ALTERATIONS IN THE NEUROTRANSMITTER FUNCTIONS OF GLUTAMATE AND GABA IN GALACTOSAMINE INDUCED FULMINANT HEPATIC FAILURE AND HYPERAMMONEMIA

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To
My Parents
&
Brother

STATEMENT

I hereby state that the work presented in mis thesis entitled **"Alterations in the neurotransmitter functions of glutamate and GABA in galactosamine induced fulminant hepatic failure and hyper-**ammonemia" has been carried out by me under the supervision of Dr.Ch.R.K.Murthy and that mis has not been submitted for any degree or diploma of any other university earlier.

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ABBREVIATIONS

α-KG	α-Ketoglutarate
AAT	Aspartate Aminotransferase
AcOH	Acetic acid
ADP	Adenosine 5'-Diphosphate
$AgNO_3$	silver nitrate
A1AT	Alanine Aminotransferase
AMPA	a-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ANSA	1-Amino-2-napthol-4-sulphonic acid
ATP	Adenosine 5'-Triphosphate
BBB	Blood Brain Barrier
BS	Brain Stem
BSA	Bovine Serum Albumin
C/P	Cholesterol/phospholipid ratio
cAMP	Cyclic Adenosine Monophosphate
CB	Cerebellum
CC	Cerebral Cortex
CCl_4	Carbon tetrachloride
cGMP	Cyclic Guanosine Monophosphate
Ci	Curie
DPH	1,6-Diphenyl-1,3,5-hexatriene
DTT	Dithiothreitol
EDTA	Ethylene Diamine Terra Acetic acid
EGTA	Ethyleneglycol-bis-(β-amino-ethyl ether)N,N'-Tetra Acetic acid
EtOH	Ethyl alcohol
F-1,6-P2	Fructose-1,6 diphosphate
FHF	Fulminant Hepatic Failure
GABA	Gamma Amino Butyric Acid
GABA-T	Gamma Amino Butyric Acid Transaminase
GALN	Galactosamine-hydrochloride
GGTP	Gamma glutamyl transpeptidase
GS	Glutamine Synthetase
HAUS	ffigh Affinity Uptake System

HCOH	Formaldehyde
HE	Hepatic Encephalopathy
HEPES	N-[2-Hydroxyethyl)piperazine-N-2-[ethanesulphonic acid]
KA	Kainic Acid
kDa	Kilo Dalton
LAUS	Low Affinity Uptake System
MeOH	Methyl alcohol
MK-801	(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclo-hepten- 5,10-iminemaleate
Na_2CO_3	Sodium carbonate
$Na_2S_2O_3$	Sodium thiosulphate
NAD	Nicotinamide Adenosine Dinucleotide
NADH	Nicotinamide Adenosine Dinucleotide Reduced
NADP	Nicotinamide Adenosine Dinucleotide Phosphate
NADPH	Nicotinamide Adenosine Dinucleotide Phosphate Reduced
NTF	Neurotransmitter functions
NH3	Non ionic form of ammonia
$NH4^+$	Ionic form of ammonia
NMDA	N-Methyl-D-Aspartaic acid
PCA	Perchloric acid
POPOP	1,4-Bis[5-phenyl-2-oxazolyl]benzene;2',2'-p-phenylene bis[5-phenyloxazole]
PPO	2,5-Diphenyl oxazole
rpm	Rotations Per Minute
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate - Poly Acrylamide Gel Electrophoresis
SODH	Sorbitol dehydrogenase
TAA	Thioacetamide
TCA	Trichloroacetic acid
TMA-DPH	1-[4-(Trimethylammonio)phenyl]6-phenyl-1,3,5-hexatriene
v/v	Volume/volume
w/v	Weight/volume

CHAPTER 1

INTRODUCTION

Derangement in cerebral functioning is a consistent finding in man in conditions of impaired liver function. Wide spectrum of neurological and neuropsychiatric alterations seen in conditions of hepatic inadequacy are termed as Hepatic **Encephalopathy** (HE). Initial stages of HE can be associated with personality and emotional disturbances. As encephalopathy progresses, hyperventilation, confusion, drowsiness, hyperthermia and coma are exhibited. In later stages of encephalopathy, decerebrate rigidity, decortical posture, convulsions and finally death may occur. If proper medical care is not provided in time, such patients may become comatose and terminally ill (Schenker *et al.,* 1974). Mental retardation was also observed in certain mild cases and in patients who survived (Conn and Lieberthal, 1979; Cooper and Plum, 1987). There are also reports of structural changes in both neurons and glial cells of both patients and laboratory animals with HE (Baraldi, 1990).

In man, HE occurs in two major forms: an acute (fulminant) type and a chronic type. Fulminant hepatic failure (FHF) is a clinical syndrome resulting from severe inflammatory and/or necrotic liver disease with rapid onset. The neurological disorder progresses from altered mental status to coma, generally within hours or days. Death frequently results from increased intracranial pressure caused by massive brain oedema (Rakela, 1983; Blei *et al.,* 1992). Chief reason for acute hepatic necrosis was reported to be viral infections (Hepatitis; Saunders *et al.,* 1972). Infections related to viral hepatitis type B accounts for 74% of cases under this category. Contribution of non-A, non-B type of viral hepatitis accounts for 24% while type A accounts for 2% (Papas-Venegeleu *et al,* 1984). Drug induced hepatic

necrosis is the next major cause of FHF. Most frequently used drug, acetaminophen (paracetamol) was found to be the major culprit and the next is halothane (Barnard *et al.,* 1981). FHF may also result due to the extensive accumulation of fat in the liver as in Reye's syndrome, in pregnancy, toxicity due to drugs such as tetracycline or valproic acid and in patients with surgically constructed jejunoileal bypass for morbid obesity (Burroughs *et al*., 1982; DeLong and Glick, 1982).

Management of FHF patients is less rewarding. Supportive intensive care is very essential. Better survival rates observed at present have been attributed to improvements in the intensive care methods rather man any specific treatment (Hoyumpa and Schenker, 1985).

Therapeutic procedures adopted to treat FHF are several and are based on several principles. These include administration of lactulose, lactulose + neomycin sulphate, infusion of synthetic amino acid mixtures, dialysis and providing artificial liver support and finally liver transplantation (Saunders *et al.,* 1972; Schenker *et al*., 1974; Conn and Lieberthal, 1979; Cooper and Plum, 1987).

Actual mechanism in the encephalopathy in FHF is still enigmatic and uncertain. Reasons for this uncertainty are :

(i) Lack of appropriate animal model which closely resemble the human disease condition.

(ii) Regional and cellular heterogeneity of the brain which renders the studies more difficult, if not impossible. This heterogeneity is seen in the

structure, function and metabolism at cellular and subcellular levels of cerebral organization.

(iii) Multiplicity of factors implicated in the aetiology of cerebral dysfunction in HE has further complicated the issue. These factors include ammonia, false neurotransmitters, mercaptans and short chain fatty acids etc., (Zieve, 1981). Though none of these factors can satisfactorily explain the aetiology of all the symptoms in HE, a brief description is given about these factors.

*AMMONIA**:

Intestine is the major site of ammonia production in the body (Weber and Veach, 1979). Bacterial action on dietary nitrogenous compounds (proteins, amino acids, nucleic acids etc.,) and intestinal metabolism of glutamine are responsible for the production of ammonia in the intestine. Ammonia, so formed, is transported to liver via portal blood and is converted to urea in periportal hepatocytes and to glutamine in perivenous hepatocytes (Haussinger *et ai,* 1984). In conditions of hepatic insufficiency, ammonia is not detoxified in the liver and enters the systemic circulation. Elevation in blood and brain ammonia level is known to be neurotoxic (Schenker *et al.*, 1974; Conn and Lieberthal, 1979; Cooper and Plum, 1987; Rukmini and Murthy, 1993).

Ammonia exists in two forms - unprotonated NH_3 form and protonated NH_4 (ionic) form. Interconversion of these two forms is extremely rapid and depends on pH of the medium. At physiological pH, about 97% of ammonia is in ionic form which is impermeable across the biological membranes. Unprotonated NH_3 form is lipid soluble and is highly permeable. However, in the present study, unless otherwise mentioned specifically, the term ammonia represents both the forms.

Once again, several mechanisms have been proposed to explain cerebral dysfunction in hyperammonemic states and none of the proposed mechanisms are equivocal (Cooper and Plum, 1987). Some investigators believe that excess ammonia levels in the brain exerts an adverse influence on cerebral energy metabolism (Bessman and Bessman, 1955), either by depleting α-ketoglutarate (a-KG) from citric acid cycle or by disrupting the transport of reducing equivalents from cytoplasm to mitochondria through malate-aspartate shuttle (Siesjo, 1978). It has also been proposed that ammonia disrupts Blood-Brain Barrier (BBB) and affects the transport of several substances including ions resulting in cerebral oedema (Ware *et al*., 1971). Another group of investigators believe that ammonia might be exerting its toxic effects by affecting the synthesis, release, reuptake and post-synaptic action of neurotransmitters (Schaffer and Jones, 1982; Rao and Murthy, 1991; Rao *et al*., 1992). Though, there are some studies in this direction no reports are available in the condition of FHF.

Plasma Amino Acids And False Neurotransmitters :

In some patients with HE and in animal models, a decrease in the level of branched-chain amino acids (BCAA; leucine, isoleucine and valine) and an increase in the levels of aromatic amino acids (AAA; phenylalanine, tyrosine and tryptophan) were observed (James *et al.,* 1979). As these amino acids are transported across the BBB by a single carrier, it was postulated mat mere would be an increased influx of AAA into brain in the absence of functional liver. As a consequence, cerebral AAA content would exceed the Km of the AAA hydroxylases (rate limiting reactions in the conversion of AAA into biogenic monoamines) and are directly decar-

boxylated to aromatic amines, such as tyramine, tryptamine, β-pheylethylamine, octopamine etc., which are called False Neurotransmitters (FNT).

These amines displace the real neurotransmitter amines (dopamine, epinephrine, norepinephrine and serotonin) from nerve terminals and alter the neurotransmitter balance in brain and thus cerebral function. A decrease in the dopamine and norepinephrine levels and an accumulation of false neurotransmitters have been found in brain during GALN induced FHF (Ferenci *et al.,* 1984). As FNT might competitively inhibit the binding of dopamine to its receptors, it has been suggested that abnormal dopaminergic neurotransmission might be implicated in the mediation of HE (Fischer and Baldessarini, 1971). Similarly, cerebral levels of serotonin were reported to be increased and the number of serotonin receptors on neurons were observed to be decreased in HE and this might contribute to the neural inhibition in HE (Cummings *et at.,* 1976; Riederer *et al.,* 1982). It was observed that in some human subjects with hepatic inadequacy and in experimental animals that perfusion of BCAA or their keto analogues resulted in an improvement of clinical picture (Beaubernard *et al.,* 1984).

However, in some cases of HE, no changes were observed in the levels of dopamine, noradrenaline and other minor amines, inspite of a neurological impairment (Fischer and Baldessarini, 1971; Cuilleret *et al.*, 1980). Moreover, this hypothesis fails to explain some of the changes observed in the brain in hepatic insufficiency such as Alzheimer astrocytosis II.

Role of other etiological factors, such as mercaptans (Phear *et al.,* 1956; Chen *et al.*, 1970; Zeive, 1980; Zeive and Brunner, 1985), short

chain fatty acids (Samson *et al,* 1956; Zeive, 1983; 1985) etc., have not been worked out in detail. Increased blood levels of short chain fatty acids have been reported in the patients with hepatic insufficiency (Zieve, 1981). The reason for this increase is not clear and many factors seem to be involved. Short chain fatty acids uncouple oxidative phosphorylation and were shown to decrease the ATP and creatine phosphate content in the brainstem regions which is the seat for reticular activating system (McClain *et al,* 1980; Pappas-Venugeleu, 1984; Cooper and Plum, 1987). Injection of short chain fatty acids is known to induce coma (Zieve *et al,* 1974).

It is believed that other neurotransmitters are involved in the pathophysiology of HE. Altered metabolism of GABA, the major inhibitory neurotransmitter, has been implicated in the pathogenesis of HE. GABA hypothesis was proposed by Schaffer and Jones (1982) to explain HE. It is believed that GABA is produced not only in CNS but also in the intestine due to bacterial action on dietary glutamic acid. During liver dysfunction, GABA produced in the intestine is not detoxified in liver and enters into systemic circulation. Moreover, under these conditions, due to a selective disruption of the BBB, GABA enters the brain, binds to cerebral GABA receptors thus inhibiting the neuronal activity. In support of this hypothesis, these investigators observed an increase in the serum concentrations of GABA like compound in patients with HE (Maddison *et al.,* 1987; Mullen *et al.,* 1988). Schaffer and Jones (1982) reported an increase in the number of GABA receptors in rabbits with HE due to galactosamine (GALN) induced FHF. Baraldi and Zeneroli (1982) reported an increase in the number of GABA binding sites in a rat model during GALN induced FHF. An

increase in the number of cerebellar GABA binding sites in hyperammonemic states with a functional liver was reported by Rao *et al*., (1991).

It has been suggested that ammonia and other toxins accumulated in the absence of a functional liver might alter the functions of biological membranes (Hoyumpa and Schenker, 1985). Neuronal membranes are known to be the seats of the neurotransmitter receptors, various types of ionic channels (ion gated, voltage gated) and carrier proteins for the transport of amino acids (Amara and Kuhar, 1993). However, there are no conclusive evidences or systematic studies in this area.

This may be vital as the neurotransmitter receptors mediate communication between the neurons, while the ionic channels maintain the proper ionic environment in and around the neuron for its efficient functioning. In fact, opening of ionic channels is mediated by binding of neurotransmitters to the receptors. Alteration in neurotransmitter receptors (affinities and densities) would lead to the disruption of neuronal communication and cerebral functions.

It is believed that derangement of cerebral function is usually due to alterations in cerebral energy metabolism or in the electrical activity of brain. These two parameters are interrelated and influence each other. Earlier studies have shown that liver dysfunction results in (i) an elevation in brain ammonia levels (Hoyumpa and Schenker, 1985) (ii) ammonia disposal in brain is by way of glutamate and glutamine fonnation and mis requires energy and also drains α-ketoglutarate from citric acid cycle (Weil-

Malherbe, 1950) (iii) these reactions deplete cerebral energy stores either directly or indirectly thus disrupting cerebral function (Bessman and Bessman, 1955).

However, studies on cerebral energy metabolism gave contradictory results (Hawkins *et al.*, 1973 Schenker *et al,* 1974; Bates *et al.,* 1989). Ratnakumari *et al.,* (1986) showed that failure of cerebral energy metabolism may not be the chief reason for cerebral dysfunction at least in acute hyperammonemic conditions.

Above information, in summary, indicates that (i) hepatic insufficiency is of common occurrence in man and has variable clinical consequences (ii) the precise aetiology of the cerebral dysfunction under these conditions is not known (iii) various toxins accumulate under these conditions, of which ammonia is still supposed to be the chief culprit and (iv) survival in this condition depends on the proper management of patients and intensive care techniques rather than on a rational therapeutic mechanism.

In the present study, efforts were made to develop an animal model for FHF using galactosamine (GALN) as a hepatotoxin. Since the initial report of Keppler and Decker (1969) on the hepatotoxicity of GALN, considerable amount of work has been done to using this compound.

Mechanism of Galactosamine Toxicity:

Galactosamine, an aminosugar, is a naturally occurring component of heteroglycans in cells. However, very little amount of free GALN is detectable either in the liver or in other tissues. In liver, administered GALN is first phosphorylated to GALN-1-phosphate by galactokinase This is con-

verted to UDP-GALN by UDPglucose:galactose-1-phosphate uridyltransferase. UDP-GALN is further converted to UDP-N-acetylGALN or epimerized to UDP-glucosamine.

UDP-hexosamines, so formed during GALN administration, do not act as uridylate donors and prevent the replenishment of UTP levels in liver. When UTP content is decreased to a critical level (20% the normal values), degenerative reactions are initiated in the hepatocytes. Moreover, UDP-GALN and UDP-glucosamine are also incorporated into glycogen resulting in the formation of aminoglycogen (Mandl *et al.,* 1979). It has been shown that aminoglycogen precipitates ribosomal subunits and acts as an effective inhibitor of protein synthesis (Mandl *et al.,* 1979). However, despite the knowledge of the biochemical events, leading to GALN toxicity, it is not exactly known as to how the changes in the uridine nucleotide levels and of the inhibition of protein synthesis brings about hepatocellular degeneration.

In the later part of this work, another animal model for hyperammonemia was used. In this model, ammonium acetate was administered to animals with a functionally normal and active liver. This model was used to elucidate the role of ammonia in the changes observed in neurotransmitter functions. In some experiments, animals (with functionally active liver) starved for 36 hr were also used. This is to delineate the effects of starvation from those of FHF (as animals with FHF stop feeding).

Neurotransmitter Functions of Glutamate and GABA :

Using these animal models, studies were carried out on the neurotransmitter functions of glutamate and GABA. These two ammo acids have

both metabolic and functional roles in brain (Mayer and Westbrook, 1987). They occupy a unique position in brain biochemistry as they constitute more than 50% of the total amino nitrogen present in brain (McIlwain and Bachelard, 1971). Moreover, their metabolism is closely associated with that of glucose and they (especially glutamate) are supposed to serve as alternate fuels in brain (Erecinska and Silver, 1990). Besides these, glutamate and GABA also play a pivotal role in neurotransmission - glutamate as an excitatory neurotransmitter (Fonnum, 1984) and GABA as an inhibitory neurotransmitter (Roberts, 1986). As neurotransmitters, they have been implicated in long term potentiation and long term depression, memory and learning (Kennedy, 1989; Ito, 1989). Another interesting aspect of glutamate is its neurotoxicity. Prolonged exposure to glutamate (or its analogues) leads to neuronal degeneration and death and this has been implicated in the aetiology of several neurodegenerative disorders (Choi, 1988; Wroblewski and Danysz, 1989, Choi and Rothman, 1990; Blackstone *et al.*, 1992). A brief survey of literature pertaining to major metabolic and functional aspects of these two amino acids is given below.

Metabolic Aspects of Glutamate and GABA :

Studies carried out on glutamate metabolism revealed that it is closely related to the metabolism of glucose. Carbons from glucose rapidly equilibrate with the carbons of glutamate and GABA (Balazs *et al.*, 1972). Further studies revealed that in brain, glutamate is present in two pools - a large pool and a small pool. Large pool of glutamate contains most of the (80-92%) tissue glutamate and has a slow turnover rate while small pool of glutamate contains less glutamate (8-20%) and has a rapid turnover rate.

These two pools do not mix with each other. Glutamate in the large pool is metabolized by oxidative deamination while in the small pool it is involved in the synthesis of glutamine and in transamination. Rate of synthesis of glutamine was found to be higher in the small pool than in the large pool. Precursor for the larger pool of glutamate has been shown to be glucose while in small pool glucose, β-hydroxybutyrate, acetate, CO_2, butyrate, propionate, citrate, leucine etc., would serve as precursors (Balazs *et al.*, 1972; Clarke *et al*, 1974; Mohler *et al*., 1974; Van den Berg *et al*, 1975).

Further studies revealed that larger pool of glutamate is localized in neurons and the small pool in glia (Garfinkel, 1966, 1972; Benjamin and Quastel, 1972; Clarke *et al*.,1974; Van den Berg *et al*, 1974). Later studies revealed the existence of subcompartments in these two pools of glutamate. For e.g., the large compartment was shown to have a subcompartment from which glutamate is released on depolarization (releasable pool of glutamate; Benjamin and Quastel, 1972). This is most probably localized in the synaptic vesicles of glutamatergic neurons. Similarly, a subcompartment of the large compartment was believed to be involved in the synthesis and release of GABA (most probably) in the GABAergic neurons (Fyske and Fonnum, 1988). It is suggested that glutamate metabolism might be different not only in neurons and glia but even among different types of neurons.

Glutamate or GABA is released (from a subcompartment of the large compartment), especially in response to depolarizing stimuli. Glutamate/ GABA that is released (referred as extracellular glutamate/GABA) is transported to the astrocytes. In these cells, major portion of glutamate is converted to glutamine and the rest is transaminated to a-KG and oxidized in

the citric acid cycle (Benjamin and Quastel, 1974). Glutamine, so formed, in the glia, would be transported to nerve endings where it serves as a precursor for the releasable pool of glutamate and GABA (Ward and Bradford, 1979). This process of recycling of glutamate (thus of α-KG/glucose) carbons between glia and neurons would prevent the perpetual loss of glutamate carbons from neurons (Benjamin and Quastel, 1974). This concept received support from histochemical and biochemical studies on the localization of the enzymes - glutamine synthetase and glutaminase in astrocytes and nerve terminals, respectively (Martinez-Hernendez *et al.*, 1977; Norenberg and Martinez-Hernandez, 1979; Weiler et al., 1979).

