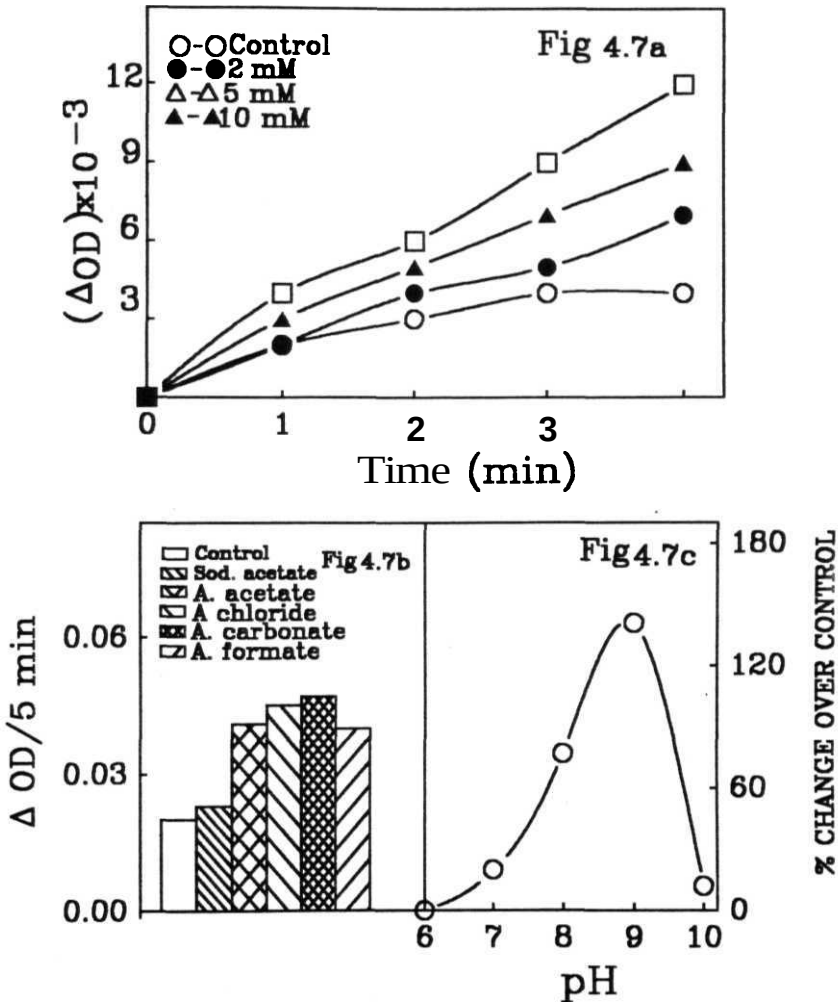


Fig. 4.7: (a) Effect of different concentrations of AA on non enzymic hydrolysis of acetylthiocholine, (b) non-enzymic hydrolysis of acetylthiocholine in presence of ammonium ions (c) effect of pH on non-enzymic hydrolysis All the values are Mean of three experiments done in duplicates