GABA, like glutamate, has two major functions in brain tissue - it serves as a neurotransmitter and also as an intermediate in energy metabolism. In synaptic terminals, it functions as a neurotransmitter whereas in the non-synaptic regions (perikarya and dendrites) GABA functions as a metabolic intermediate (Martin and Rim vall, 1993). Studies carried out on GABA metabolism revealed that it is synthesized in brain at least in two compartments, commonly called as the transmitter and metabolic compartments (Garfinkel, 1966; Baxter, 1970; Patel *et al.*, 1970; Balazs *et al.*, 1972; Van den Berg, 1972; Iadarole and Gale, 1981).

In brain, GABA is synthesized principally from glutamate in a reaction mediated by glutamate decarboxylase, which uses pyridoxal-5-phosphate as a cofactor. In the subsequent step, GABA transaminates with a-KG to form succinic semialdehyde. This is oxidized to succinate by a mitochondrial NAD^+ dependent succinic semialdehyde dehydrogenase, thus providing a

pathway for the entry of GABA into the TCA cycle. This alternate route is called the *GABA shunt* In brain, about 10-20% of the α-KG is diverted from TCA cycle through GABA shunt (Baxter, 1970; 1976). The transmitter compartment contains ~30% of tissue GABA and the metabolic compartment contains the rest (~70%) of GABA (Patel *et ai,* 1970; Balazs *et ai,* 1972). Although presence of metabolic pool of GABA is well established, its physiological significance is unclear.

Glutamate and GABA as Neurotransmitters

Release :

In isolated neural preparations, release of glutamate is evoked by electric pulses (deBellaroche and Bradford, 1972; Potashner, 1978), by depolarizing chemical stimuli such as high external K^+ levels (30-56 mM; Naddler *et ai,* 1978), by ergot alkaloids (like veratridine; Toggenburger *et ai,* 1982), by A23187 (calcium ionophore) along with Ca^{2+} (Levi *et al.*, 1976), tityus toxin, p-bungarotoxin (Smith *et al.*, 1980), verrucologen (Norris *et ai,* 1980). It is believed that glutamate is released from the nerve terminals by two processes (Haycock *et ai,* 1978; Sandoval *et ai,* 1978). The first process is the Ca^{2+} dependent exocytosis of amino acids from the synaptic vesicles (Douglas, 1974; Strom-Matheison *et ai,* 1983; Nicholls *et al,.* 1987). Second one is the Ca^{2+} independent release. This is believed to be mediated by the high affinity uptake system acting in reverse direction - transporting cytosolic pool of glutamate to cell exterior (Erecinska, 1987; Nicholls and Attwell, 1990). Ca^{2+} dependent exocytosis of glutamate is ATP dependent (Nicholls et al., 1987) whereas the Ca^{2+} independent release occurs when energy levels (ATP/ADP) are decreased and ion gradi-

ents across plasma membranes are reduced (Sanchez-Prieto and Gonzalez, 1988). During normal neuronal activity, Ca^{2+} dependent system is likely to dominate while Ca^{2+} independent release occurs during pathological conditions associated with low intracellular ATP levels.

GABA is released from nerve terminals by a K^+ stimulated, Ca^{2+} dependent mechanism (deBellaroche and Bradford, 1972; Osborne *et al.*, 1973; Redburn and Cotman, 1974) which is blocked by Tetanus toxin (Osborne *et al,* 1973). Sustained release of GABA from cortical slices or synaptosomes is elicited by raising the extracellular K^+ concentration in the absence of Ca^{2+} (Redburn *et al.*, 1976; Vargas *et al,* 1977; Haycock *et al.*, 1978). GABA released under these conditions is derived from recently accumulated GABA pools, whereas the Ca^{2+} dependent K^+ stimulus facilitate the release from both pools (Haycock *et al,* 1978).

Receptor Binding :

Glutamate and GABA, once released from the nerve terminals into the synaptic cleft, bind to their receptors and exert their action. These receptors are membrane proteins present either on the post-synaptic or on the pre-synaptic neuron. Recently, receptors have also been reported to be present on astrocytes (Pearce, 1993). When neurotransmitter binds to the receptors on the post-synaptic neuron, then information transfer is accomplished. However, binding of neurotransmitter to a receptor on the pre-synaptic receptor (autoreceptor), it regulates the release of neurotransmitter itself. Binding of neurotransmitter to the receptor may open up ion channels associated with the receptor (ionotropic action) or may result in the synthe-

sis of a second messenger (metabotropic action). As the binding of glutamate results in the depolarization of the neuronal membranes and the genesis of an action potential, this amino acid is called as excitatory neurotransmitter (Fonnum, 1984). In contrast to mis, GAB A binding results in hyperpolarization of neuronal membranes and suppresses the neuronal activity. Hence, it is considered as an inhibitory neurotransmitter (Roberts, 1986; McGeer and McGeer, 1989). A brief description of glutamate and GAB A receptors is given below.

Glutamate Receptor :

Based on pharmacological specificity, several subtypes of glutamate receptors have been described. This classification is based on their preference for agonists and antagonists of glutamate. These include N-methyl-D-aspartate (NMDA), kainate (KA) and quisqualate (QA) receptors (Watkins and Evans, 1981; Honore *et ai,* 1989). More recently the QA receptor has been renamed as AMPA receptor, as quisqualate is non-selective when compared to the selectivity of AMPA (a-amino-3-hydroxy-5-methylisoxazole-4-propionic acid) as an antagonist for this receptor subtype (Watkins *et ai,* 1990). In addition, evidence for two more glutamate receptor subtypes has been obtained. One of the two new receptor subtypes (termed the L-AP4 receptor) has been defined on the basis of its sensitivity to low concentrations of glutamate analogue, L-2-amino-4-phosphonobutanoate (L-AP4) (Monaghan *et ai,* 1989). The second (referred as Glu_G receptor) is linked to an intracellular second messenger systems via a GTP binding protein (G protein) in the post-synaptic neuron (Schoepp *et al.*, 1990). Molecular biological studies, conducted in the past few years, have

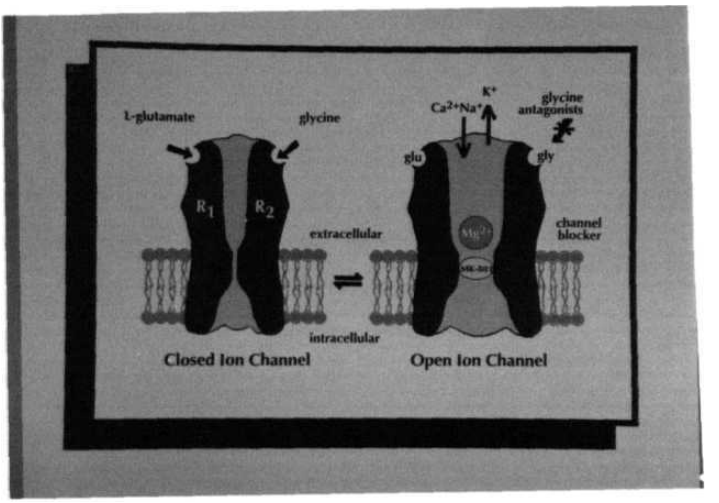
confirmed the existence of all five glutamate receptor subtypes in brain and lead to the identification of multiple isoforms of each receptor subtype with distinct functional properties (Foster and Fagg, 1993).

NMDA receptor is the best characterized acidic amino acid receptor subtypes. This receptor is associated with an ionic channel (hence an ionotropic receptor). This channel is opened when glutamate and other related agonists bind to the receptor. When opened, the channel is permeable to both Na^+ and Ca^{2+} (in addition to K^+; MacDermott *et al,* 1986).

NMDA receptor complex consists of four different domains: (a) an NMDA recognition domain with a unique high affinity for glutamate, (b) a glycine recognition domain, (c) a polyamine recognition domain, and (d) an ion channel domain permeable to Ca^{2+} and Na^+. Ion channel domain has four independent sites which are sensitive to inhibition by Mg^{2+}, Zn^{2+}, H^+ and allosteric blockers such as MK-801 and TCP (Nowak *et al,* 1984).

Ca^{2+} permeability of the NMDA receptor has been implicated in the physiological and pathological roles of NMDA receptor. Activation of NMDA receptor is believed to play a role in the synaptic plasticity associated with learning and memory (Iversen, 1994) and in information processing (Daw *et al,* 1993). Entry of Ca^{2+} through NMDA receptor channel has also been shown to play a crucial role in long term potentiation in many neural pathways in the brain (Collingridge and Bliss, 1995), epileptiform activity (Dingledine and McBain, 1994) and excitotoxic neuronal degeneration (Choi, 1988; Meldrum, 1990) and cell death (Ghosh and Greenberg,

Fig. 1: NMDA receptor ion channel complex.


L-glutamate
glycine
R_1
R_2
extracellular
intracellular
Closed Ion Channel
K^+
$Ca^{2+}Na^+$
glycine antagonists
glu
gly
Mg^{2+}
channel blocker
Open Ion Channel

1995). A recent investigation indicated that activation of a protein tyrosine kinase followed by phosphorylation of a putative microtubule-associated protein (MAP2) kinase might be one of the early consequences of Ca^{2+} entry via the NMDA receptor channel (Bading and Greenberg, 1991). Increased formation of arachidonic acid and synthesis of nitric oxide have also been reported to occur due to NMDA receptor stimulation (Dumuis *et al.*, 1988).

Several antagonists, each acting by a different mechanism, have been formulated and identified for NMDA receptors. Competitive antagonists compete for binding at the glutamate recognition sites (e.g. AP-5, CPP); MK-801 and related non-competitive antagonists (e.g. ketamine, phencyclidine) act by occupying a site within the ion channel (distinct from the Mg^{2+} sites). They act most effectively when the receptor is activated (i.e., when the associated channel is open). Binding of non-competitive antagonists such as MK-801 is a good biochemical index of the activity of the open NMDA channel in brain synaptic membranes. It was shown that phosphorylation of one or more amino acid residues on the NMDA receptor might be necessary for sustained activation of the receptor (Dingledine and McBain, 1994).

NMDA receptor was shown to have five subunits. NMDAR1, a subunit of NMDA receptor, was the first characterized subunit from rat brain has a molecular mass of 103 kDa cDNAs for these subunits have been cloned and characterized for both mouse and rat brain (Nakanishi *et al.*, 1990; 1992; Moriyoshi *et al.*, 1991). The open reading frame of the cDNA sequence for NMDAR1 predicts a total of 938 amino acids with a

series of 5 hydrophobic sequences which are thought to represent 4 transmembrane domains. A second gene family of NMDA receptor subunits with four members sharing only 18-20% amino acid sequence homology to NMDAR1 was isolated from rat. Four subunits of this family share 38 - 53% amino acid sequence identity and were termed NR2A - NR2D (Monyer *et al.*, 1992; Ishii *et al.*, 1993).

Kainate receptors are a subtype of glutamate receptors associated with ionic channels which are permeable to Na^+ and K^+ (Mayer and Westbrook, 1987; Collingridge and Lester, 1989). Overstimulation of neurons through kainate receptors induces neurodegenerative response in discrete populations of neurons (Monaghan *et al,* 1989). Kainate can also activate the AMPA receptors (Honore *et al,* 1988). Screening cDNA libraries for clones resulted in the identification of five proteins, GluR5, 6 and 7, and KAl and 2, which showed properties expected of kainate receptors (Bettler *etal,* 1991; 1992; Egebjerg et al., 1991; Werner *et al.,* 1991).

AMPA receptor associated ionic channels are permeable to Na^+ and K^+ but not to Ca^{2+} This receptor is activated by quisqualate and this can be partially be blocked by the lectin, concanavalin A (Fagg and Foster, 1993). In the absence of other excitatory activity, AMPA receptors may mediate fast depolarizing responses at most excitatory synapses in the central nervous system. Four subunits for AMPA receptors have been cloned. They were identified as GluR1, 2, 3 and 4. cDNA sequencing indicates that GluR1 - GluR4 are 862-889 amino acids in length and are about approximately 100 kDa (Keinanen *et al,* 1990; Boulter *et al,* 1990; Hollmann *et al.*, 1991).

Recent work using whole cell recording techniques indicated that L-AP4 receptor may act as a G protein coupled receptor which directly inhibits high threshold Ca^{2+} currents and thereby suppresses transmitter release (Trombley and Westbrook, 1992).

Glu_G receptors are the only acidic amino acid receptors that have been shown to be directly linked to intracellular second messenger systems. This receptor activates phospholipase *C* to cause the hydrolysis of phosphoinositides to inositol 1,4,5-triphosphate (IP3), which in turn releases Ca^{2+} from intracellular stores, increases arachidonic acid and nitric oxide production. Glu_G receptors have been proposed to participate in excitotoxic neurodegeneration (Choi, 1988; Fagg and Foster, 1993). The first member of the G protein linked receptors for glutamate (mGluR1α) was isolated by expression cloning from rat CB (Masu *et al.,* 1991). This protein contains 1199 amino acids (about 133 kDa) and seven putative membrane spanning domains. Recently, four additional mGluR clones have been identified. These proteins (mGluR2-5) are 872-1171 amino acids in length and share sequence homology with mGluR1 (Abe *et al.,* 1992; Aramori and Nakanishi, 1992; Tanabe *et al,* 1992; 1993).

Investigations using a variety of animal species indicate that excitatory amino acid receptor antagonists may be of primary value for the treatment of epilepsy and ischemic neurodegeneration (e.g., stroke). Therapeutic role of glutamate antagonists in Parkinson's disease, Schizophrenia and chronic neurodegenerative disorders is also being persued (Lodge and Collinridge, 1990; Meldrum, 1991).

GABA Receptors :

GABA is the major inhibitory neurotransmitter in mammalian central nervous system. Altered GABAergic function is involved in neurological and psychiatric disorders such as Huntington's chorea, epilepsy, tardivne dyskinesia, alcoholism, schizophrenia, sleep disorders and Parkinson's disease. Pharmacological manipulation of GABAergic transmission is being used for the treatment of anxiety (Enna and Mohler, 1987). GABA receptors are currently classified into three groups. $GABA_A$ receptors are associated with a Cl^- ion channel (ionotropic receptors) and are antagonized by bicuculline. They are also modulated by a variety of ligands such as benzodiazipines and barbiturates. GABA and benzodiazipine binding sites were found to copurify and benzodiazipine binding site cross react with the antibodies of GABA binding site, indicating that this is an integral part of the GABA receptor-Cl^- channel complex (Siegel *et al.*, 1983). GABA receptors are activated by muscimol and THIP (4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridine-3-ol; Enna, 1988).

$GABA_A$ receptor was reported to be made up of 4 subunits -α, 3, y, and 5. Isoforms of receptor arise by the assembly of subunits in different combinations, for e.g. isoforms α1-α6, β1-β4, γ1-γ3, and 5 have been identified (Burt and Kamatchi, 1991; Wisden and Seeburg, 1992). Each of the polypeptides is encoded by a separate gene which arose by duplication of a common ancestor (Lasham *et ed.*, 1991).

$GABA_B$ receptors, for which baclofen is a specific agonist, are coupled to G proteins and acts through the stimualtion of phospholipase activity (Feltz *et ed.*, 1987) and gate K^+ or Ca^{2+} or bom the channels (Bowery *et*

a!., 1988). $GABA_C$ receptors also gate Cl^- channels and are blocked by picrotoxin but are insensitive to bicuculline and baclofen; they are activated by agonists TACA and CACA *(trans-* and *cis-* 4-aminocrotonic acid, respectively). A fourth type (baclofen resistant, G protein coupled) of GAB A receptor might also exist (Djamgoz, 1995).

Termination of Neurotransmitter Activity by Uptake:

Neurotransmitter activity of both glutamate and GABA is terminated by uptake mechanisms. These uptake mechanisms are very efficient and maintain very low extracellular levels (<1 μM) of amino acids under physiological conditions (Nicholls and Attwell, 1990). Investigations on the transport of these amino acids revealed the existence of two types of uptake mechanisms, viz., one with a high affinity and low capacity and other with a low affinity and high capacity.

The high affinity uptake system has an absolute requirement for sodium (Bennet *et al.,* 1973). This system is saturated at very low concentrations of the amino acid and is supposed to be involved in the removal of neurotransmitter glutamate from the synaptic cleft. Low affinity uptake system is not Na^+ dependent and is saturated at relatively high levels of extracellular amino acids (Km 1-2 mM). Presence of these uptake mechanisms in the brain is well documented in the literature (Logan and Snyder, 1972; Campbell and Shank, 1978; deBary *et al.,* 1982; Levi et al., 1982; Gordon and Balazs, 1983; Hansson, 1986).

Mechanism of glutamate transport by high affinity uptake system is yet to be elucidated. It is not clear whether this process is same in nerve end-

ings and glia. Energy required for this process is provided by a simultaneous downhill movement of sodium ions. Stoichiometry of mis process is two or more sodium ions and one H^+ for each molecule of glutamate transported (Stallcup *et al,* 1979; Drejer *et al.,* 1982; Kanner and Marva, 1982; Kimelberg *et al.,* 1989). The proton is supposed to neutralize the charge of the amino acid (Erecinska *et al.,* 1983). Transport of glutamate through this carrier seems to be bidirectional and the direction of transport varies with the physiological state. When the magnitude of the driving force (which is a combination of the membrane electrical potential and the Na^+ concentration gradient) falls below equilibrium value, outward flux will be greater than inward flux and the carrier will mediate net glutamate release from the cell.

Presence of low affinity transport system for glutamate has been demonstrated in synaptosomes (Campbell and Shank, 1978; Weiler *et al.,* 1979). This system transports glutamate with a low affinity but with a high capacity. It may be responsible for the transport of glutamate into GABA-ergic terminals which lack sodium dependent high affinity uptake system (Erecinska and Silver, 1990). Not much information is available on the properties and distribution of these carrier proteins (transporters). The glutamate transporter has been solubilized and partially purified (Gordon and Kanner, 1988; Amara and Kuhar, 1993). In general, studies have shown that these carriers are glycoproteins with apparent molecular weights ranging from 60-85 kDa.

GABA is also transported by two transport systems, the high affinity system and a low affinity systems. Mechanism for the uptake of GABA were shown to be present in the cerebellar neurons, glial cells (Hokfelt and

Ljungdahl, 1970), synaptosomes (Weinberger and Cohen, 1983) and oligodendrocytes (Reynolds *et al.,* 1987). GABA is taken up principally into inhibitory neurons viz., Purkinjee, Golgi, stellate and basket cells (East *et al.,* 1980). Uptake of GABA by granule cell population is approximately 6% of that of glutamate. Accumulation of GABA by glial cells was about 20 times of that of granule cells (Campbell and Shank, 1978). High affinity uptake of GABA was more intense in the GABAergic cortical neurons than in cerebellar granule cells and in astrocytes (Yu and Hertz, 1982). Transport of GABA into synaptic vesicles is by a Mg-ATP dependent mechanism which are different from that of the plasma membrane (Fyske and Fonnum, 1988). GABA transporter has been solubilized and reconstituted in proteoliposomes (Radian and Kanner, 1986; Kanner *et al.,* 1989). Studies have shown that these transporters have an apparent molecular weight ranging from 60-85 kDa (Amara and Kuhar, 1993).

A brief survey of literature indicated alterations in the neurotransmitter (glutamate and GABA) functions during hepatic encephalopathy. Decreased release of GABA was shown in portacaval shunted rats (Mans *et al.,* 1979; 1990). Diaz-Munoz and Tapia (1988) reported decreased release of GABA in striatum and cerebellum and a significant increase in the hippocampus during CCl_4 induced hepatic coma. Rao and Murthy (1991) reported increased in the evoked release of glutamate from cerebellar synaptosomes of hyperammonemic rats. Bosnian *et al.,* (1992) reported a 4-5 fold increase in glutamate release in conditions of complete liver devascularization

Baraldi and Zenroli, (1982) reported that membrane preparations from rats with mild GALN induced hepatic encephalopathy had an increase in

the number of low and high affinity GAB A binding sites, whereas only high affinity binding sites were present in those with severe encephalopathy. Increased number of GABA receptors in the brain during GALN induced FHF was also reported by Schafer and Jones (1982). Ferenci *et al,* (1984) reported that the development of hepatic encephalopathy in rabbits correlates with a decrease in the density of receptors for the excitatory amino acids. Binding of GABA to its receptors was reported to be unchanged in brains of portacaval shunted rats (Zanchin *et al,* 1984) while loss of low affinity sites for the GABA binding in brains of GALN induced FHF was reported by Zeneroli *et al,* (1984). Butterworth *et al.*, (1986, 1987) reported increased binding of glutamate in cerebral cortex of portocaval shunted rats. However, Maddison *et al.,* (1987) reported that GABA binding and function in brain, were unaltered in rats with thioacetamide induced hepatic encephalopathy. Olasmaa *et al.,* (1990) reported a 10 fold increase in the cerebral concentrations of endogenous ligands for benzodiazipine receptors in rats suffering from FHF, but not in rats with portacaval shunts. Selective loss of NMDA sensitive [^{3}H]glutamate binding sites without alterations in the kainic acid (KA) and quisqualate sensitive binding sites in rat brain following portocaval anastomosis was also reported (Peterson *et al.*, 1990). Rao *et al,* (1991) reported a decrease in the glutamate binding and an increase in the GABA binding in the cerebellum of rats treated with ammonium acetate.