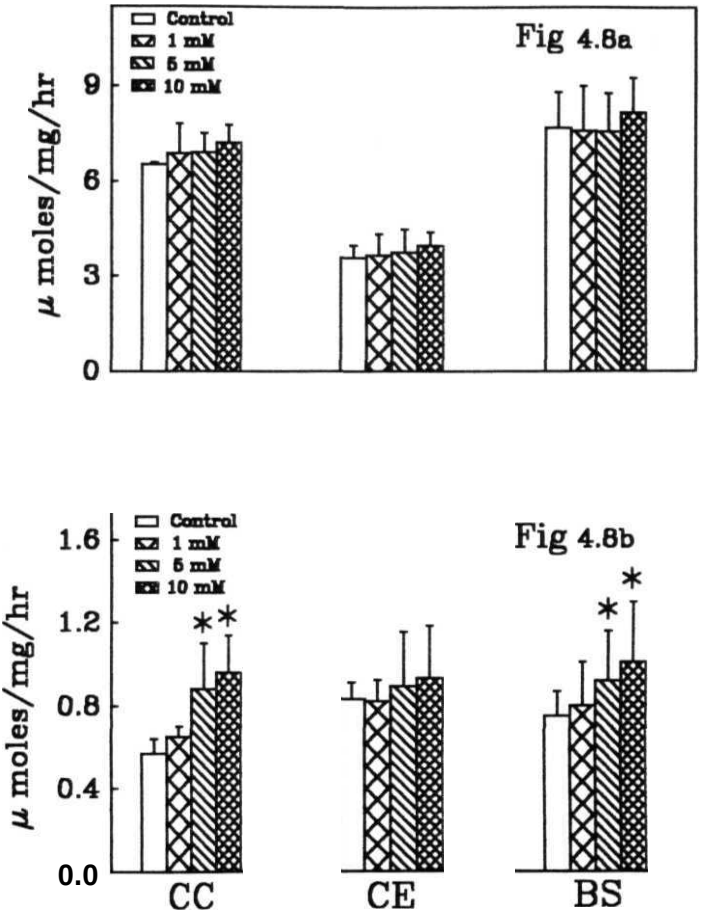


Fig 4.8; *In vitro* effects of AA on synaptosomal (a) AChE and (b) PChE activities in CC, CE and BS Values are Mean ± S D. of three experiments done in duplicates *: Statistically significant compared to controls $p < 0.05$

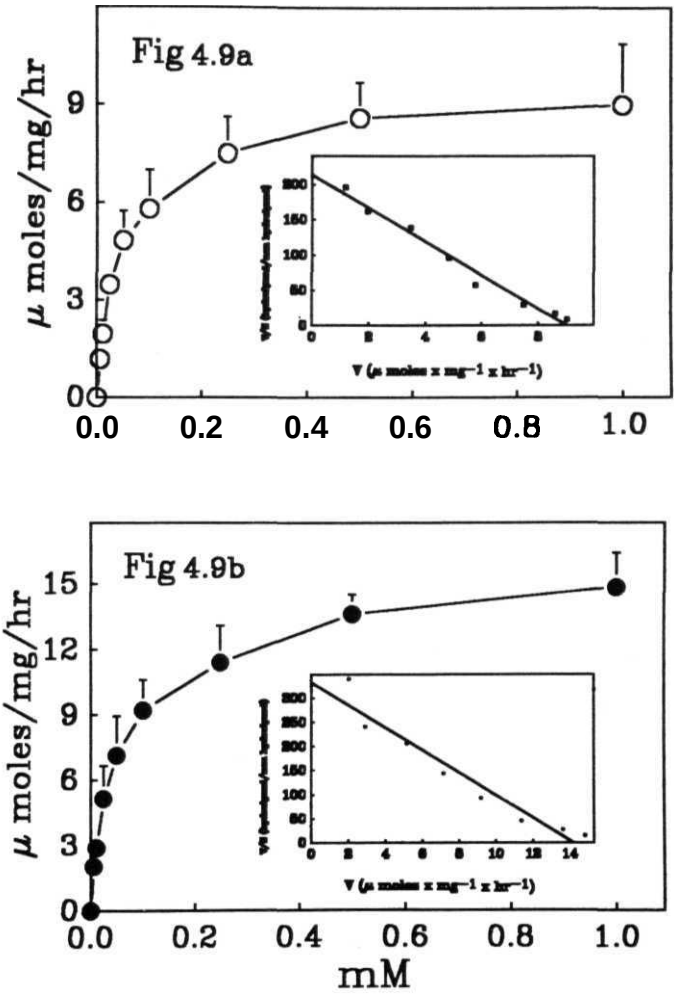


Fig 4.9: Saturation isotherms for AChE activity in synaptosomes isolated from CC of control (a) and rats administered with acute dose of AA (b) Insert: Scatchard plot Values are Mean ± S.D of three experiments done in duplicates