Rao and Murthy, (1991) reported an increase in synaptosomal high affinity uptake systems for glutamate during acute hyperammonemia. uptake was reported to be increased in most of the brain

regions after CCl_4 treatment and this was independent of the presence of coma (Diaz-Munoz and Tapia, 1988).

Review of literature has shown that during acute liver damage the neurotransmitter functions of glutamate and GABA are altered and the information on this aspect during FHF is incomplete. Complete study of temporal and region specific changes in neurotransmitter functions of glutamate and GABA is not yet done during conditions of FHF. Also studies are not yet carried out on the kinetics of these changes (binding and uptake). It is not yet known whether these alterations are occurring due to changes in receptors (upregulation/down regulation or due to conformational changes) and transporters or whether these changes are associated with membrane architecture etc. Similarly, it is not yet worked out whether the changes observed in neurotransmitter functions is due to the action of a single factor (modulator) or a synergistic action of more than one factor. Therefore it was felt essential to understand the changes in the excitatory and inhibitory amino acid neurotransmission (mediated by glutamate and GABA) in conditions of FHF.

Three aspects of neurotransmitter functions were studied viz., (i) release of glutamate from nerve terminals (ii) binding of glutamate and GABA to their respective receptors (representing post-synaptic action of these amino acid neurotransmitters) (iii) uptake of glutamate into nerve terminals (representing the inactivation of glutamate released by nerve terminals). As mentioned earlier, alterations in these neurotransmitter functions of glutamate have been implicated in several neurological disorders, such as Alzheimer's disease, Huntington's chorea, epilepsy, Parkinsonism,

AIDS encephalopathy and dementia complex (Gasic and Hollman, 1992; Kitamaru *et al*., 1993; Dingledine and McBain, 1994; DeLorey and Olsen, 1994). As neurotransmitter functions are membrane associated phenomena, alterations in the membrane functions due to the changes, if any, in the membrane composition or the organization were also studied.

Scope of the Present Work

Hepatic failure occurs due to viral infections (Hepatitis) and hepatotoxins. A wide spectrum of neurological and neuropsychiatric disturbances are observed during this condition. Though therapeutic measures are available, survival depends on better intensive care facilities, monitoring and proper management of the patients in the hospitals. This is because of lack of complete knowledge of pathophysiological mechanisms involved in the disease process. Development of animal model for FHF would aid in carrying out temporal and regional studies on the alterations in the cerebral functions and the mechanisms responsible for these changes, which is not possible in human samples. Moreover, pilot studies of drug treatment can also be successfully carried out in animal model.

Present work is aimed to study the alterations in the neurotramsmitter functions of glutamate and GABA during FHF and in hyperammonemic states associated with or without a functional liver. This work will help in the design and development of drugs for the altered neurotransmitter functions which might help in preventing the cerebral dysfunction. This will open up new avenues for the development of rational therapeutic mechanisms for FHF.

CHAPTER 2

MATERIALS AND METHODS

CHEMICALS

ADP, alanine, aldolase, aspartate, aspartate aminotransferase, ATP, bovine serum albumin, brilliant blue-G250, bromophenol blue, cholesterol, citrate, citrate lyase, diacetyl monoxime, dithiothreitol, EDTA, EGTA, fructose, fructose-1,6-diphosphate, galactosamine hydrochloride, glucose-6-phosphate dehydrogenase, glutamate (monosodium salt), glutamate dehydrogenase, y-amino butyric acid, γ-glutamyl hydroxamate, γ-glutamyl-*p*-nitroanilide hydrochloride, glyceraldehyde-3-phosphate dehydrogenase, glycylglycine, HEPES, hexokinase, hydrazine, hydroxylamine hydrochloride, imidazole, kainic acid, α-ketoglutarate, lactate, lactate dehydrogenase, malate, malate dehydrogenase, β-mercaptoethanol, NAD^+, NADH, $NADP^+$, NADPH, N-methyl-D-aspartate, *p*-nitroaniline, phosphoenol pyruvate, PPO, POPOP, pyruvate kinase, SDS-PAGE marker proteins, thiosemicarbazide, triethanolamine hydrochloride, triose phosphate isomerase, Tris and urea were from Sigma Chemical Co., USA. Ficoll-400 was purchased from Pharmacia Fine Chemicals, Sweden. Sucrose was puchased from BDH, England. Ammonium acetate, ammonium molybdate, silver nitrate was purchased from Merck, Germany. Triton X- 100 was purchased from Kochlight, England. SDS, acrylamide, bis-acrylamide, glycine, naphthalene were obtained from Spectrochem Chemicals, India. MK-801 (RBI Chemicals, USA) was a generous gift from Dr. Yukio Yoneda, Japan. DPH and TMA-DPH was purchased from Membrane Probes Inc., USA . Other chemicals were Anala R/GR grade and were procured locally. [^{3}H]Glutamate (56 Ci/mmole) was purchased from Amersham International, England. Another batch of [^{3}H]glutamate (30 Ci/mmole) and [^{3}H]GABA (30 Ci/mmole) were purchased from American Radio

Chemicals Inc., USA. [^{3}H]Kainic acid (58 Ci/ mmole) and [^{3}H]MK-801 (20 Ci/mmole) was purchased from DuPont, NEN Products, USA. [^{32}P]ATP was purchased from Bhabha Atomic Research Centre, Bombay, India.

ANIMALS

Inbred albino rats of Wistar strain weighing about 175-225 g (3 months old) were used throughout this study. Animals of either sex were maintained under natural light and dark cycles (12 hr light and 12 hr dark) in groups of 6-8 per cage in an air conditioned room at 21 ± 1°C. Food (balanced pellet food supplied by Hindustan Lever Ltd., India) and water were provided *ad libitum.*

DRUG ADMINISTRATION

Induction of Fulminant Hepatic Failure

Fulminant hepatic failure was induced in rats as per the method of Groflin *et al*., (1978). A single dose of galactosamine hydrochloride (2.5 gm/kg body weight; dissolved in saline and pH adjusted to 7.4) was injected intraperitoneally.

Induction of Hyperammonemia

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

Acute Hyperammonemia

Ammonium acetate (dissolved in saline, pH 7.4) was injected intraperitoneally at a single dose of 25 mmoles/kg body weight. Animals were decapitated during convulsive phase which usually appears between 5-10 minutes after the treatment.

Subacute Hyperammonemia

For inducing subacute hyperammonemia, a single intraperitoneal dose of 2.5 mmoles/kg body weight of ammonium acetate was injected. In this group no convulsions were observed even after 24 hr. However, animals in this group were decapitated between 25-30 minutes after treatment.

In Vitro Effects

For *in vitro* studies, ammonium acetate was added to the incubation medium consisting of preparations from normal animals. Two different concentrations of ammonium acetate were used viz., 1 and 5 mM concentrations.

SAMPLE COLLECTION/PREPARATION

Plasma

At specified time periods, blood was drawn by cardiac puncture into a syringe (rinsed with trisodium citrate) and transferred into centrifuge tubes which were rinsed earlier with trisodium citrate. In some instances, blood was collected from the neck wound after decapitating the animal. Blood was immediately centrifuged at 5,000 rpm for 10 min at 4°C to obtain plasma. This was used for the estimation of enzymes, glucose, proteins and urea

For ammonia estimation, plasma was deproteinized with PCA. To the plasma, an equal volume of 10% cold PCA (w/v) was added. This was allowed to stand for 15 min for complete precipitation of proteins. The tubes were centrifuged at 5,000 rpm for 10 min at 4°C. The supernatant was neutralized with solid potassium carbonate till the pH was 7.0 - 7.2. Tubes were kept in ice for 15 min and centrifuged at 10,000 rpm for 15 min at 2°C. Ammonia content was determined in the supernatant.

Liver

Liver was collected only from normal and GALN treated animals. After decapitation, liver was excised and transferred to a beaker containing ice-cold 0.32 M sucrose. The tissue was gently pressed between two Whatman No. 1 filter papers wetted with sucrose to remove the blood present in the tissue. Tissue was cut to required size taking care that every time the same liver lobe was used.

ISOLATION OF BRAIN REGIONS

Immediately after decapitation, brains were removed from the cranial vault and transferred to ice-cold saline. They were gently pressed between two wet Whatman No. 1 filter papers to remove excess of water, meninges and surface capillaries etc. Cerebral cortex (CC), cerebellum (CB) and brain stem (BS) were separated.

PREPARATION OF HOMOGENATES

A 10% (w/v) homogenate of liver, CC, CB and BS was prepared individually in 0.32 M sucrose using a motor driven Potter-Elvehjem homogenizer with a serrated Teflon pestle operating at 800 rpm with 8-10 up and down strokes. The temperature was maintained at 0-2^{o}C.

PREPARATION OF BRAIN AND LIVER EXTRACTS :

(FOR DETERMINING INTERMEDIATES **OF CEREBRAL GLUCOSE METABOLISM AND** AMMONIA)

Rats were decapitated and the head was allowed to fall into liquid nitrogen and frozen at this temperature for 10-15 min. Brains were chiselled out with a stainless steel chisel (pre-cooled in liquid nitrogen) and were powdered with a stainless steel pestle in a stainless steel mortar (both the mortar and pestle were pre-cooled with liquid nitrogen). For ammonia determination, liver tissue was excised and plunged into liquid nitrogen.

After 10 min, tissue was powdered as described above. Powdered tissue was rapidly transferred into pre-weighed tubes containing 3 ml of ice-cold 10% (w/v) PC A and the tubes were weighed again. The powder was dispersed well and homogenized as described above. Samples were allowed to stand on ice for 15 min and centrifuged at 5,000 rpm for 10 min at 4°C. The supernatant was neutralized with solid potassium carbonate till pH was 7 to 7.2 and was centrifuged at 10,000 rpm for 10 min at 0-4°C. This supernatant was used for the assay of metabolites and ammonia. Metabolites were estimated in the brain extracts while ammonia was determined both in liver and brain extracts.

SAMPLE PREPARATION FOR LIPID ANALYSIS

Lipids were extracted by the method of Folch *et al*., (1959). Different regions of brain were homogenized (1g in 40 ml) in chloroform : methanol (1:1; v/v) and allowed to stand for 5 min for complete extraction of lipids. Homogenates were centrifuged at 3,000 rpm for 5 min. The supernatant was saved and the pellet was extracted twice with 5 ml of chloroform : methanol (1:1; v/v). The pellet was washed twice with chloroform: methanol (1:2; v/v) and all the supernatants were combined and evaporated under reduced pressure. Dried residue was extracted with known volume of chloroform : methanol (1:1; v/v) and used for lipid analysis.

PREPARATION OF SYNAPTOSOMES

Synaptosomes were isolated from the CC, CB and BS of control and GALN treated rats by the method of Cotman, (1974) as described by Rao and Murthy (1991). Tissues were homogenized individually in 0.32 M sucrose (pH 7.4) to obtain a 10% (w/v) homogenate. This was centrifuged at l,000g for 5 minutes to obtain pellet having unbroken cells, crude nuclei

and capillaries. Supernatant (S_1) was centrifuged at 15,000 g for 15 minutes to obtain a crude pellet (P_2) containing synaptosomes, myelin and mitochondria. The P_2 pellet was suspended in 5 ml of 0.32 M sucrose. This was layered on top of the preformed discontinuous density gradient (consisting of 10 ml each of 4%, 6% and 13% (w/v) Ficoll-400 in 0.32 M sucrose (pH 7.4) and centrifuged at 63,500g for 45 minutes. This resulted in the separation of myelin (in 4% Ficoll layer), synaptosomes (at the interface of 6%-13% Ficoll) and mitochondria (pellet at the bottom of the tube). Myelin layer was discarded. Synaptosomal layer was aspirated carefully with a Pasteur pipette and was diluted with 5 volumes of 0.32 M sucrose and centrifuged at 20,000g for 20 minutes. Pellet was suspended in Krebs Ringer, protein content was adjusted to 1 mg/ml and oxygenated till further use. Centrifugations were carried out in Beckman J2-21 centrifuge and the density gradient separation was done in Beckman LS-80M ultracentrifuge.

MEMBRANE PREPARATION FOR RECEPTOR BINDING :

Membranes were isolated from normal, GALN treated and hyperammonemic rats according to the method described by Rao *et al.*, (1991). Different regions of brain were isolated as described above and homogenized individually in 10 volumes of 0.32 M sucrose containing 5 mM Tris-HCl buffer (pH 7.4) and centrifuged at l,000g for 10 minutes at 4°C. The supernatant was diluted with 3 volumes of ice-cold double distilled water and centrifuged at 36,400g for 15 minutes at 4°C. Pellet was homogenized in 5 mM Tris-HCl buffer (pH 7.4) and centrifuged at 36,400g for 15 min at 4°C. Resulting pellet was homogenized again in 40 mM Tris-HCl (pH 7.4) and centrifuged at 36,400g for 15 minutes at 4°C. This step was repeated for one more time. The final pellet was suspended in 1.5 to 4

ml (depending on the brain region) of 40 mM Tris-HCl buffer and protein content was adjusted to 1 mg/ml. This preparation was used for receptor binding studies, enzyme assays, electrophoresis, protein phosphorylation, lipid analysis and fluorescent studies.

Glutamate binding was performed within 3-4 hours of membrane isolation. For studying GABA binding, membrane preparations were frozen at -80°C for 24 hr were used. On the day of assay, membranes were thawed, washed twice with 40 mM Tris-HCl buffer (pH 7.4) and treated with 0.05% Triton X-100.

For studying kainic acid binding, membranes were prepared as described for GABA binding except that they were washed 4 times after thawing and Triton X-100 treatment was omitted. Protein content was adjusted to 3 mg/ml. A similar procedure was adopted for preparing membranes for MK-801 binding.

BIOCHEMICAL METHODS

Protein Estimation

Protein content present in an aliquot of the brain and liver preparations was estimated by the method of Lowry *et al.*, (1951). Bovine serum albumin was used as standard. Protein content in the plasma was estimated using biuret reagent by the method described by Varley (1969). For electrophoretic studies, protein content in the membrane samples were estimated by the method of Bradford (1976).

Phospholipids

Phosopholipd content was determined by the method of Summer *et al.*, (1944). Lipid extract (50 µl) was evaporated to dryness under nitrogen and the phospolipids were estimated. To the residue, 1 ml of digestion mixture

(20 ml of sulphuric acid, 25 ml of 70% PC A and 50 ml of water) was added and heated in a sand bath at 250°C for 2 hours. The sample was cooled and 1 ml of distilled water was added followed by 1 ml of 50% sodium acetate and the volume was made up to 10 ml with water. To this, 1 ml of 2.5% ammonium molybdate was added and mixed well. This was followed by the addition of 1 ml of metol (p-methylaminophenol sulphate - 1 g in 100 ml of 3% sodium bisufite). Absorbency of the sample was measured at 700 nm after 10 min. Potassium dihydrogen orthophosphate (10-100 ug) was used as standard. In blanks, sample was replaced with chloroform : methanol.

Cholesterol

Cholesterol was estimated by the method of Natelson *et al*., (1971). A suitable aliquot of the sample was evaporated under nitrogen and 2 ml of glacial acetic acid was added to the dried sample. This is followed by the addition of 2 ml of acetic anhydride : concentrated sulphuric acid (10:1) and incubated at 37°C for 20 min. The colour intensity was measured at 625 nm against a blank in which sample was replaced with chloroform : methanol. Cholesterol (10 to 100 ug) was used as standard.

Ammonia

Ammonia was estimated in the neutralised PCA extracts of plasma, liver and brain by method of Ratnakumari and Murthy (1990). To 1 ml of the supernatant 1.5 ml of phenol-nitroprusside reagent (containing 50 g phenol and 250 mg sodium nitroprusside in 3.75 L water) and 2 ml of sodium hypochlorite (8.4 g NaOH, 89.2 g disodium hydrogen phosphate and 10 ml of 5% sodium hypochlorite/L) solutions were added. After 20 min, the colour intensity was measured at 630 nm against a distilled water blank. Ammonium chloride (0. 1 to 1. 0 µmoles) was used as standard.

Urea

Urea content in the plasma and liver samples was estimated by diacetyl monoxime method of Wybenga *et al.*, (1971). To 5 ul plasma or liver homogenate (10%; w/v), 3 ml of urea reagent (2:1 mixture of reagents A and B - reagent A contains 100 ml of phosphoric acid, 300 ml of sulphuric acid and 100 mg of ferric chloride while reagent B contains 500 mg of diacetyl monoxime and 10 mg of thiosemicarbazide in 100 ml of water). Samples were kept in a boiling water bath for 5 min. After cooling the samples, colour intensity was measured at 525 nm against a distilled water blank. The standard contains 100 nmoles of urea in 5 ul of water.

Plasma Glucose

Glucose was estimated by glucose oxidase method of Barham and Trinder (1972) using the kit supplied by Ranbaxy Laboratories, Bombay (India) Supplied enzyme powder was dissolved in 250 ml water and refrigerated. At the time of assay, a working solution was prepared by adding 1 ml of phenol (supplied) to 10 ml of enzyme solution. To 3 ml of working solution 20 ul of plasma or liver homogenate was added and incubated at $37^{o}C$ for 15 min and the absorbency of the sample was measured at 505 nm against water as blank. For standards 100 nmoles of glucose was used.

ASSAY OF METABOLITES :

Metabolites present in the neutralized PC A extract were assayed by spectrophotometnc method (using commercial enzymes) using a Shimadzu spectrophotometer (Model No. UV-160A). Metabolite assays were coupled to an appropriate NAD(P) or NAD(P)H dependent dehydrogenase (in a final volume of 750 ul) and change in absorbency was recorded at 340 nm till four successive measurements of absorbency were constant at 21 ± 2°C.

Usually, assays were done using two different volumes of PCA extract in duplicate. Authentic standards (different concentrations) were used for preparing a standard curve and for the calculation of metabolite concentrations. Unless otherwise mentioned, methods for the metabolite assays were according to that of Lowry and Passonneau (1972).

Glucose (Brain)

Reaction mixture for glucose determination consists of 250µl of 150 mM of Tris-HCl buffer (pH 8. 0), 25 ul of 30 mM $MgCl_2$, 10 ul of 38 mM ATP, 25 ul of 15 mM NADP, 10µl of hexokinase (0.28 U/ml; 2 ug/ml) and 10 ul of glucose-6-phosphate dehydrogenase (0.08 U/ml; 2 µg/ml). The final volume was made up to 750 ul with water. The reaction was started with 10-20 ul of sample and the change in the absorbency was recorded.

Fructose-1, 6-Diphosphate

Reaction mixture consists of 100 ul of 375 mM of imidazole-HCl (pH 7.5), 25 ul of 30 mM NAD, 25 ul of 30 mM sodium arsenate, 25 ul of 30 mM EDTA, 25 µl of 60 mM β-mercaptoethanol, 10 µl of glyceraldehyde-3-phosphate dehydrogenase (1.8 U/ml; 50 µg/ml). Final volume of the reaction mixture was made up to 750 ul with water. Reaction was allowed to complete. After the completion of the reaction, 10 ul of triosephosphate isomerase (2.4 U/ml; 1 µg/ml) was added and the reaction was once again allowed to proceed till completion and the change in absorbency was noted. After the completion of the reaction, 10 ul of aldolase (0.09 U/ml; 10 µg/ml) was added and the reaction was allowed till completion and the change in the absorbency was taken as a measure of fructose-1,6-diphosphate content.

Phosphoenol Pyruvate

Reaction mixture consists of 100 ul of 250 mM phosphate buffer (pH 7.0), 50 ul of 30 mM $MgCl_2$, 10 ul of 15 mM ADP, 20 ul of 4 mM NADH, 5 ul of pyruvate kinase (0.15 U/ml; 1 ug/ml), 10 ul of lactate dehydrogenase (0.2 U/ml; 1 µg/ml). Final volume was made up to 750 ul with water. Reaction was started with the addition of 10-20 ul of sample and the change in absorbency was recorded.

Pyruvate

Reaction mixture consists of 100 µl of 250 mM phosphate buffer (pH 7.0), 20 µl of 4 mM NADH, 5 µl of lactate dehydrogenase (0.1 U/ml; 0.5 µg/ml). Final volume was made up to 750 ul with water. Reaction was started with 10-20 ul of sample and change in absorbency was recorded.

Lactate

Reaction mixture consists of 250 ul of 600 mM hydrazine buffer (pH 9.6), 50 ul of 30 mM NAD and 10 ul of lactate dehydrogenase (20 U/ml; 100 µg/ml). Final volume was made up to 750 ul with water. Reaction was started with the addition of 10-20 ul of sample and the change in the absorbency was recorded.

Citrate

Reaction mixture consists of 500 µl of 150 mM Tris-HCl (pH 7.6), 20 ul of 4 mM NADH, 25 ul of 1.2 mM $ZnCl_2$, 15 ul of citrate lyase (0.12 U/ml; 15 µg/ml), 2 ul of malate dehydrogenase (0.3 U/ml; 0.4 ug/ml). Final volume was made up to 750 ul with water. Reaction was started with of 10-20 ul of sample and the change in the absorbency was recorded.

α-Ketoglutarate

Content of α-ketoglutarate in the sample was measured as per the method of Lowenstien *et al*.,(1969). Reaction mixture consists of 200 µl of 250 mM phosphate buffer (pH 7.4), 25 ul of 4 mg/ml NADH, 25 ul of 30 mM NH_4Cl, 20 ul of glutamate dehydrogenase (3 U/ml; 10 mg/ml). Final volume was made up to 750 ul with water. Reaction was started with 10-20 ul of sample and the change in the absorbency was followed.

Malate

Reaction mixture consists of 250 ul of 600 mM hydrazine buffer (pH 9.3), 50 ul of 30 mM NAD, 10 ul of malate dehydrogenase (2.1 U/ml; 3 µg/ml). Final volume was made up to 750 ul with water. Reaction was started with the addition of 10-20 ul of sample and the change in the absorbency was recorded.

Glutamate

Reaction mixture consists of 250 ul of 150 mM Tris-acetate buffer (pH 8.4), 25 ul of 30 mM NAD, 5 ul of 15 mM ADP and 25 ul of glutamate dehydrogenase (4.5 U/ml; 100 µg/ml). Final volume was made up to 750 ul with water. Reaction was started with the addition of 10-20 ul of sample and the change in the absorbency was recorded.

Aspartate

The reaction mixture consists of 100 ul of 375 mM imidazole-HCl, 20 µl of 4 mM NADH, 25 ul of 6 mM α-KG, 20 ul of aspartate aminotransferase (1.8 U/ml; 10 µg/ml) and 10 ul of malate dehydrogenase (1.4 U/ml; 2 µg/ml). Reaction was started with the addition of 10-20 ul of sample. Final volume was made up to 750 ul with water. After the reaction was

completed 20 ul of aspartate aminotransferase was added and the change in the absorbency was recorded.

ATP

Reaction mixture consists of 250 ul of 150 mM Tris-HCl (pH 8.1), 25 ul of 30 mM MgCl2, 10 ul of 37.5 mM DTT, 25 ul of 15 mM NADP, 25 ul of 30 mM glucose, 10 ul of hexokinase (0.28 U/ml; 2 ug/ml), 10 ul of glucose-6-phosphate dehydrogenase (0.07 U/ml; 0.15 ug/ml). Final volume was made up to 750 ul with water. Reaction was started with the addition of 10-20 ul of sample and the change in the absorbency was followed.

APR

Reaction mixture has 100 ul of 375 mM imidazole buffer (pH 7.0), 50 µl of 30 mM MgCl2, 25 ul of 2.25 M KCl, 20 ul of 3.75 mM NADH, 25 ul of 9 mM phosphoenol pyruvate, 10 µl of pyruvate kinase (0.3 U/ml; 2 µg/ml), 20 ul of lactate dehydrogenase (0.4 U/ml; 2 µg/ml). Final volume was made up to 750 ul with water. Reaction was started with the addition of 10-20 ul of sample and the change in the absorbency was followed.

ENZYME ASSAYS

Na^+, K^+-ATPase

Na^+, K^+-ATPase was assayed by the method of Sadasivudu *et al.*, (1977). The assay mixture (2 ml) for total ATPase consisted of 0.8 ml of 0.4 M Tris-HCl buffer (pH 7.4), 0.05 ml 0.2 M $MgCl_2$, 0.15 ml 2 M NaCl, 0.2 ml 0.15 M KC1 and 0.1 ml 0.06 M ATP. In the assay mixture for Mg^{2+}-ATPase, 0 1 ml 10 mM ouabain was used, sodium and potassium chlorides were replaced with equal amounts of distilled water. 100 µg/100 ul of membrane protein was added to the assay mixture. After 20 min of incubation at 37°C, reaction was terminated with 0.5 ml of 22.5% trichloroacetic

acid (TCA) and the samples were centrifuged. Inorganic phosphate was determined in 1 ml aliquot of the supernatant following the addition of 1 ml of 5N H_2SO_4, 1 ml of 2.5% ammonium molybdate and 0.1 ml of reducing reagent (1.28 g sodium sulphite, 1.28 g sodium metabisulphite, 0.2 g ANSA (1-amino-2-napthol-4-sulphonic acid, 25 mg/ml dissolved in water just before use). The solution was made up to 10 ml with distilled water and the absorbency was measured at 660 run after 20 min. KH_2PO_4 (0.1 to 1 umol) was used as standard. Na^+, K^+-ATPase activity was calculated as the difference between the activities of total and Mg^{2+}-ATPases and expressed as umol Pi/mg protein/hr.

γ-Glutamyl Transpeptidase(GGTP)

This enzyme was assayed by the method of Tate and Meister, (1974). Reaction mixture consisted of 0.8 ml of 0.1 M Tris-HCl (pH 9.0), 0.4 ml of 0.025 M γ-glutamyl-*p*-nitroanilide hydrochloride, 0.2 ml of 0.25 M glycyl-glycine, 0.4 ml of 0.375 M NaCl and 0.1 ml of 10% liver homogenate or plasma or cerebral membrane preparation and the final volume was adjusted to 2.0 ml with distilled water. Reaction was initiated by the addition of enzyme. After incubation for 30 min at 37°C, reaction was stopped by addition of 1 ml of 5% TCA. After centrifugation at 4,000 rpm for 30 min, absorbency of supernatant measured at 410 nm In blanks, 5% TCA was added before the addition of the enzyme. *p*-nitroaniline (0.3 mM) was used as standard.

Sorbitol Dehydrogenase (SOPH)

The method described by Bergmeyer and Brent (1974) was used for the assay of this enzyme. One ml of the reaction mixture consists of 500 ul of 0.2 M triethanolamine hydrochloride (pH 7.4), 30 ul of 12 mM NADH

and a suitable aliquot of the enzyme. The reaction was initiated by the addition of 100 ul of 4 M fructose. Change in the absorbency at 340 nm was measured at 21°C for 10 min at 1 min intervals. Fructose was omitted in the blanks.

Aspartate Aminotransferase (AAT)

Activity of this enzyme was measured as per the method of Bergmeyer and Brent (1974). Reaction mixture (final volume 1.0 ml) consists of 500 ul of 160 mM of potassium phosphate buffer (pH 7.4), 25 ul of 20 mM aspartic acid (pH 7.4), 50 ul of 18 mM a-KG (pH 7. 4), 25 ul of 0. 4 mM NADH, 5 ul malate dehydrogenase (0.5 mg protein/ml) and suitable amount of plasma or liver homogenate (10-20 ul). Reaction was started by the addition of a-KG. Change in the absorbency was measured at 340 nm at 21°C for 10 min at one min intervals. Enzyme activity was calculated using the formula :

$$\frac{\text{Change in absorbency min} \times \text{Vol. of reaction mixture} \times 60 \times 1000}{0.0062 \times 1000 \times \text{time} \times \text{mg tissue protein or } \mu\text{l plasma/homogenate}}$$

Enzyme activity in liver were expressed as mmoles of NADH oxidised/gm tissue/hr or umoles of NADH oxidized/mg protein/hr. Plasma AAT activities were expressed as umoles of NADH oxidized/ml serum/hr or as umoles of NADH oxidized/mg protein/hr.

Alanine Amino Transferase (ALAT)

The method of Bergmeyer and Brent, (1974) was used for the assay of this enzyme. Reaction mixture (final volume 1.0 ml) contained 500 ul of 160 mM potassium phosphate buffer (pH 7.4), 25 ul 40 mM alanine (pH 7.4), 25 ul of 18 mM a-KG (pH 7.4), 25 ul of 0. 4 mM NADH, 5 ul lactate dehydrogenase (0.5 mg protein/ml) and a suitable quantity of plasma or

liver homogenate (10-20 µl). Reaction was started by the addition of a-KG. Change in absorbency (due to NADH oxidation) at 21°C was measured at 340 nm for 10 min at one min intervals. Activity of the enzyme was calculated and expressed as mentioned above for AAT activity.

Glutamine Synthetase(GS)

Glutamine synthetase in the liver was assayed by the method of Rowe *et al.,* (1970). The assay mixture (1 ml) contained 50 mM imidazole-HCl buffer (pH 7.2); 20 mM magnesium chloride; 20 mM β-mercaptoethanol; 50 mM sodium glut am ate; 100 mM hydroxylamine hydrochloride (freshly neutralised) and 10 mM ATP. ATP was omitted in blanks. Reaction was initiated by the addition of 0.1 ml of the 5% (w/v) homogenate and incubated for 15 min at 37°C. Reaction was terminated by the addition of 1.5 ml of ferric chloride reagent (6% ferric chloride containing 3.35% trichloroacetic acid and 6.6% hydrochloric acid). Contents of the tubes were centrifuged at 5,000 rpm for 10 min at room temperature and the absorbency of the supernatant containing the colored complex formed between the y-glutamylhydroxamate (formed during the reaction) and ferric chloride reagent was measured at 535 nm. Commercially supplied γ- glutamylhydroxamate was used as standard.

RECEPTOR BINDING ASSAY

Glutamate and GAB A receptor binding studies were performed as per the method described by Rao *et al.,* (1991). For studying [^{3}H]glutamate binding, membranes (100-200 µg protein equivalent) were incubated in 0.5 ml of 40 mM Tris-HCl buffer (pH 7.4) containing 20 nM [^{3}H]glutamate (56 Ci/mmole - procured from Amersham; 30 Ci/mmole - procured from American Radio Chemicals) at 37°C in a shaker water bath. After 15

minutes of incubation, binding was terminated by the addition of 5 ml of ice-cold 40 mM Tris-HCl buffer. Samples were rapidly filtered under negative pressure through Whatman GF/C glass microfibre filters placed in a Millipore 12 place filter manifold. Filters were washed twice with 5 ml ice-cold buffer. They were dried at 45°C and transferred to a scintillation vial containing Bray's scintillation fluid (60 g naphthalene, 4 g PPO, 200 mg POPOP, 100 ml methanol and 20 ml of ethylene glycol made up to 1 litre with 1,4-dioxan) and the radioactivity was determined. Non-specific binding was determined in parallel assays performed in the presence of large excess of unlabelled (mm) glutamate. Specific binding was calculated from the difference between total and the non-specific binding.

GAB A binding was also determined by the same method using 12 nM [^{3}H]GABA (30 Ci/mmole - procured from American Radio Chemicals). In these experiments non-specific binding assays were performed in the presence of 1 mm unlabelled GABA.

For the preparation of saturation isotherms, ligand (glutamate) concentration was varied from 10 nM to 2,000 nM. For 10 and 20 nM concentrations, radioactive glutamate was used without diluting with unlabelled glutamate. In other cases 40 nM of [^{3}H]glutamate was used and the concentration was made up with unlabelled glutamate.

Two methods were followed for studying the binding of [^{3}H]glutamate to the receptor subtypes. In **Receptor Blocking Assay,** membranes were preincubated for 15 min with 0.2 mM NMDA (N-methyl-D-aspartate) or KA (kainate) at 37^{o}C before starting the assay. Assay was started by the addition of [^{3}H]glutamate (30 Ci/mmole). Difference in specific binding in the

presence and absence of the NMDA or KA was taken as binding to these subtypes of receptors.

In the second method, ligands specific to glutamate receptor subtypes (MK-801, 20 Ci/mmole; and KA, 58 Ci/mmole) were used. MK-801 binding assay was performed as described by Akinci and Johnston (1993). For MK-801 binding 300 ug of membrane suspension was incubated in 40 mM Tris-HCl buffer at pH 7.4 for 45 min at 37°C with a fixed concentration of [^{3}H]MK-801 (5 nM) which was diluted to required concentration with unlabelled ligand (10 concentrations between 0.5-500 nM). The final volume of assay mixture was 500 µl. Non-specific binding was determined in the presence of 100 uM unlabelled MK-801. Kainic acid binding assays were performed as described by Akinci and Johnston, (1994). For kainate binding, the membrane preparation (300 µg/ml) was incubated at 37°C for 30 min with a fixed concentration of [^{3}H]kainic acid (5 nM) and a range of concentrations of unlabeled ligand (10 concentrations between 0.5 to 500 nM). The assay volume was 500 ul. Non-specific binding was determined in the presence of 100 uM kainate. All the above binding experiments were performed in duplicates.

Scatchard analysis was performed from the above data by first order linear regression using Sigma plot program. Binding kinetics (Kd and Bmax) were calculated from these plots using plot statistics option. The correctness of the kinetic constants were cross-checked with least squares method. For this purpose, a computer program was developed.

In the receptor binding studies, buffers and all other reagents were prepared in fresh glass distilled water just before the experiment. These reagents were filtered through 0.45u Millipore membrane filters before the

assay to avoid any artefacts due to microbial contamination (Yoneda and Ogita, 1989).

To find the purity of [^{3}H]glutamate, paper chromatography was done using n-butanol : acetic acid : water (65:15:25 v/v) as solvent. [^{3}H]glutamate and unlabelled glutamate (20 nmoles) were spotted separately on the same Whatman no. 1 chromatographic paper (20 x 12 cm). Ascending chromotography was performed. When the solvent front was 1 cm from the top edge, paper was removed and dried at room temperature. Chromatogram was cut along the length into two strips, one containing unlabelled glutamate and other having labelled glutamate. The strip containing unlabelled glutamate was sprayed with ninhydrin reagent (250 mg ninhydrin, 1 ml of pyridine and 99 ml of acetone) and dried at 50°C. Position of glutamate spot was marked. The strip containing labelled glutamate was cut into 1 cm pieces (from origin and edge of solvent front) and the radioactivity was measured using Bray's scintillation fluid. Contaminants and the spot containing labelled glutamate were eluted separately into 10 ml of 0.01 N HC1. These samples were lyophillized to minimal volume and diluted to 1 ml with 40 mM Tris-HCl buffer (pH 7.4). This was centrifuged at 15,000 rpm for 10 min at room temperature to remove particulate material. These supernatants were used to find the interference of contaminants with the glutamate binding assay which was performed as described above.

STUDIES ON GLUTAMATE UPTAKE

High affinity and low affinity uptake of glutamate into synaptosomes were carried out as described by Rao and Murray (1991). Well oxygenated Krebs-Ringer-phosphate-glucose-HEPES medium (0.5 ml final volume), consisting of 120 mM NaCl; 2.5 mM KC1; 10 mM glucose; 1.3 mM

$MgSO_4$; 20 mM $NaHCO_3$; 10 mM sodium dihydrogen ortho phosphate; 10 mM HEPES and 0.5 mM $CaCl_2$, was used in incubation medium. All the uptake studies were carried out within 3-4 hours of isolation of synaptosomes. For high affinity uptake, final concentration of glutamate was in the range of 1 uM to 100 uM. For low affinity uptake, glutamate concentration was varied from 0.1 to 5 mM. In all the studies, 0.1 uCi of [^{3}H]glutamate was added per tube. Uptake was initiated by the addition of 100 µg of synaptosomal protein. Incubations were carried out at 37°C for 5 minutes with continuous shaking and oxygenation. Uptake was terminated by rapidly cooling the tubes to 4°C followed by centrifugation at 10,000g for 1 minute at 4°C. Supernatant was carefully removed and the pellet was quickly washed twice with 2 ml of ice-cold medium having same concentration of unlabelled amino acid. The final pellet was digested in 100 ul of 0.1 N NaOH and was transferred to a scintillation vial having 5 ml of scintillation fluid (60 gm naphthalene, 4 gm PPO, 200 mg POPOP, 100 ml methanol, 20 ml ethylene glycol in 1 L of 1,4-Dioxan). Radioactivity was determined with Beckman LS 1800 liquid scintillation counter.

In all these experiments, non-specific uptake was determined by carrying out incubations at 0°C for 5 minutes. Non-specific uptake values were subtracted from total uptake to obtain specific uptake.

Scatchard analysis was performed to determine the kinetic constants (Km and Vmax) using Sigma plot program with plot statistics option.

GLUTAMATE RELEASE STUDIES

The method adopted was that of Rao and Murthy (1991). For studying the depolarisation induced release of glutamate, synaptosomes were preloaded with radioactive glutamate under nondepolansing conditions using

incubation conditions similar to those mentioned for uptake studies except that the time for incubation was 15 minutes. Same incubation medium was used but without $CaCl_2$. After loading, the synaptosomes were pelleted and resuspended in a fresh medium having either 1.2 mM $CaCl_2$ or 2 mM EGTA. Both the samples were incubated with or without 50 mM KCl at 37°C in a shaker water bath for a period of 5 min. Immediately after the incubation, the contents of the tubes were chilled and centrifuged rapidly at 10,000g in cold. Supernatants were collected and the radioactivity was determined in an aliquot of the supernatant. Pellets were briefly washed with ice-cold medium and digested in 0.1 N NaOH and radioactivity was determined. Results were expressed as % of the total radioactivity released at 5 min time period.

SDS-PAGE ANALYSIS OF MEMBRANE PROTEINS

Membrane proteins were separated on SDS-PAGE by the procedure of Lamelli (1970) on a 1 cm 3% stacking gel (pH 6.8) was followed by a 15 cm 10% separating gel (pH 8.8). Tris-glycine buffer (0.025 M) with 0.1% sodium dodecyl sulphate (pH 8.3) was used as electrode buffer.

PREPARATION OF SAMPLE FOR ELECTROPHORESIS

An aliquot of membrane protein (2 mg/ml) was mixed with an equal volume of 2X sample buffer containing 0.125 M Tris-HCl (pH 6.8), 4% sodium dodecyl sulphate (SDS), 20% glycerol, 10% β-mercaptoethanol and 0.002% bromophenol blue. The sample was denatured at 100°C for 5 min. Electrophoresis was carried out at a constant voltage of 120 volts until the tracking dye reached 1 cm above the base of the resolving gel.

SILVER STAINING

Silver staining of the gels were done according to the method of Blum *et al.*, (1987). The procedure for silver staining is as follows :

	STEPS	SOLUTIONS	TIME OF TREATMENT
1.	Fix	50% MeOH; 12% AcOH; 0.5 ml. 37% HCOH/L	> lh
2.	Wash	50% EtOH	3 X 20 min
3.	Pretreat	$Na_2S_2O_3.5\,H_2O$ (0.2 g/L)	1 min
4.	Rinse	H_2O	3 X 20 sec
5.	Impregnate	$AgNO_3$ (2 g/L) 0.75 ml; 37% HCOH/L	20 min
6.	Rinse	H_2O	2 X 20 sec
7.	Develop	Na_2CO_3 (60 g/L); 0.5 ml 37% HCOH/L; $Na_2S_2O_3$. 5 H_2O (4 mg/L)	10 min
8.	Wash	H_2O	2X2min
9.	Stop	50%MeOH; 12% AcOH	10 min
10.	Wash	50% MeOH	> 20 min

MOLECULAR WEIGHT DETERMINATIONS ON SDS-PAGE :

Molecular weight of electrophoretically separated polypeptides were determined by coelectrophoresing SDS-PAGE marker proteins from a high molecular weight calibration kit (high range Mol. Wt. kit 29-205 KD) obtained from Sigma Chemical Co.