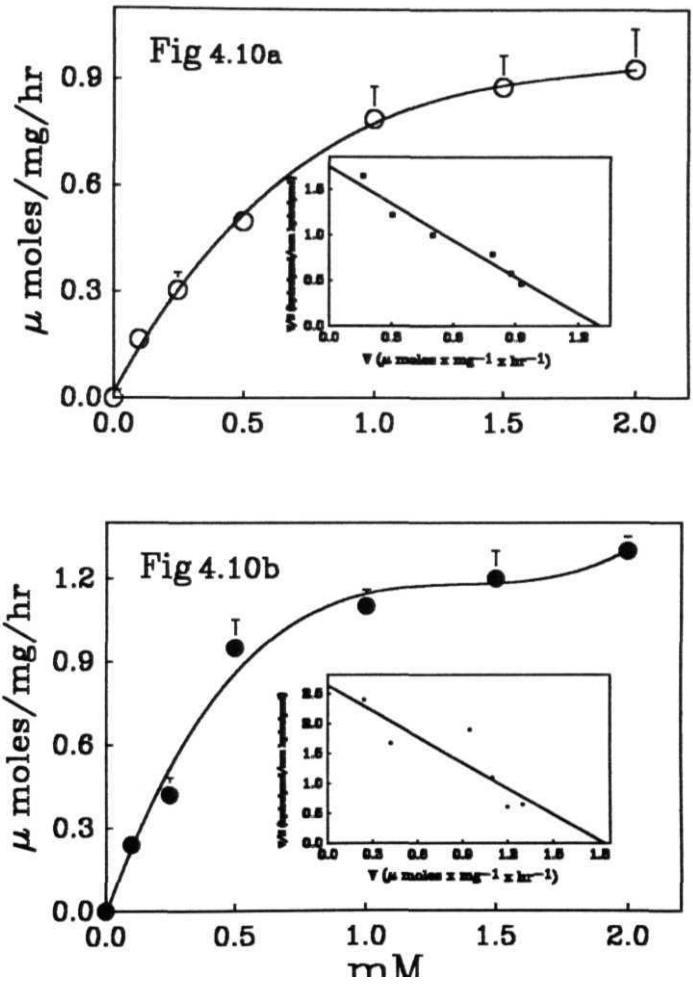


Fig 4 10: Saturation isotherms for PChE activity in synaptosomes isolated from CC of control (a) and acute AA administered rats (b). Insert: Scat chard plot Values are Mean ± S D of three experiments done in duplicates

Table: 4.1 Effect of AA on purified AChE activity

AA (mM)	OD AT 420 nm/5 min
0	0.296 ± 0.005
1	0.302 + 0.009
2	0.326 ± 0.004
5	0.331 ±0.006

All the values are Mean ± S.D. of three experiments done in duplicates

Table: 4.2 Kinetic constants for cholinesterases in synaptosomes isolated from *CC* of control rats and rats administered with acute dose of AA.

	CONTROL		ACUTE	
	K_m	V_{max}	K_m	V_{max}
AChE	34 ±9	8.3 ±0.8	29 ±3	11.9±2.4*
PChE	592 ±126	1.1 ±0.1	654 + 78	1.8 ±0.1*

All the values are Mean ± S.D. of four experiments done in duplicates. K_m values are expressed as μM and V_{max} values as μ moles of acetylthiocholine hydrolyzed/mg/hr. *: Statistically significant compared to controls $p < 0.05$.

Summary and Conclusions:

1. Hyperammonemia was induced by intraperitoneal administration of either AA or MSI. Patency of these models was tested by estimating ammonia levels.

2. Plasma and brain ammonia levels were increased in rats administered with AA/MSI. In rats administered with acute dose of AA, plasma and brain ammonia levels increased with respect to time.

3. Hyperammonemia had no significant effect on the activity of choline-acetyltransferase, an enzyme involved in the synthesis of acetylcholine.

4. V_{max} of both high and low affinity transport systems for choline was decreased without significant alterations in K_m values in rats administered with acute dose of AA. K_m for high affinity choline uptake was significantly increased in rats administered with acute dose of MSI.

5. $^{14}CO_2$ production from [U-^{14}C]glucose was significantly decreased, when incubation was prolonged for 60 min. in synaptosomes of rats administered either with AA or MSI.

6. Hyperammonemia had no effect on acetylcholine synthesis when [U-^{14}C]glucose was used as a precursor for the acetyl moiety of acetylcholine.

7. As high affinity choline uptake is supposed to provide choline for acetylcholine synthesis, the observed changes in present study indicated that acetylcholine synthesis might be affected in hyperammonemic states by interfering with supply of one of the precursor, choline (through the high affinity uptake system) but not through the enzyme involved in its synthesis or the supply of the other precursor, acetyl Co A.

8. There was a significant loss of M_1 muscarinic receptors in CC and BS of rats administered with acute dose of AA. In MSI induced hyperammonemia loss in M_2 muscarinic receptors was observed. Since all the mAChRs function with second messengers, a decreased muscarinic function might also affect the production of second messengers and the processes mediated by these second messenger systems. It has been suggested that the cholinergic neurons in the brain are involved in initiation and execution of motor functions and in the regulation of sleep wake cycles. Dysfunction in cholinergic system in hyperammonemic states might be partially responsible for such behavioral changes reported in hyperammonemic states.

9. Results of the present study indicated an increase in nicotine binding. As, these receptors are excitatory in nature their stimulation might lead to depolarization of the neurons and might keep them in state of activation. This could be one of the reasons for the observed convulsions in hyperammonemic states.

10. Both acetyl and pseudocholinesterase activities were increased in hyperammonemic states in all the three regions of brain and increase in

cholinesterase activity was correlated well with increase in brain ammonia levels.

11. Since most of the changes were observed in membrane associated phenomena, it was suggested that these changes might be due to alterations in membrane architecture.

12. It can be concluded that the observed changes in present study might represent altered behavioral and physiological changes during hyperammonemic states and might contribute to the better understanding of mechanism of pathogenesis in hyperammonemic states.

PART IV

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