*MEMBRANE PROTEIN **PHOSPHORYLATION** (in vitro) :*

Assay mixture (5 ul) for protein phosphorylation consists of 40 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 1 mM DTT, 1 mM $CaCl_2$ (in $+Ca^{2+}$ experiments and in $-Ca^{2+}$ experiments 4 mM EGTA was used). Final volume of the reaction mixture was 20 ul. After the addition of protein (10 µg/10 ul), samples were preincubated at 30°C for 5 min. Reaction was started by the addition of 2 µCi (5ul) of [^{32}P]ATP. After incubating for 30 sec, reaction was terminated by the addition of 10 µl 2X sample buffer containing 0.125 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% β-mercaptoethanol and 0.002% bromophenol blue. Samples were denatured at 100°C for 5 min. SDS-polyacrylamide gel electrophoresis was carried out (10% gel) at a constant voltage of 120 volts until the tracking dye reached 1 cm above the base of the resolving gel. Gels were silver stained and dried between two cellophane sheets in a gel dryer. Incorporation of [^{32}P] label was revealed by autoradiography by exposing the gels to X-ray films for 40-48 hrs depending on the amount of radio activity.

For studying the glutamate effect, membranes were preincubated with 100 uM glutamate for 10 min at 37°C before starting the phosphorylation assay. Similarly for NMDA effect, 100 uM NMDA was used. Membranes were initially incubated with 10µM of MK-801 for 5 min and then a final concentration of 100 uM glutamate was added and incubation continued. Phosphorylation assay was performed later.

***FLUORESCENCE POLARIZATION STUDIES** :*

Fluorescence polarization studies were performed according to the method of Revathi *et ed.*, (1994). Membrane preparation (100 ug) was suspended in 50 mM Tris-HCl buffer (pH 7.4) and incubated for 30 min at

37°C with 1 uM membrane probes DPH, (1,6-dipheny-1,3,5-hexatriene) or TMA-DPH (1 -[4-(trimethyammonio)phenyl]-6-phenyl-1,3,5-hexatriene). DPH was dissolved in dimethylformamide while TMA-DPH was dissolved in water : dimethyl form amide (1 : 1 ; v/v). All the incubations were carried out in dark and the probes were freshly prepared and stored in dark. Fluorescence measurements of the sample were made with a Hitachi F-4000 steady state spectrofluorimeter using 1 cm path-length quartz cuvettes. Excitation and emission slits with a nominal bandpass of 5 nm were used for all experiments. All the spectra were recorded using the correct spectrum mode at an excitation wave length of 358 nm at 23°C. To reverse any photoisomerization of DPH, samples were kept in dark in the fluorimeter for 30 seconds before excitation shutter was opened and fluorescence was measured. Background intensities of the samples without fluorophore were negligible. Polarization measurements were made using Hitachi polarization accessory. Polarization values (P) were calculated from the equation:

$$P = (I_{VV} - GI_{VH}) \div ((I_{VV} + GI_{VH})$$

where I_{VV} and I_{VH} are the measured fluorescence intensities with the excitation polarizer vertically oriented and the emission polarizer horizontally oriented, respectively and G is the grating correction factor which is equal to I_{HV}/I_{HH}. The polarization values are mean values of measurements made with at least three different samples in duplicates.

HISTOLOGY OF LIVER

Liver histology was studied in control and in GALN treated rats at different time periods (6, 12, 18, and 24 hrs) after the administration of drug. Animals were anaesthetised with ether and the portal vein was cannulated with Viggo Venflon-2 I.V. cannula with an injection valve and PTFE

catheter (0.8 mm O.D; 22 G). Leur-lock end of the catheter was connected to 0.9% (w/v) ice-cold saline reservoir while the injection valve was connected to 10% buffered formaline (pH 7.4) through a two way teflon valve. Both saline and formaline were allowed to flow under gravitational force (35-40 ml/min). Initially, liver was perfused with 0.9% saline till the colour of the tissue turns to pale brown (5-8 min of perfusion). At this juncture the two way valve was opened in such a way that formaldehyde reservoir was connected and saline reservoir was disconnected. Perfusion with formaline was continued for 10 min to achieve total fixation of the tissue. Liver tissue was then excised, cut into 1 mm cubes and stored in Bouin's fluid for 3-4 days.

Tissue was passed through graded series of aqueous alcohol (ascending) with 70% alcohol (24-48 hrs), 90% alcohol (3 changes of 1 hr each) and 95% alcohol (1 change - 1 hr). Tissue was stored overnight (14-18 hrs) in methyl benzoate and transferred to benzene (10-15 mins). Tissue was transferred to benzene : wax mixture (1:1 v/v - kept at 60-62^{o}C) and left for 30 mins. Tissue was transferred to wax bath and wax was changed after every one hour for three hours. Blocks made in fresh wax.

Preparation Of Wax

Wax (60-62^{o}C) was seasoned by melting (60-62^{o}C) and cooling for at least for 4-5 times. To 100 gm of seasoned wax, lg of bees wax was added and allowed to melt. This was thoroughly mixed and stored in frozen condition. Requisite quantities of this wax was melted just before use.

Sections (5-7 urn) were cut with a rotary microtome. Ribbons were placed on slides pre-coated with Meyer's albumin and spread by slight warming of the slides.

Staining Of Sections

Slides with tissue sections were placed successively in xylene (twice - 5 min each), absolute alcohol (5 min), 90% alcohol (5 min), 70% alcohol (5 min), 50% alcohol (5 min), 30% alcohol (5 min), distilled water (10-15 min), 3% iron alum (30 min), tap water (10 min), distilled water (2 min), heamatoxylene (1 hr), 1% iron alum (allowed to differentiate), running tap water (30 min), distilled water (5 min), 30% alcohol (5 min), 50% alcohol (5 min), 70% alcohol (5 min), alcoholic eosin (2 min), 90% alcohol (2-3 min), absolute alcohol (2-3 min), 1:1 alcohol and acetone (5 min), acetone (5 min), acetone and xylene (5 min), xylene (5 min), and finally mounted in DPX. Photographs of these slides were taken.

STATISTICAL ANALYSIS:

The data in this thesis are reported as Mean ± SD. Unpaired Student's 't test' **(Student,** 1908) for determining the statistical significance between the means of two groups was used **(Mattson, 1981)**. Comparisions between multiple groups were carried out using Newman-Keul's multiple range test **(Keul,** 1952).

CHAPTER 3

ANIMAL MODEL

Fulminant hepatic failure (FHF) is a clinical syndrome with a rapid onset of severe inflammatory and necrotic liver disease. Neurological disturbances under these conditions range from altered mental status to coma and finally death. These changes occur within hours or days. Frequently death results from brain herniation caused by increased intracranial pressure resulting from brain oedema.

To identify and understand the neural dysfunction in FHF, it is desirable to carry out studies in human samples. However, availability of the material would be problematic due to ethical considerations. This will be a serious restriction in carrying out temporal studies to follow the course of disease. Even if the material is available, it would be mostly post-mortem material wherein the methods employed to collect the tissue, time lapse between death and collection of material and the mode of preservation are highly variable from sample to sample. As a result, various degrees of post-mortem changes might occur in the material that may or may not influence the results. In addition, drugs used for the treatment, methods adopted in the management of disease might also affect the tissue to various degrees. These problems can be overcome by developing a suitable animal model which mimics closely the disease conditions observed in humans. Development and availability of a suitable animal model of FHF would also facilitate a better understanding of the disease process. This might also aid in designing new and rational therapies for human FHF. Development of an animal model would also help in carrying out pilot studies on the drug safety prior to trials on man.

Rationale behind selecting rat as an animal model is mat, it can be

easily bred and handled in nonnal laboratory conditions and also availability of enough samples for the biochemical and morphological characterization. Animal models of acute liver failure have been developed by earlier investigators along two lines :

(i) Excision of the liver (hepatectomy; Lee and Fisher, 1961) and
(ii) Administration of a specific hepatotoxin (Keppler *et al.,* 1968; Gazzard *et al*., 1975; Zimmerman *et al*., 1989).

The later model, where a hepatotoxin is used to induce FHF, the necrosis of liver cells proceeds over a period of time, thus decreasing amounts of functionally active liver. This helps to follow course of the disease. Carbon tetrachloride (CCl_4), acetaminophen, aflotoxin, thioacetamide (TAA), and galactosamine (GALN) have been used previously as hepatotoxins to induce hepatic failure (Blei *et al.,* 1992). There are some important differences in the action of these drugs. GALN is a direct hepatotoxin (Keppler *et al.,* 1968; Chojkier and Fierer, 1985) whereas CCl_4, acetaminophen and TAA are to be converted to toxic metabolites within the liver via the microsomal P450 system (Blei *et al.,* 1992). Consequently, the dosage of CCl_4, acetaminophen and TAA needed to induce FHF and HE varies from animal to animal, even if it is in the same species (Proctor and Chatamra, 1984). In selecting a hepatotoxin certain precautions are to be observed. to fact criteria for developing an animal model for acute liver failure have been proposed (Blei *et al*., 1992) and are as follows :

1. *A lesion with a potential for reversibility*
2. *A reproducible clinical picture*
3. *Hepatic failure as a cause of death*
4. *A large animal model*
5. *Minimum hazard to personnel*

Galactosamine fulfils this criteria because it selectively damages the liver and does not affect any other organ. The reversibility of the lesion and the type of the clinical picture obtained depends on the dose of the drug administered. Keppler and Decker (1969) and several others working with galactosamine have established the fact that this drug causes selective liver damage and this as the cause of death. They have also shown that the galactosamine induced liver injury resembles human viral hepatitis in its morphologic and functional features. As a large dose of galactosamine is required to induce hepatotoxicity even in rats, hazards to the personnel due to accidental ingestion would be minimal. Hence, in the present study GALN has been used as an agent to induce FHF in rats.

The first animal model for FHF using GALN was developed in rabbit by Blitzer *et a!.,* (1978) . An adequate neurological description of this model has been provided. Encephalopathy in GALN treated animals was characterized by gradually increasing stupor with poor righting ability, loss of head support and diminished pain response. Food and water intake decreased considerably over the first 24 hr. Schafer *et ah,* (1984) described 4 stages of encephalopathy in the rabbit model of FHF induced by GALN:

Stage I : lethargy
Stage II: hyper-reflexia, at ax i a and asterixis
Stage III: marked ataxia and stupor; head turning (Pappas *et ah,* 1984).
Stage IV: deep coma

Although the neurological stages of this model are reproducible, there are certain disadvantages. There is a variability between the time of onset of syndrome and the duration of encephalopathy from animal to animal. Traber *et ah,* (1986) reported a range of onset of neurological symptoms in

rabbit from 6-60 hr after intraperitoneal injection of GALN with a mean of 23 hr. The large size of the animal also creates certain problems - it requires large amounts of the drug (which is relatively expensive) and it is difficult to arrest post-mortem changes in brain especially when labile metabolite levels are to be determined.

The rat model, unlike the rabbit model, is not uniformly fatal. Different neurobehavioural staging systems for encephalopathy in this model have been proposed. Zeneroli, (1985) describes a two-stage system (mild, severe) based on clinical signs and changes of visual evoked potentials. Others (Dixit and Chang, 1990) divide galactosamine induced HE in rats into four grades - (1) lethargy; (2) confusion, stuporous but awake; (3) sleeping most of the time but arousable; and (4) unarousable and unresponsive to painful stimuli. Even though rat model of FHF was developed previously, complete characterization was not done.

A brief study was conducted on the dose dependency of GALN induced FHF. For this purpose three different doses were selected viz., 1.2 g, 1.6 g and 2.5 g/Kg body weight. When 1.2 g of GALN was administered intraperitoneally, the symptoms developed were very feeble and the animals survived for more than one week. There were not many changes in the liver morphology. When 1.6 g of GALN was administered, there was an inconsistency in the time course with which the symptoms were observed as well as the mortality rate of the animals. Hence, 2.5 g/Kg body weight was selected for further studies. This dose was also used by Groflin and Tholen, (1978). Adult Wistar strain of rats of either sex were given a single intraperitoneal dose of GALN-HC1 (2.5 g/Kg body weight).

Preliminary standardization experiments have shown that most of the animals injected with galactosamine (2.5 g/Kg body weight) die between 40-50 hr after the injection. Food and water intake decreased markedly during the first 12-18 hr and ceased completely after this time period. From 24 hr onwards, animals become increasingly lethargic, sleepy and inactive. Prior to death, the animals passed through phases of immobility of varying duration. Violent trembling and hyperactive phases wherein excessive and uncontrolled motor activity with frequent and sudden jumps with severe convulsive spasm were also observed. Later, these animals go into deep coma. These animals die between 40-50 hr and the mortality rate was high during this time period. As the exact time of death was different in different animals, the last time period selected in this study was 36 hr after GALN administration. At this time period, death was observed very rarely and the mortality rate was almost insignificant.

***LIVER** FUNCTION TESTS:*

Functional integrity of liver, after GALN administration, was followed by performing liver function tests at various time periods.

Plasma and liver ammonia levels were determined in normal and FHF rats. Ammonia levels in the liver were about 5 folds higher than that of plasma in normal animals. There was a sharp increase in plasma ammonia levels at different time periods after GALN treatment. By the end of 18 hr, there was a 10 fold increase in plasma ammonia levels and it remained more or less stable thereafter. Liver ammonia levels were also elevated by 8 fold at 18 hr time period and there was a decrease there-after (Table 1.1). Ammonia formed in the intestine, is usually transported to liver, where it is

converted either to urea or glutamine. After the administration of GALN, liver necrosis occurs which in turn decreases the ammonia utilizing capacity of liver and this might explain the rise in plasma ammonia level.

Liver and plasma urea levels also increased significantly in rats with FHF. There was a 10 fold increase in liver urea by 6 hr and this remained constant in the subsequent time periods. In the plasma, urea content increased by 2 folds by 18 hr after the drug treatment (Table 1.2). Increase in urea level might be attributed to hyperammonemic condition during FHF. Studies on metabolic zonation by Haussinger and bis associates indicated differentia] utilization of ammonia in penportal and pen venous hepatocytes (Haussinger *et al*., 1984) . In the former cell types, ammonia is converted to urea and these cells are enriched with complete compliment of urea cycle enzymes. In contrast, perivenous cells convert ammonia to glutamine and these cells were shown to be poor in urea cycle enzymes but are endowed with a high activity of glutamine synthetase. Elevated urea levels in FHF suggested that the periportal cells were spared while the perivenous hepatocytes might be affected in GALN toxicity. The surviving periportal cells would then convert excess ammonia to urea. Such differential effect of administered hepatotoxins on liver cells has been reported earlier. For example, CCl_4 was shown to affect only the perivenous cells but not the periportal cells (Haussinger and Gerok, 1984). Further evidence for the preferential necrotic effect of GALN on perivenous hepatocytes was obtained from the studies on hepatic glutamine synthetase (GS) activity in the liver of experimental animals. At 12 hr time period, liver GS activity was decreased by 55% and a 77% decrease by 36 hr of GALN treatment

(Table 1.3). This confirmed the above tenet on the necrosis of perivenous cells in galactosamine toxicity.

If the effect of GALN is specifically on the perivenous cells which produce glutamine then this should be reflected in plasma glutamine levels. However, earlier reports on human and animal models of FHF as well as HE indicated an elevation in plasma glutamine levels (Haussinger, 1983). This apparent anomaly might be due to the contribution of glutamine by muscle tissue. Though the activity of GS per unit weight of tissue is lower in muscle cells than in hepatocytes, the total contribution of the muscle tissue to the body weight would be much higher than any other tissue.

Liver and plasma glucose levels were observed to decrease in FHF. Liver glucose level decreased by one-third of control value within 6 hr of treatment. However, at this time period, mere was a marginal but significant increase in plasma glucose levels. Thereafter, there was a significant decrease **(-65%)** till 24 hr and it remained unaltered at this level till 36 hr (Table 1.4). The initial increase observed in plasma glucose levels under these conditions might be due to increased glycogenolysis *and/or* decreased glycogen synthesis in liver. Decreased food intake coupled with glycogen depletion and hepatic necrosis might be responsible for the fall of plasma glucose levels at subsequent time periods **(Wagle** *et al.*, 1976; Hoyumpa and Schenker, 1985).

Values obtained for protein content of liver and plasma are given in (Table 5). There was no change in the liver protein content at the end of 6 hr after the administration of GALN. Eighteen hr after the administration of

GALN, there was a 20% reduction in liver protein content. By the end of 24 hours, there was a marginal but statistically significant reduction in liver protein content and by the end of 36 hr it decreased by 40%. Plasma protein content was also decreased significantly by 36 hr of FHF and this was observed even in early periods (6 hr) of GALN toxicity. Magnitude of decrease in protein content was higher in plasma than in liver. Results similar to those reported in the present study were obtained earlier. Mukherjee (1989) observed a decrease in liver protein content, *al beit* of a smaller magnitude following the administration of GALN to the rats. Discrepancy in the magnitude of change in these studies might be due to the differences in the dose of the drug administered. Mukherjee (1989) administered a dose of 1.6 g/Kg body wt, while a dose of 2.5 g/Kg body wt was used in the present study. The decrease in liver protein might be due to a decrease in the synthesis *and or* increase in the degradation as well as leakage of tissue proteins into the blood. Evidences are available to this effect. Decreased protein synthesis in GALN toxicity might be due to synthesis of aminoglycogen in the liver tissue which precipitates the ribosomes and microsomes. This would adversely affect the liver protein synthesis. This has been repeatedly demonstrated both *in vivo* and *in vitro* conditions (Mandl *et al.,* 1979). Direct evidences are not available for the increase in the protein degradation in GALN toxicity. However, the degenerative changes observed by light microscopy (present study) and the reported presence of autophagic vacuoles in the liver of GALN treated animals are in support such a contention (Koff *et al.,* 1971).

Following the administration of GALN, there was a considerable

decrease in plasma protein content. As liver is the primary source for proteins in plasma, the decrease in the plasma protein may be attributed to the decreased protein content of the liver and also the reduction in its ability to synthesize proteins. However, while discussing the decrease in liver protein it was mentioned that leakage of proteins from degenerating liver tissue into blood as one of the causative factors. If this were true, there should have been an increase in plasma protein rather than decrease. This anomaly might be due to the release of selective, rather than all the proteins, from the liver during GALN toxicity. Similar reports on changes in the plasma protein content are available in literature. Mukherjee (1989) reported 23-35% decrease in serum protein in GALN treated animals. Further, semi-quantitative densitometric analysis of the serum proteins separated by SDS-PAGE by Mukherjee is also in agreement to the above suggestion (Mukherjee, 1989).

It is well established that proteins present in the plasma contribute to plasma osmolality to a large extent. Hence, any changes in the plasma protein content would affect plasma osmolality. In the present study, GALN administration resulted in a decreased plasma protein content. As a result, there might also be a decrease in the plasma osmolality. This would affect glomerular filtration rate in the kidney and water retention in intra- and extra- cellular compartments. Moreover, changes in plasma osmolality were also reported to affect the integrity of the blood brain barrier, which in turn influences cerebral functions. However, changes in plasma osmolality can be alleviated by making suitable alterations in the ionic compositions as well as non-ionic osmotically active compounds such as urea. The

increased plasma urea content in GALN treated rats may have such an effect.

Gamma glutamyl transpeptidase (GGTP) activity in plasma also serves as a marker of liver integrity (Goldberg, 1980). This is a membrane bound enzyme involved in the metabolism of glutathione and the transfer of γ-glutamyl moieties from glutathione to other amino acids peptides Glutathione is known to be involved in the detoxification of the drugs, in maintaining the sulphydryl groups, especially of membrane, in reduced state and in scavenging the free radicals (Rosalski, 1975). Moreover, changes in the activity of this enzyme in liver tissue is known to represent the physiological status of the tissue (Goldberg, 1980). Hence, activity levels of this enzyme were determined in the liver of normal and GALN treated rats.

Following the administration of GALN, there was a decrease in the activity of this enzyme in liver at all the time periods studied. At 6 hr time period, activity of this enzyme was decreased by 50% in liver. By the end of 18 hr of GALN treatment, its activity in liver was one-third of that in the normal animals (Table 1.6). Results of the present study, on the changes in the activity of hepatic GGTP suggests not only of hepatic damage but also of alterations in the integrity of hepatocyte membranes. Under such conditions, proteins (including enzymes) might be released selective from liver into the blood. Hence, changes in the activity of this enzyme in liver should be reflected in the plasma of GALN treated animals.

There was a significant rise in the activity of GGTP in the plasma of

experimental animals indicating that this membrane bound enzyme might be released from the liver into blood during GALN induced hepato-toxicity.

Aspartate aminotransferase (AAT) was another enzyme used as a marker for assessing hepatic damage in GALN toxicity. Administration of GALN resulted in a decrease in the activity of this enzyme in liver. The magnitude of decrease was prominent even at 6 hr time period. By the end of 36 hr, AAT activity in liver was decreased by 60% of that in the normal animals (Table 1.7). Such a decrease might be due to the extensive damage of the liver and the leakage of the enzyme from the tissue into the blood. To verify mis tenet, AAT activity was measured in the plasma following the administration of GALN. There was a significant increase in the activity of this enzyme in the plasma of the drug injected animals. There was very little increase at the initial stages of toxicity (6 and 12 hr). However, at 24 hr after the administration of the drug, activity of the enzyme increased by about 12 fold over the control value. The increase in plasma AAT paralleled more or less with the fall in the enzyme activity in liver. From these results, it appeared that the enzyme might be leaking out of the liver cells during GALN toxicity.

The enzyme, AAT, was reported to be localized both in cytosol and in mitochondria. It was shown earlier that the cytosolic and mitochondrial enzymes differ in their electrophoretic mobility and in their afGnities for substrates (Boyd, 1961; Demetriou *et al.*, 1974). From the results of the present study, it is difficult to conclude which of this two enzymes (i.e., cytosolic or mitochondrial AAT) was affected. However, presence of auto-

phagic vacuoles and increased activities of not only AAT but also of other mitochondrial enzymes such as glutamate dehydrogenase, carbomoyl phosphate synthetase I in the serum of GALN treated rats suggests extensive damage to the mitochondria (Maier and Gerok, 1984). Therefore, some of the serum AAT might be coming from degenerating mitochondria of liver.

Studies were also conducted on the changes in the activity of alanine aminotransferase (A1AT), another marker enzyme for hepatic damage. This enzyme transfers a-amino group from alanine to α-KG to form glutamate. It is believed that the alanine released by the skeletal muscle is transported to liver and pyruvate is regenerated (glucose-alanine cycle). The a-amino group of alanine will be ultimately incorporated into one of the nitrogens of urea and the pyruvate is converted to glucose (Felig, 1975; Rodwell, 1993).

Results obtained for this enzyme in GALN toxicity were more or less similar to tnose of AAT. Activity levels of A1AT decreased in liver by 24 hr of GALN treatment while it was increased in plasma (Table 1.8). However, the magnitude of change in the A1AT activity in me plasma was much higher tiian tiiat of AAT at all the time periods. As suggested earlier, it appears that this enzyme might also be released from the liver of the rats treated with GALN and the release was in much larger quantities when compared to mat of AAT.

Studies were conducted on yet another marker enzyme, sorbitol dehydrogenase (SODH), to assess liver function in normal and GALN treated rats. Activities of SODH were studied in liver and plasma. Activity of this enzyme was below the level of detection (by me method adopted in

the present study) in the plasma of normal animals. However, its activity increased in the plasma of GALN treated rats (Table 1.9). At the end of 6 hr, activity of this enzyme was detectable in the plasma and it increased by 15 fold by 12 hr and at the end of 36 hr, it increased by about 87 fold in the plasma when compared to the 6 hr sample. When the activity of the enzyme was expressed per ml of plasma, more or less a similar change was observed except that the magnitude of increase at the end of 36 hr was about 35 fold. However, changes observed in the activity of this enzyme in liver were not commensurate with changes observed in plasma. Irrespective of the mode of expression, activity of this enzyme was unaltered in the liver up to 18 hr. In the subsequent two time periods there was a marginal but statistically significant decrease in the activity of this enzyme. It is suggested that this enzyme is released into the plasma during hepatic necrosis and at the same time it might also be synthesized in the tissue, especially during reactive phase.

Results of the present study pertaining to the liver function tests indicated that GALN administration induced reactive changes and necrosis of hepatocytes. Further evidence for this was obtained from studies on the histopathological changes in liver tissue by light microscopy. This might provide more information about the nature of the damage and structural changes at the cellular level. Control animals showed normal architecture (Fig. 1.1 A). Not many structural changes were seen in liver 6 hr after GALN treatment (Fig. MB). At 12 hr time period, wide spread necrosis, inflammation and variation in the size of cells were seen and some cells were bigger in size tiian otner cells (Fig. 1.1C). Balloon cells and acido-

phillic degeneration were observed at this time period. Sinusoids were dilated with acidophillic debris. At the end of 24 hr, intracytoplasmic lipid vacuoles and fatty changes were evident in liver cells (Fig. 1.1D). Infiltration of liver with polymorphonuclear cells, mononuclear cell infiltrate and wide spread necrosis and inflammation were very common at this stage. Light microscopic studies by Decker *et al,* (1972) also revealed similar changes in the liver histology. Four hr after a single dose of GALN (1.8 mmole/Kg body wt), hepatocellular injury as evidenced by acidophillic degeneration of the cytoplasm was observed. By me end of 6 hr, necrosis of hepatocytes, enlargement of Kupffer cells were observed. At 24 to 26 hr after drug injection, hepatocytes were replaced by inflammatory infiltrates consisting chiefly of segmented leukocytes, lymphocytes and plasma cells. Hepatocytes exhibited variations in nuclear and cytoplasmic size, shape and staining quality. Balloon cells and acidophillic degeneration was also observed. Decker *et al,* (1972) reported that the liver iistology in GALN treated rats resemble to those seen in viral hepatitis. Results obtained presendy from biochemical and histological studies indicated that GALN induced hepatic necrosis resembles human viral hepatitis to a large extent.

As mentioned earlier, food and water intake by the animals was reduced in initial phases and ceased totally at later stages of GALN toxicity. It is now established beyond doubt that brain derives its energy from glucose metabolism and me brain glucose levels are affected by changes in plasma glucose levels. Glucose enters brain by a saturable transport mechanism with stereospecificity. Under normal conditions, rate of entry for glucose was reported to be 0.28-0.35 μmole/min/gm (Bachelard *et al.,*

1973). The glucose transport mechanisms of brain was observed to have a Km of 6-7 μmole/min/gm while the normal influx rate of glucose was found to be 0.76 μmole/min/gm while the net uptake was only 0.28-0.35 μ mole/ min/gm. It was, therefore, suggested that there might be a significant efflux of glucose from brain. Thus, brain cannot accumulate glucose and synthesize glycogen as in other tissues. As mentioned earlier, there was a considerable decrease in plasma glucose levels in different stages of GALN induced FHF. It was suggested that the observed decrease in plasma glucose under these conditions might be due to (i) decreased food intake in the initial phases followed by complete cessation, (ii) decreased liver glycogen levels due to the depletion of UTP levels in GALN toxicity, and (iii) decreased gluconeogenesis due to hepatic inadequacy. Irrespective of the precise mechanism involved in decreasing the plasma glucose levels, this would affect the cerebral energy metabolism. Hence, it was felt appropriate to study the effects of GALN toxicity on cerebral energy metabolism.

Levels of glycolytic and citric acid cycle intermediates in brain were determined 36 hr after GALN induced FHF (Table 1.10). There was a significant decrease in the brain glucose levels (75%) at this time period when compared to the normal animals. Levels of fructose-1,6-diphosphate, phosphoenol pyruvate and pyruvate were decreased significantly. Lactate content in the brain was not altered significantly during FHF. Citrate, malate and glutamate contents also decreased considerably while aspartate level remained more or less unaltered. a-KG level was increased. The ATP and ADP levels were also decreased during FHF.

As mentioned earlier, the animals stop feeding and drinking water almost 6-12 hr after GALN injection. Therefore, it was felt appropriate to verify whether the changes observed in brain energy metabolism during GALN treatment were simply due to starvation or it is due to acute liver failure. Hence, the levels of glucose metabolites in brain were estimated in rats starved for 36 hr (Table 1.10).

During starved conditions there was a depletion of cerebral glucose content by 50%. At this time period, there was a pronounced energy failure associated with decrease in the concentration of ATP. Profile of changes in the levels of metabolites of cerebral glucose metabolism during starvation was entirely different from that of GALN induced FHF. Hence, the alteration in brain energy metabolism observed was due to FHF and not due to glucose deprivation.

In conclusion, it is suggested mat administration of GALN to rats may be used as a model to study the process of hepatic necrosis and as a model for FHF. Development of this animal model of FHF would help in studying the mechanisms of the neuronal dysfunction during hepatic failure. This model is positively reproducible and not hazardous to personnel unlike any virus models.

TABLE 1.1

AMMONIA LEVELS IN THE PLASMA AND LIVER OF NORMAL AND GALACTOSAMINE TREATED RATS

	PLASMA	LIVER
NORMAL	0.19 ±0.03 (6)	A. 0.74 ± 0.04 (4) B. 0.98 ± 0.06 (4)
6 HRS	0.42 ± 0.05* (5)	A. 4.72 ± 0.06 (4) B. 6.29 ± 0.08 (4)
12 HRS	0.91 ±0.10* (6)	A. 4.78 ±0.13* (6) B. 6.37 ±0.18* (6)
18 HRS	1.92 ±0.09* (5)	A. 6.01 ± 0.28* (5) B. 8.01 ±0.37* (5)
24 HRS	1.89 ±0.07* (5)	A. 4.61 ±0.28* (5) B. 6.15 ±0.38* (5)
36 HRS	1.95 ±0.15* (3)	A. 3.57 ± 0.31* (3) B. 4.76 ±0.41* (3)
48 HRS	1.88 ±0.10* (6)	A. 3.37 ± 0.27* (6) B. 4.49 ± 0.36* (6)

UNITS: Plasma: mM. Liver - A: µmoles/g wet wt. B: mM. * = P<0.01. All the values are Mean ± S.D. Number in parenthesis indicates the number of experiments. While converting the values to mM liver water content was assumed to be 75%.

TABLE 1.2

UREA LEVELS IN THE PLASMA AND LIVER OF NORMAL AND GALACTOSAMINE TREATED RATS

	PLASMA	LIVER
NORMAL	3.49 ± 0.69 (8)	A. 0.21 ± 0.03 (6) B. 0.28 ± 0.04 (6)
6 HRS	2.96 ± 0.29 (5)	A. 2.36 ± 0.53* (6) B. 3.15 ±0.71* (6)
12 HRS	5.05 ±0.51* (4)	A. 1.57 ±0.14* (6) B. 2.09 ±0.18* (6)
18 HRS	6.52 ±0.39* (6)	A. 2.25 ± 0.26* (7) B. 3.00 ± 0.34* (7)
24 HRS	5.48 ± 0.46* (8)	A. 2.13 ± 0.17* (5) B. 2.85 ± 0.22* (5)
36 HRS	5.46 ± 0.79* (3)	A. 2.09 ± 0.56* (3) B. 2.79 ± 0.75* (3)
48 HRS	4.56 ± 0.32◆ (6)	A. 2.40 ±0.18* (6) B. 3.20 ±0.24* (6)

UNITS: • = P<0.01; • = P < 0.05. Rest of the legend same as in Table 1.1

TABLE 1.3

ACTIVITY LEVELS OF GLUTAMINE SYNTHETASE IN THE LIVER OF NORMAL AND GALACTOSAMINE TREATED RATS

GROUP	ACTIVITY
NORMAL	1.882 ±0.123 (5)
12 HRS	0.848 ± 0.054* (3)
24 HRS	0.775 ± 0.157*(3)
36 HRS	0.427 ± 0.099*(3)

UNITS: umoles/ mg protein/ hr. * = P<0.01. All the values are Mean ± S.D. Number in parenthesis **indicates the number of experiments**.

TABLE 1.4
GLUCOSE LEVELS IN THE PLASMA AND LIVER OF NORMAL AND GALACTOSAMINE TREATED RATS

	PLASMA	LIVER
NORMAL	7.62 ± 0.49 (5)	A. 111 ±6.84(6) B. 148 ±9.12 (6)
6 HRS	8.54 ± 0.55* (4)	A. 41.67 ± 4.16* (4) B. 55.45 ± 5.68* (4)
12 HRS	4.78 ± 0.52* (6)	A. 30.35 ± 4.59* (6) B. 40.47 ± 6.12* (6)
18HRS	4.78 ±0.61* (4)	A. 35.39 ± 2.66* (4) B. 47.19 ± 3.55* (4)
24 HRS	2.62 ±0.23* (6)	A. 34.30 ± 3.69* (5) B. 45.73 ± 4.92* (5)
36 HRS	3.14 ±0.45* (6)	A. 41.59 ±5.89* (3) B. 59.49 ± 3.09* (3)

UNITS: Legends same as in Table 1.1

TABLE 1.5
PLASMA AND LIVER PROTEIN IN NORMAL AND GALACTOSAMINE TREATED RATS

	PLASMA	LIVER
NORMAL	38.9 ± 2.4 (6)	143 ± 3.5 (3)
6HRS	33.0 ± 5.0* (5)	145 ± 7.8 (3)
12 HRS	29.0 ± 1.5* (5)	136 ± 8.7 (6)
18 HRS	27.5 ±1.7* (5)	115±8.2*(5)
24 HRS	18.9 ± 0.8* (7)	104 ± 6.2* (6)
36 HRS	ND	84 ± 1.5* (3)

UNITS: Plasma: mg/mL; Liver: mg/g wet wt. • = P<0.01. ND - Not Determined. Rest of the legends same as in Table 1.1.

TABLE 1.6

GAMMA GLUTAMYL TRANSPEPTIDASE LEVELS IN THE PLASMA AND LIVER OF NORMAL AND GALACTOSAMINE TREATED RATS

	PLASMA	LIVER
NORMAL	17.37 ±1.26 (5)	96.60 ± 6.40 (4)
6 HRS	16.21 ±0.90(6)	56.50 ±8.54* (3)
12 HRS	40.38 ± 8.0* (5)	38.67 ± 2.31* (4)
18 HRS	50.47 ± 3.07* (5)	28.52 ±2.43* (5)
24 HRS	104 ± 8.10* (6)	32.14 ±2.70* (5)

UNITS: nmoles/mg protein/hr. All the experiments were done in duplicates. * = P<0.01. All the values are Mean ± S. D. Number in parenthesis indicates the number of experiments.

TABLE 1.7

ACTIVITY LEVELS OF ASPARTATE AMINOTRANSFERASE IN PLASMA AND LIVER OF NORMAL AND GALACTOSAMINE TREATED RATS

	PLASMA	LIVER
NORMAL	0.11 ±0.02(7)	22.7 ± 4.5 (5)
6HRS	0.10 ±0.01 (5)	8.79 ± 2.02* (6)
12 HRS	0.38 ± 0.06* (4)	4.00 ± 0.60* (5)
18 HRS	0.66 ± 0.10* (6)	6.46 ±1.44* (4)
24 HRS	1.36 ±0.11* (7)	6.18 ± 0.37* (5)
36 HRS	1.15 ± 0.016* (3)	5.07 ± 0.88* (3)

UNITS: µmoles/mg protein/hr. Rest of the legends same as in Table 1.6

TABLE 1.8

ACTIVITY LEVELS OF ALANINE AMINOTRANSFERASE IN THE PLASMA AND LIVER OF NORMAL AND GALACTOSAMINE TREATED RATS

	PLASMA	LIVER
NORMAL	0.02 ± 0.006 (6)	4.13 ±0.43 (3)
6HRS	0.02 ± 0.002 (4)	1.59 ±0.23* (4)
12 HRS	0.15 ± **0.02*** (6)	3.04 ±0.58* (5)
18 HRS	0.20 ± **0.02*** (5)	1.80 ±0.04* (5)
24 HRS	**0.41** ± **0.05*** (5)	1.59 ±0.18* (5)
36 HRS	0.37 ± **0.06*** (3)	**3.24**± **0.11*** (3)

UNITS: **μmoles/mg** protein/hr. Rest of the legends same as in Table 1.6

TABLE 1.9

ACTIVITY LEVELS OF SORBITOL DEHYDROGENASE IN PLASMA AND LIVER OF NORMAL AND GALACTOSAMINE TREATED RATS

	PLASMA	LIVER
NORMAL	BLD	3.03 ± 0.52 (5)
6HRS	0.02 ± 0.002 (3)	ND
12 HRS	0.30 ± **0.04*** (4)	3.85 ± **0.62♦** (4)
18 HRS	0.67 ± 0.08* (5)	2.72 ± 0.53 (5)
24 HRS	**1.58 ± 0.11*** (5)	2.94 ± **0.15♦** (6)
36 HRS	1.76 ±0.17* (3)	**3.81 ± 0.106♦** (3)

Units: • = P<0.01; • = P<0.05. BLD - Below the level of determination, ND - Not Determined. μmoles/mg protein/hr. Rest of the legends same as in Table 1.6

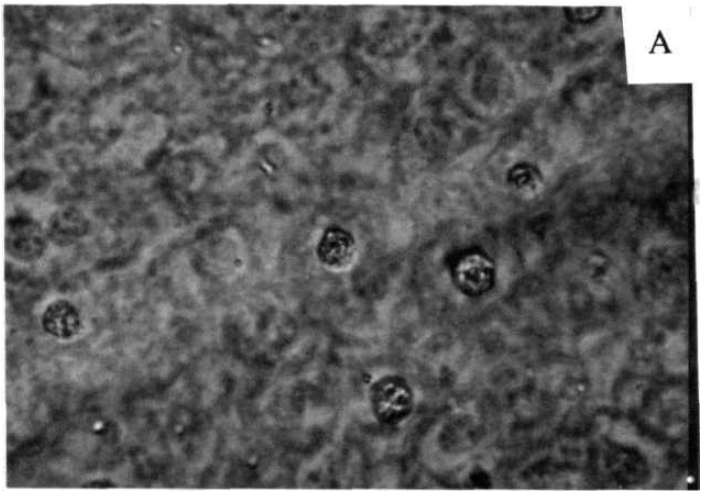
TABLE 1.10

PERCENTAGE CHANGE IN THE LEVELS OF ENERGY METABOLITES IN THE BRAIN OF NORMAL, 36 HRS GALACTOSAMINE AND 36 HRS STARVED RATS

METABOLITE	NORMAL	36 HRS GALN	STARVATION
GLUCOSE	100±5	24.70 ±0.89*	47.92 ± 4.22*
F 1,6-P2	100±8.1	60.36 ± 3.12	127 ± 35
PEP	100 ± 7.45	50.31 ± 4.4*	54.83 ± 6.71*
PYRUVATE	100 ± 18.45	39.85 ± 2.42*	71.4518.18*
LACTATE	100 ± 3.33	89.9 ± 1.98	539 ± 53.93*
CITRATE	100 ± 16.88	23.2 ± 3.54*	102 ± 24
a-KG	100 ± 23.81	185 + 38.66	8.32 ± 0.63
MALATE	100 ± 15.48	26.23 ± 6.39*	109 1 5.02*
GLUTAMATE	100 ± 14.47	59.48 ± 2.11*	97.48 1 7.64
ASPARTATE	100 ± 7.89	95.67 1 13.2	211 ± 6.02*
ATP	10018.6	88.30 ± 18	47.74 ± 5.19*
ADP	100 ± 16.88	23.20 ± 3.54*	102 ± 24*

All values are expressed as μmoles/gm wet weight tissue. * = P<0.01. Each value is Mean ± S.D of three different experiments done in duplicates. F 1,6-P2 : Fructose 1,6-diphosphate; PEP : phosphoenol phosphate

Fig. 1.1: Photomicrographs of GALN treated rat liver. (A) Normal (B) 6 hrs after GALN treatment.


A

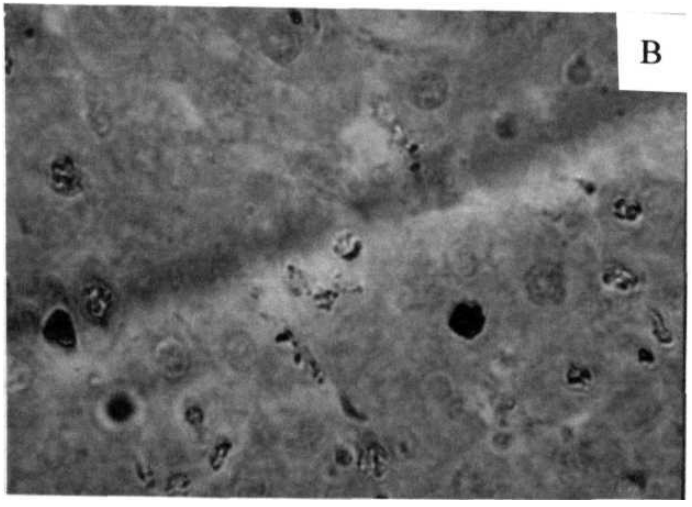
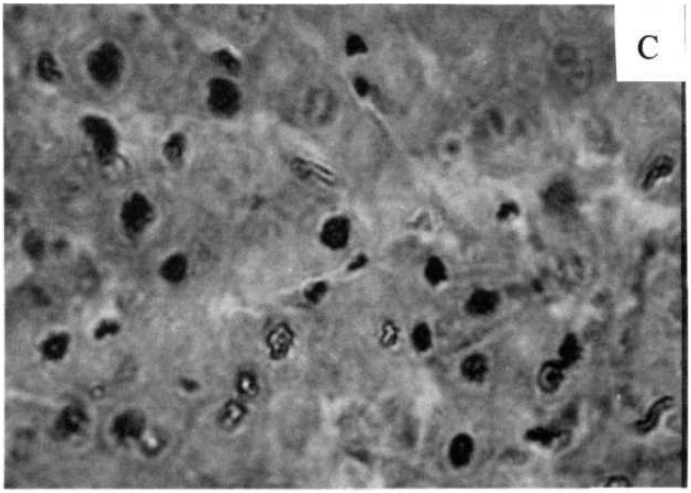
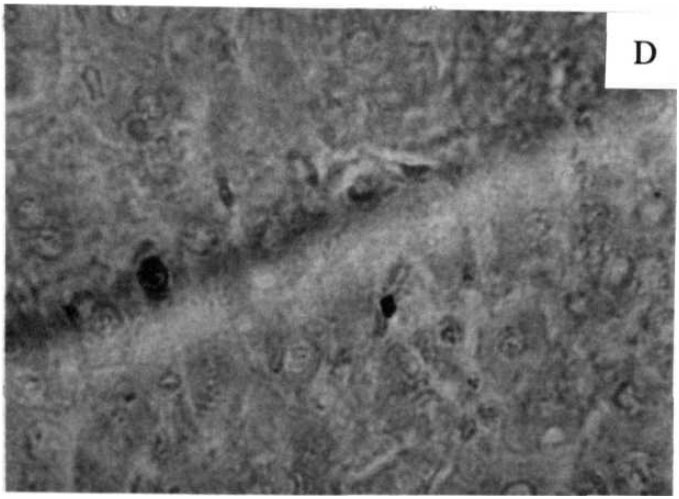

B

Fig. 1.1: Photomicrographs of GALN treated rat liver (C) 12 hrs (D) 24 hrs after GALN treatment.


C


D

CHAPTER 4

NEUROTRANSMITTER FUNCTIONS

Hepatic encephalopathy (HE) following hepatic failure occurs when overall liver function is grossly subnormal as a consequence of massive and acute necrosis of liver as seen in cirrhosis of liver, hepatitis and Reye's syndrome (Adams *et al*., 1953; Rao *et al.,* 1992). Several neurological and neuropsychiatric changes have been reported in man and several experimental animal models under these conditions (Hoyumpa and Schenker, 1985). Biochemical basis of cerebral dysfunction in FHF is still under debate and several mechanisms have been proposed as of today. As was mentioned earlier, in recent years, attention is being focused on the involvement of neurotransmitters and their receptors in the aetiology of cerebral dysfunction in FHF and other hyperammonemic disorders.

Ferenci *et al.,* (1983) reported alterations in neurotransmitter functions in hyperammonemic states. These investigators reported that the dietary glutamate is converted to GABA by the gut bacteria and is transported into portal blood. In the absence of a functional liver, to detoxify the gut derived GABA (liver has very high activity of GABA-T), it enters systemic circulation. In fact, Ferenci *et al.,* (1983) reported an elevation in the blood levels of GABA (or GABA like substances) in patients with hepatic encephalopathy. Further, they predicted that due to a selective disruption of blood brain barrier (BBB), gut derived GABA enters the brain and enhances the GABAergic transmission. They have also reported an increase in the benzodiazipine receptor activity (associated with GABA receptors) under these conditions. This was in concurrence with the earlier reports that patients with HE develop an increased sensitivity to benzodiazipine (Fowler and Schafer, 1981). Subsequently, Baraldi and Zeneroli (1982) and

Schafer and Jones (1982) reported an increase in muscimol (GABA agonist) binding and Peterson *et al.,* (1990) reported a decrease in NMDA binding in animals with portocaval shunts. Rao *et al.,* (1991) reported a decrease in glutamate binding and an increase in muscimol binding in CB of rats rendered hyperammonemic by direct injection of ammonium acetate.

Neurotransmitters are substances that are released from nerve terminals upon stimulation. They act on receptor sites and produce either excitation or inhibition of the target cell. Chemically mediated neurotransmission involves the following processes :

1. *Synthesis of the neurotransmitter at the* ***presynaptic*** *terminal*
2. *Storage of the neurotransmitter in the synoptic vesicle.*
3. *Release of the neurotransmitter into the synoptic* ***cleft.***
4. *Recognition and binding of the neurotransmitter to their receptors.*
5. *Post synoptic action of neurotransmitter.*
6. ***Inactivation*** *and termination of the action of the neurotransmitter by uptake into the pre- or* ***post-synaptic*** *neurons or into the surrounding* ***glial*** *cells.*

Presently, three aspects of neurotransmitter functions were studied in GALN induced hepatic failure. They are (1) release of glutamate from nerve terminals (2) binding of glutamate and GABA to respective receptors (representing post-synaptic action of these amino acid neurotransmitters) and (3) uptake of glutamate into terminals (representing inactivation of glutamate released). At this juncture, it must be reiterated that (i) glutamate and GABA are the predominant neurotransmitter mediating excitatory and inhibitory neurotransmission, respectively, in mammalian central nervous system (Curtis and Watkins, 1960; Curtis and Johnston, 1974), and (ii) alterations in these neurotransmitter systems have been implicated in several neurological disorders, such as Alzheimer's disease, Huntington's

chorea, epilepsy, Parkinson's disease, amyotropic lateral sclerosis, AIDS encephalopathy and dementia complex (Gasic and Hollman, 1992; Kamboj *et al.*, 1994; Dingledine and McBain, 1994; DeLorey and Olsen, 1994). Though other neurotransmitters do mediate excitatory or inhibitory neurotransmission in brain, their contribution is quite low when compared to glutamate and GABA (Ito, 1984). Therefore, it was felt appropriate to study the region specific alteration, if any, in neurotransmitter functions of glutamate and GABA in cerebral cortex (CC), cerebellum (CB) and brainstem (BS) of GALN treated rats.

RELEASE OF GLUTAMATE

Preliminary standardization on the release of glutamate was carried out with synaptosomes isolated from the cerebral cortex of normal animals. Prior to the initiation of release, synaptosomes were loaded with radioactive glutamate by incubating them for 15 min in non-depolarizing conditions (2.5 mM K^+ in medium). The total amount of glutamate loaded into synaptosomes under such conditions was 32.52 nmoles/mg protein. Results were expressed as % of glutamate released from the amount of radioactivity present in the synaptosomes after the completion of preloading.

One of the prerequisites for a substance to be called as neurotransmitter is its release from the nerve terminals when they are depolarized. Such evoked release of the neurotransmitter should, however, occur in the presence of Ca^{2+} (Blaustein, 1975). Release of glutamate from brain cortex *in vivo* after activation through neuronal pathways was first demonstrated by Jasper and Koyama (1969). Depolarization induced release of glutamate from different types of brain preparations (such as whole brain, synapto-

somes, cells in cultures etc.,) in *in vivo* and *in vitro* conditions from different species has been reported (Levi *et al.,* 1976; Naddler *et al.,* 1978; Potashner, 1978; Rao and Murthy, 1991). However, calcium dependency of glutamate release has become controversial due to the reported release of glutamate both in the presence and absence of calcium (Erecinska *et al.,* 1987; Haycock et al., 1978; Wheeler and Wise, 1983; Rao and Murthy, 1991). Nicholls and Attwell (1990) reviewed this aspect and concluded that glutamate present in the synaptic vesicles is released from the depolarized nerve terminals by exocytosis in a calcium dependent manner. This release is highly dependent on the maintenance of high energy levels and any decrease in ATP/ADP ratio inhibits this release. They further suggested that calcium independent release occurs by the reversal of high affinity uptake system for glutamate. They suggested that this glutamate belongs to non-vesicular pool which resides in the synaptic cytoplasm. Release of mis pool occurs when the energy levels of the nerve terminals are low due to the decreased ATP/ADP ratio.

Prior to describing the results, it is worth defining the term "basal release". Some investigators consider basal release of the neurotransmitter as the release that occurs under nondepolarized conditions in the absence of Ca^{2+} (Foster and Roberts, 1980; Levi *et al.,* 1982). However, in the present study, basal release is considered as the release of neurotransmitter under nondepolarised conditions in the presence of Ca^{2+}. The rationale behind such a definition is that under physiological conditions, Ca^{2+} is present in the extracellular medium whether the nervous system is depolarized or not.

In the present study, glutamate release was observed even in nondepolarizing conditions (Fig. 2.1 A). Such a basal release has been reported earlier (Drejer *et al.,* 1982; Wheeler and Wise, 1983; Rao and Murthy, 1991). Increasing the extracellular potassium concentration is known to depolarize the neurons and bring about the release of neurotransmitters (Wheeler and Wise, 1983).

A small but statistically significant increase was observed in the release of glutamate when the extracellular K^+ was increased to 50 mM. This phenomenon was observed only in the presence of Ca^{2+}. Mere depolarization (by increasing extracellular K^+) had no effect on the release of glutamate (Fig. 2.1 A). These results were in concurrence with the earlier reports on the Ca^{2+} dependent release of neurotransmitter, especially of glutamate, from different types of brain preparations such as slices, synaptosomes and cell cultures (Bosley *et al.,* 1983; Levi and Gallo, 1986; Rao and Murthy, 1991). However, the magnitude of increase observed in the present study was less when compared to that reported by others.

Alterations in the release of glutamate under depolarized and non-depolarised conditions in the presence and absence of Ca^{2+} was determined at different time periods in normal and GALN treated rats. As was mentioned earlier, mere depolarization in the absence of Ca^{2+}, (- K^+, + EGTA Vs + K^+, + EGTA), had no effect on the release of glutamate in normal animals. However, this was not the case in GALN treated animals. In these animals glutamate release was enhanced by depolarization even in the absence of Ca^{2+}. Another interesting observation was the loss of Ca^{2+}

effect in the 12 and 36 hr samples and its reappearance in 24 hr samples. In other words, there was no statistically significant difference in the release of glutamate in the presence and absence of Ca^{2+} in the former two time periods (Fig. 2.1B).

A comparison was also made between the differences in the release of glutamate at different time periods after drug treatment. Basal release of glutamate at the end of 24 hr of drug treatment was enhanced when compared to the normal, . At this time period, release of glutamate was also higher than that in the previous time period i.e., 12 hr. However, this effect was not persistent as it was normalized by the end of 36 hr. More or less a similar phenomenon was observed in the depolarization induced release in the GALN induced rats.

In summary, GALN treatment resulted in a small but statistically significant transient increase in the release of glutamate under stimulated (depolarized) and unstimulated (nondepolarized) conditions.

From the results obtained in the present study, the precise mechanism involved in the transient increase of glutamate release in GALN treated rats is not clear. However, it is pertinent at this juncture to cite the work of Erecinska *et al*, (1987) and that of Nicholls and Attwell (1990). The earlier group of investigators reported an increase in the release of glutamate in the presence of pathophysiological concentrations of ammonia. They predicted that the neuronal membrane might be depolarized under such conditions. A similar hypothesis was proposed earlier by Hawkins *et al.*, (1973) and Sadasivudu *et al.*, (1977). Under such prolonged depolarizing conditions,

an initial increase followed by a decline (due to the exhaustion of the neurotransmitter pool) in the release of glutamate might be expected. It has been shown that neurotransmitter pool of glutamate is derived predominantly from glutamine. Glutaminase, responsible for the genesis of this pool of glutamate, was reported to be inhibited by pathophysiological concentrations of ammonium ions (Bradford *et al.,* 1978; Van Den Berg *et al*., 1978; Schousboe and Hertz, 1983). This might be one of the reasons for the depletion in the neurotransmitter pools of glutamate.

As mentioned above, Nicholls and Attwell (1990) reported that a decrease in ATP/ ADP ratio would inhibit Ca^{2+} dependent release of glutamate and at the same time enhance the release of cytosolic pool of glutamate by the reversal of high affinity uptake system. As cerebral ATP/ADP levels are supposed to be decreased in the presence of pathophysiological concentrations of ammonia (Hindfelt, 1983; Pusinelli and Cooper, 1994), it is possible that the above said mechanism for the release of glutamate might become operative under hyperammonemic conditions. The results of these two investigators are pertinent to the present study as liver failure is known to be associated with an increase in cerebral ammonia levels. An increase in the release of glutamate was also reported by Moroni *et al.,* (1983); Rao and Murthy (1991); Butterworth *et al*., (1991) in hyperammonemic states.

Though the above hypothesis seems to be attractive, it is applicable only to *in vivo* release of glutamate. However, in the present study glutamate release was studied under *in vitro* conditions. In these experiments, synaptosomes (nerve terminals) isolated from normal and experimental

animals were provided with glucose and oxygen. Moreover, the endogenous ammonia content, even it was elevated in *in vivo* conditions, would have been diluted to a very great level during the preparation of synaptosomes. Hence, the above said mechanisms may not be operative under present experimental conditions It is therefore suggested that changes that have occured in the release mechanisms under *in situ* conditions have persisted the rigours of synaptosomal preparation and manifested during the assay. It is quite likely that the changes would have occured in the GALN treated rats at the level of neuronal membrane and such changes might have altered the release of glutamate. Further evidence for this tenet (i.e., altered membrane architecture) will be presented in the next chapter.

Whatever may be the mechanism, it appears that the release of neurotransmitter pool of glutamate was affected during FHF. It is not known at present whether this change is specific to glutamate or all other neurotransmitters

RECEPTOR BINDING STUDIES :

Neurotransmitter (glutamate and GABA) once released, binds to its receptors present on the pre- and post-synaptic membranes. Alteration in the binding of these neurotransmitters affects neurotransmission at the nerve terminals and neuronal pathways. For e.g. GABA receptor abnormalities have been found in Parkinson's disease and Huntington's Chorea (DeLorey and Olsen, 1994). Similarly, decreased GABA binding was found in the hippocampus and substantia nigra of Parkinson's patients (Lloyd and Dreksler, 1978). Changes in the neurotransmission mediated by excitatory amino acid have been implicated in neurological disorders, such as

Alzhiemer's disease, Huntington's chorea, epilepsy, Parkinson's disease, amyotropic lateral sclerosis, AIDS encephalopathy, dementia complex and the chronic neurodegenerative diseases (Choi, 1988; Gasic and Hallman, 1992; Kitamura *et a!.,* 1993; Dingledine and McBain, 1994).

In the present study, an attempt was made to study alteration in the binding of glutamate and GABA to their receptors during GALN induced FHF. As mentioned earlier, reports are available which indicate that the binding of glutamate and GABA to their receptors is altered during FHF. Baraldi and Zeneroli (1982) reported an increase in the number of low and high affinity GABA binding sites in membrane preparations from rats with mild GALN induced FHF and a decrease in the high affinity binding sites in the rats with severe encephalopathy. Ferenci *et at.,* (1984) reported an increase in GABA receptors and decrease in glutamate receptors in GALN induced FHF in rabbits. Peterson *et al.*, (1990) measured kainate, quisqualate and NMDA subclass of glutamate receptors in adult rat brain by quantitative receptor autoradiography in rats with portacaval anastomosis and found a decrease in the NMDA receptors in outer and inner layers of cerebral cortex, thalamus and striatum when compared to sham operated animals. Despite these reports, the information available is meagre and incomplete. Regional and temporal specificity in the response of these receptor in FHF is not known. Similarly, mechanisms responsible for these changes and their physiological significance have not been investigated. An attempt was made in the present study on these aspects.

Prior to standardization, purity of the labelled ligand was assessed. This might be altered due to deterioration of the compound (due to

inherent radiation energy) over a period of time even when stored at -20° C. In fact, it was indicated in the supplied technical information, that the rate of deterioration would be 1% per month under standard conditions of storage. In such a case, it is possible that the products formed due to deterioration might interfere with the binding of the labelled ligand. Hence, it is imperative to check this before performing the actual experiments.

Purity of the labelled glutamate was checked by paper chromatography as described in the methods section. About 60% of the applied radioactivity was recovered in an area corresponding to authentic glutamate (Rf value is 0.27). Rest of the radioactivity was distributed all along the length of the paper. Of this, about 20% of radioactivity was localized to two spots (Rf values of 0.562 and 0.72) and rest was diffusely distributed (Fig. 2.2). Areas corresponding to 60% of radioactivity of (glutamate) and 20% radioactivity (might be a contaminant) were eluted (described in methods) and binding studies were performed (Fig. 2.3A). These results were compared with the values obtained by using unpurified labelled ligand.

After normlizing the specific activities to the amount of glutamate applied, the values obtained for receptor binding with purified and unpurified glutamate were more or less same. Since the nature and content of contaminant was unknown, its binding to membranes was expressed as percentage of cpm of glutamate binding. This was observed to be ~ 5%. These results demonstrated that contaminant was not interfering with the binding of glutamate and purification of glutamate prior to performing binding studies had no significant influence either on the binding or on the values obtained. Hence, in subsequent studies labelled glutamate was used

without any purification. Due to this it is possible that the value obtained presently may be having an offset of about *5%*.

Preliminary standardization of glutamate binding was performed with the membranes from CC of normal animals. These experiments include the optimization of incubation time and the reversibility of the binding (Fig. 2.3B-2.3C). Glutamate binding to the membranes increased with time of incubation till 15 min and did not show any significant increase till 30 min. Reversibility of glutamate binding was determined by incubating the membranes for 30 min with 20 nM **[^{3}H]glutamate** followed by the exposure of these membranes to a large excess (1 mM) of unlabelled glutamate. Aliquots were taken at various time periods and filtered. These filters were washed and radioactivity was determined. Addition of unlabelled glutamate decreased the amount of label bound to the membranes indicating that binding was specific, saturable and reversible. Possibility of glutamate binding to its transport site was ruled out as binding was performed in the absence of sodium ions, which is essential for the binding of glutamate to its transport sites (Roberts, 1974). In all these studies, non-specific binding was 20% of total binding (Fig. 2.4A).

In normal animals, glutamate binding was higher in CB **than** in CC and BS (Fig. 2.4B). Extent of glutamate binding to the membranes of CC and BS in these animals was more or less identical. Such regional heterogeneity in glutamate binding to its receptor might be due to the differences in the number of receptor sites and their properties in these regions.

Glutamate binding was studied in animals treated with GALN. As the animals survived for 40-50 hr, 12, 24 and 36 hr of time period of treatment were selected for further studies. Region specific and time dependent alterations were observed in glutamate binding in the brains of GALN treated rats (Fig. 2.4C). There was a progressive increase in glutamate binding in the CC till 36 hr after GALN treatment. Peak elevation (+54%) was observed at 36 hr. A similar change but of a lesser magnitude (+33%) was observed in BS except that maximal elevation was observed earlier (at 24 hr) than in CC. There was no further increase in the glutamate binding at 36 hr in BS.

However, magnitude of elevation was not same throughout the time periods in these two regions. In CC and BS, the magnitude of elevation between 0 and 12 hr was 26% and 21%, between 12 and 24 hr it was 10% and 9% and between 24 and 36 hr it was 10% and 2%, respectively. In both these regions maximum elevation has occurred between 0 and 12 hr and thereafter the magnitude of elevation was decreased.

In contrast to the above two regions, glutamate binding in CB decreased in GALN treated rats. Though the decrease was progressive with time, maximal decrease occurred between 12 to 24 hr after GALN administration. At 36 hr, glutamate binding in CB of these rats was 19% of controls. As was observed in CC and BS, the magnitude of decrease was not uniform at all the time periods. Between 0 and 12 hr mere was a 33% decrease in glutamate binding, between 12 and 24 hr it was 56% and between 24 and 36 hr it was only 9%. In other words, maximal decrease in glutamate binding in CB occurred between 24 and 36 hr.

As mentioned , Ferenci et al., (1984) reported a decrease in glutamate binding in the whole brain membrane preparations of rabbit following the administration of GALN. Results of the present study on glutamate binding in GALN toxicity, especially of CB, are in agreement with the results of Ferenci *et al.*, (1984). No reports are available on the regional and time dependent changes in the glutamate binding in GALN toxicity.

Observed alterations in the binding in GALN toxicity might be due to changes in the affinity of the receptor to its ligand or due to a change in the number of binding sites. These changes might be due to (i) alterations in the synthesis of the number of receptor proteins or synthesis of receptor protein with altered conformation *and or* (ii) direct effect of the drug or some other toxic compounds (generated during the course of action of the drug) on the receptor protein *and or* (iii) changes in the membrane architecture affecting lipid-protein interactions. This might be particularly relevant as the receptors are integral proteins of the plasma membrane and span the entire bilayer including the hydrophobic lipid core of the plasma membrane *and'or* (iv) post-translational modifications of the existing receptor sites (such as phosphorylation/dephosphorylation) *and or* sensitization/desensitization due to exposure to ligand (Nantel and Bouvier, 1993; Nestler and Greengard, 1994). Keeping this in view, efforts were made in the present study to understand the possible mechanism involved in the region specific alterations in glutamate binding in GALN toxicity.

Kinetics of glutamate binding to its receptors was studied in the membrane preparations of CC, CB and BS of normal and GALN treated animals. In both these groups, nonlinearity of the Scatchard plots indicated

the existence of more than one binding site for glutamate - a low affinity binding site and a high affinity binding site (Fig. 2.5-2.6). By convention, high affinity binding sites were considered to be the receptor binding sites (Michaelis *et al.*, 1974). In addition to the receptor sites, glutamate also binds to the uptake sites which transports the amino acid across the membrane. However, these two sites can be distinguished from the Kd (1.87 and 17 μM for receptor and uptake sites, respectively; Tables 2.1 and 2.6), Bmax values (42 pmoles/mg protein and 283 nmoles/mg protein/hr, respectively; Tables 2.1 and 2.6) and their sodium dependency. The uptake sites, especially the high affinity uptake site, requires sodium ions for the transport while the receptor sites do not require this ion. In the present study, all the receptor binding assays were performed in the absence of sodium ions.

In normal animals, there was no statistically significant difference in the Kd of low affinity binding sites in different regions of brain (Table 2.1). Bmax of low affinity binding sites were more or less same in CC and BS. and were greater than that in CB. Unlike the low affinity sites, significant differences in the Kd and Bmax values in high affinity binding sites was observed in different regions of brain.

Kd for the high affinity binding of glutamate in the CB of normal animals was 3 folds lesser than that of BS and 5 fold lesser man that of CC. Among these two regions (CC and BS), the Kd for glutamate binding was higher in CC than in BS. More or less a similar profile was obtained with respect to Bmax values. The Bmax value in CB was 3 and 2 fold lesser man those observed in CC and BS respectively. In the later two regions, Bmax for glutamate binding was higher in CC than BS.

As the affinity of the receptor site to its ligand, is inversely related to Kd, results of the present study suggested that the affinity of the receptor to glutamate in CB was higher than in CC and BS (Table 2.1). The density of receptor sites, which is directly proportional to Bmax values, was highest in CC followed by BS and CB. This is in contrast to the values reported in Fig. 2.4C for total binding wherein the amount of glutamate bound was highest in CB than in CC and BS. This anomaly might be due to the fact that 20 nM glutamate was used in those studies. As the Kd for glutamate was lower in CB than in CC and BS, the number of sites saturated with glutamate at this concentration would be higher in CB than in CC and BS.

Significant changes were noticed in the kinetics of glutamate binding sites in the brains of GALN treated animals when compared to normal animals. Scatchard analysis of binding isotherms indicated an increase in the Bmax of binding sites for glutamate in CC and BS and a decrease in the CB of GALN treated rats. All these changes were statistically significant. Magnitude of increase in Bmax was higher in BS than in CC. A similar increase was observed in the Kd values of glutamate receptors in CC and BS of GALN treated rats, while in CB, Kd was unaltered. Changes in the Kd and Bmax of low affinity binding sites in these three regions of brain of GALN treated rats were not statistically significant.

Usually, in the receptor binding studies, changes in Kd values are considered as a measure of change in the affinity of the receptor for the ligand. This depends on the conformational state of the receptor. Binding of the modulators to the receptor protein might alter the conformational state of the receptor protein and thus the architecture of binding sites. This

would eventually affect the affinity of the receptor for its ligand. At least three such modulators have been reported for glutamate receptors. These are glycine (which increase the affinity of NMDA subtype of receptor), spermidine (which has similar action as glycine) and steroids (gonadal, adrenal). Sex differences were also observed in the action of steroids on glutamate receptors (Akinci and Johnston, 1993; 1994).

As the neurotransmitter receptors are integral part of membranes, spanning the entire bilayer, any perturbation in the membrane architecture or its physicochemical properties might affect the conformation of receptor protein and thus the affinity of the receptor for its ligand. Another parameter that affects the conformational state of the receptor might be post-translational modifications of receptor, such as phosphorylation/ dephosphorylation. Therefore, alteration in the Kd values of glutamate binding in CC and BS suggested that the receptors might have undergone some conformational change which alters its affinity to the ligand.

Bmax values are usually considered as a measure of the number of binding sites available for the ligand. This might be modulated in more than one way - the binding sites might be removed or expelled from the membrane or they might be masked either permanently or transiently or they might be sensitized/desensitized (these two changes might be due to altered architecture of the receptor). Such changes would alter the amount of the ligand bound to the receptor. In contrast, synthesis and insertion of the receptor proteins, or unmasking of the existing ones increase the number of binding sites, and such an increase would be a true increase in the number of binding sites. However, it must be mentioned about the

possibility of an apparent increase in the number of binding sites as a result of decreased affinity of the receptor to its ligand. Under such conditions, large amounts of ligand would be required for the saturation of receptor site. This might result in an apparent increase in the Bmax value (which is calculated from the amount of ligand bound to the receptor) without any corresponding change in the actual number of receptor sites. Increase in the Bmax values observed in CC and BS of the GALN treated rats might be an apparent or a true increase in the number of receptor sites.

Studies on kinetics of the glutamate binding, thus, revealed a regional heterogeneity in the response of glutamate receptors to the drug induced FHF. However, from these studies it was not possible to envisage the nature of the causative factor responsible for changes in glutamate receptors. It must be mentioned that either, the drug or some other compound (generated during the drug action) might be responsible for the observed changes in glutamate binding. However, it was reported that GALN is not permeable across the BBB and is degraded completely in the liver (Groflin and Tholen, 1978; Blei *et al,* 1992). Hence, the direct action of GALN on the glutamate receptors might be a remote possibility.

Hepatic necrosis is known to be associated with changes in the levels of ammonia, mercaptans, short chain fatty acids and amino acid (Blei *et al,* 1992; Hilgier *et al,* 1994). As mentioned earlier, ammonia is still believed to be the chief culprit for the neurological dysfunction in conditions of hepatic inadequacy (Raabe, 1990; Rao *et al.*, 1992; Mousseau and Butterworth, 1994). It was mentioned earlier (page no. 60) that plasma ammonia levels were elevated by 10 fold in GALN toxicity. It has been

reported earlier that a rapid equilibrium would be established between plasma and brain ammonia levels (Cooper *et a!.*, 1979; Cooper and Plum, 1987). Cooper *et al.*, (1979) have shown that $t_{1/2}$ for the conversion of plasma ammoma to cerebral glutamine is less than 1-3 sec.

These results suggested that changes in plasma ammonia levels would be reflected in cerebral ammonia levels. Hence, ammonia levels were measured in the brains of GALN treated rats. Though it is desirable to measure the levels of ammonia in different regions of brain, it was not possible in the present study as the brains were fixed! in liquid nitrogen. Regional dissection of brain was not possible in the brains fixed in liquid nitrogen. Efforts to thaw the brain even to -20°C resulted increased the ammonia levels in brain. Hence, ammonia content was determined in the whole brain extracts of normal and GALN treated rats.

In the normal animals, brain ammonia levels were 0.23 μmoles/g wet weight or 0.3 mM (Table 2.2) which was within the range of reported values (Benjamin, 1982; Cooper and Plum, 1987; Ratnakumari and Murray, 1990; Rao and Murthy, 1991). It is interesting to note that brain ammonia levels in normal animals were about 1.5 fold higher than those of the plasma. A similar observation was made earlier by several investigators (Ratnakumari and Murray, 1990; Rao and Murthy, 1991). This was explained to be due to the differences in the pH off brain and plasma (Warren and Nathan, 1958; Stabenau *et al*, 1959; Cooper and Plum, 1987).

Galactosamine induced hepatic necrosis increased the brain ammonia levels above the normal values. Though the magnitude of increase was

observed to be 3.4 fold (235%) over the control, it was not uniform at all the time periods. By the end of 12 hr, there was a 2 fold increase (104%) in brain ammonia levels and there was no further increase between 12 and 24 hr. However, between 24 and 36 hr there was an additional 57% increase (1.6 fold) in the brain ammonia levels. This is in contrast to the changes observed in plasma ammonia levels where a gradual increase was observed till 18 hr and no further change was noticed till the death of the animals (48 hr). Interestingly, from 12 hr onwards the plasma ammonia levels were higher than brain ammonia levels, a condition different from that of normal animals.

As the increase in the blood and brain ammonia levels and changes in glutamate binding in different regions of brain were time dependent, it was felt worthwhile to investigate the relationship, if any, between these two. The correlation coefficient between the percent change in plasma and brain ammonia levels and glutamate binding in brain was observed to be =1 in all the three regions (Fig. 2.7-2.8). This suggested a close relationship between the changes in ammonia levels in both brain and plasma and the changes (increase in CC and BS and decrease in CB) in glutamate binding.

To prove the correlation between elevated ammonia levels in blood and brain and glutamate binding, it was felt to select a drug that induces hyperammonemia without inducing hepatic necrosis. For this purpose, one more animal model of hyperammonemia was selected. Studies of glutamate binding was conducted in rats injected with ammonium acetate (non-convulsive and convulsive dose) to induce hyperammonemia within a short time span of 10 min Involvement of factors (generated due to hepatic

necrosis) other than ammonia would be minimal in this animal model. Rao and Murthy, (1991) and Rukmini and Murthy (1993) reported a 3 and 6 fold elevation in brain ammonia levels in nonconvulsive and convulsive groups of animals (using same dose), respectively.

Binding experiments in normal, non-convulsive and convulsive hyper-ammonemic group of animals were performed. In normal animals, binding was more or less similar in all the three regions of brain (Fig. 2.9A). Surprisingly, this result was found to be different from that reported earlier wherein the (Fig. 2.4B) binding was higher in CB than in CC and BS. Scrutiny of experimental protocols revealed no differences except that these experiments were performed using female rats (due to the shortage of male rats). When glutamate binding was done separately in male and female rats (Fig. 2.9B). The rank order of binding in male rats was CC = BS < CB while in female rats, it was CB = BS >CC. Sex difference in the binding of glutamate and responses of glutamate receptors to stress conditions have been reported earlier (Akinci and Johnston, 1993; 1994).

When animals were treated with non-convulsive or convulsive dose of ammonium acetate and glutamate binding performed, the profile of changes in binding was same as in the GALN treatment in all the three regions of brain (Fig. 2.9C). In CC and BS, an increase in the binding of glutamate was observed while a decrease in binding was seen in CB. Changes observed in the binding were not statistically significant in the non-convulsive group. These results suggested that the increase in ammonia levels (both in blood and brain) might be responsible for the changes observed in cerebral glutamate binding.

Increase in glutamate binding in CC and BS and decrease in binding in CB in both GALN treated rats and ammonium acetate injected rats might be due to the direct effect of pathophysiological levels of ammonia accumulated in brain under these conditions. Alternately it might be due to the ammonia induced alterations in the levels of a modulator which exerts its influence on the glutamate binding sites. As the duration of hyperammonemia was less than 10 min in ammonium acetate treated rats, the possibility of ammonia induced changes in the synthesis/insertion (into membranes) of receptor proteins might be ruled out.

To resolve this, membranes from CC, CB and BS of normal animals were pre-incubated with two different concentrations of ammonium acetate (1 & 5 mM; pH 7.0) and binding assays were performed (Fig. 2.9D). Exposure of membranes from CC and BS of normal animals to ammonium acetate increased binding while a decrease in glutamate binding was observed in cerebellar membranes. The magnitude of increase (in the CC and BS) was higher with 1 mM ammonium acetate than with 5 mM ammonium acetate whereas in CB the decrease was same at both the concentrations. These results indicated that ammonium ions, at pathophysiological concentrations, might be acting directly on the glutamate receptors. However, these results, do not rule out the possibility of ammonium ions acting on the membrane and thus affecting the receptor properties.

From observations of the present study, it may be concluded that (a) hepatic necrosis is associated with hyperammonemia and (b) hyper-

ammonemia with or without hepatic necrosis alters glutamate binding. The later observation is important as it suggested that a mere elevation in ammonia level altered the glutamate binding in brain. This is important as brain ammonia levels were reported to be elevated not only in pathological states (about 32 different diseases with or without hepatic necrosis; Cooper and Plum, 1987) but also even under physiological conditions such as conditioned reflex and learning (Tsukada, 1985). In fact brain ammonia levels are considered to be an index of neuronal activity as heightened neuronal activity elevates ammonia levels in brain (Benjamin, 1982; Tsukada, 1985). As the extracellular space is limited and ammonia leaks out of the cell, ammonia levels might reach millimolar concentrations in brain under such conditions. This suggested that ammonia might be acting as a modulator for glutamate receptors.

There are several subtypes of glutamate receptors in brain. Hence, studies on total binding fail to provide information on changes in glutamate receptor subtypes in hyperammonemic states. This information is essential as each receptor subtype exerts a different post-synaptic effect. For example, binding of glutamate to NMDA type of receptor was reported to increase the permeability of membrane to Na^+ and Ca^{2+}, stimulate phosphotidlyinositol breakdown, enhance cytosolic Ca^{2+} levels and generate nitric oxide. In contrast, KA receptor is purely an ionotropic receptor and enhances Na^+ permeability of post-synaptic membrane. Metabotropic (L-AP4) subtype of glutamate receptor promoting cGMP production have also been reported (Nawy and Jahr, 1990; Dingledine and McBain, 1994). Hence, changes in the binding of glutamate to its receptor subtypes was

studied in GALN toxicity. An attempt was made to see whether the observed increase in glutamate binding in CC and BS and the decrease in CB was global (i.e., all the subtypes are affected) or was restricted to any one particular subtype of glutamate receptor.

To resolve this, glutamate binding was studied in the presence of two agonists (NMDA and KA) of glutamate in the three different regions of brain in GALN treated animals. For this purpose, agonist specific receptor was blocked by pre-incubating membranes either with NMDA or KA and glutamate binding was performed. Differences in the specific binding in the presence and absence of an agonist (NMDA or KA) was taken as a measure of agonist specific binding.

Distribution of ligand specific receptors in the brains of normal animals varied from region to region. Glutamate binding to NMDA receptors was 2 fold higher in CB than in CC and BS in which there was no significant difference in the distribution of receptor binding sites (Fig. 2.10). This profile was observed to be similar to that of total binding of glutamate in these three regions of brain. The rank order of binding of glutamate to KA receptors was observed to be CB>BS>CC. The magnitude of difference between CB and other regions was observed to be 2 to 4 folds. In the CC of normal rats, NMDA receptor population was higher than mat of KA type. In CB and BS, these two receptors occur in equal densities. In these two regions the sum of NMDA and KA binding exceeded the total binding of glutamate. A similar observation was made by Rao *et ed.*, (1991). This suggests a possibility of cross reactivity among receptor subtypes. Actually low affinity AMPA binding sites are known to

be sensitive to kainate and thus likely to represent common sites partially shared by AMPA and kainate (Morgan *et al*., 1991).

In GALN treated rats, region specific and time dependent changes were noticed in glutamate binding to NMDA receptors. In CC, there was a progressive increase in binding of glutamate to NMDA receptors. Though such an increase was observed at the end of 12 hr, it was statistically significant only from 24 hr onwards. Maximum change in this region was observed to be 89% at the end of 36 hr (Fig. 2.11 A). Unlike CC, there was a progressive decrease in glutamate binding to NMDA receptors in the CB of GALN treated rats. In this region, maximal decrease (-90%) was observed at the end of 36 hr (Fig. 2.11B). In contrast to the above, glutamate binding to NMDA receptors in BS was decreased initially (12 hr) and increased thereafter till the normalcy was established (Fig. 2.11C).

After 12 hr of GALN treatment, glutamate binding to KA receptors in CC was similar to that of normal animals. However, this was enhanced by three folds by 24 hr and then decreased by 36 hr. At this time period, the new value obtained for KA receptor binding in CC was 2 folds higher than that of normal animals (Fig. 2.11 A). More or less a similar profile of change was seen in the glutamate binding to KA receptors in BS of GALN treated rats. However, these changes were statistically not significant (Fig. 2.11C). In the CB of these rats, there was a continuous decrease in the glutamate binding to KA receptors and at the end of 36 hr. The magnitude of this decrease was 79% of control (Fig. 2.11B).

Present results, demonstrated that glutamate binding to NMDA and

KA subtype of receptors was altered in all the three regions of the brain in GALN treated rats. The pattern of changes for NMDA and KA receptors were more or less similar to that of total binding of glutamate. Precise reason for the alteration in the binding is difficult to understand unless kinetics of NMDA and KA binding are performed. This would explain whether the changes observed in binding to receptor subtypes was due to decrease/increase in the number of binding sites or due to changes in the affinity of the receptors to their ligands.

Earlier attempts to label NMDA receptors using [^{3}H]NMDA were unsuccessful and beset with various difficulties due to the low affinity of this compound for the receptor (Foster and Fagg, 1984). However, a wide spectrum of ligands were developed for this receptor and are currently available. In this respect, [^{3}H]MK-801, an antagonist of NMDA receptor operated channel, is widely used to study the NMDA receptor (Kataoka *et al*., 1993). An attempt was made to study alteration in the binding of MK-801 to NMDA receptors during GALN induced FHF.

Regional differences in MK-801 binding in normal animals was shown in Fig. 2.12. Cerebral cortex has the highest number of binding sites compared to CB and BS. In these two regions, the intensity of binding was only one-tenth of CC. Regional profile of MK-801 binding was observed to be different from the results of the masking experiments reported earlier. Results of the masking experiments indicated greater number of NMDA sites in CB than in CC and BS while the binding of MK-801 was greater in CC than in CB.

This anomaly might be due to region specific differential expression of the genes involved in the synthesis of subunits of NMDA receptors. With the availability of an NMDA receptor mRNA, presence of an unique NMDA receptor subtype was reported to be localized specifically in CB not in forebrain (Kutsuwada *et al,* 1992; Monyer *et al,* 1992). In other words, pharmacologically distinct subtypes of NMDA receptors might be present in different regions of brain.

Although NMDA receptors have been classified as a homogeneous population of receptors, some studies indicate the existence of subtypes of the NMDA receptors (Maragos *et al,* 1988; Monaghan *et al,* 1988; Honore *et al,* 1988., McDonald *et al,* 1990). This is further supported by the observation on the regional heterogeneity in the distribution of the binding sites for MK-801 and PCP, both of which are antagonists for NMDA receptors. Lesser binding of MK-801 in CB and BS man CC might be due to a pharmacologically distinct population of NMDA receptors present in these regions. Sakurai *et al,* (1993) reported absence of MK-801 binding in CB.

At the end of 36 hr, MK-801 binding increased in CC and decreased in CB of GALN treated rats. Both the changes were statistically significant. Binding of MK-801 was unaffected in me BS of FHF rats (Fig. 2.12). Results of MK-801 binding in CC, CB and BS of GALN treated rats (after 36 hr) were in agreement with the results where unlabelled NMDA was used to block mis subtype of receptor.

Having established the specific response of NMDA receptors, experi-

ments were performed to study the kinetics of MK-801 binding (Fig. 2.13A). As the magnitude of binding in CB and BS was very less, kinetic studies were performed with the membranes of CC. These studies revealed two types (high affinity and low affinity) of binding sites for MK-801 in the membranes of CC in both control and GALN treated rats (Fig. 2.13B).

There was a significant increase in the Kd and Bmax values of both high and low affinity binding sites during GALN induced FHF (Table 2.3). These changes in Kd and Bmax values of MK-801 binding sites in FHF rats were similar to that of total glutamate binding except for low affinity binding sites. As mentioned earlier, changes in MK-801 binding in GALN treated rats might be due to a change in the number of binding sites (Bmax) *and or* in the affinity of the receptor (Kd).

Similar studies were performed with kainate in different regions of rat brain by using the specific agonist [^{3}H]kainic acid. In normal rats, KA binding was highest in CC compared to that of CB and BS. Brain stem had the lowest intensity of binding to KA subtype of receptor (Fig. 2.14). These results demonstrated a regionally distinct regulation/expression of kainate receptors.

Kinetics of KA binding was performed in the normal and 36 hr GALN treated rats. Unlike MK-801, a single type of receptor was noticed for KA (Fig. 2.15) i.e., there were no low and high affinity binding sites for KA (Fig. 2.16). Akinci and Johnston (1994) also have shown a single affinity binding site in the mouse forebrain. There was a marginal but not significant, increase in the Kd and Bmax values in the CC of GALN treated rats

to that of normal animals (Table. 2.4). This result was not similar to the previous result where this receptor was blocked with unlabelled KA and [^{3}H]glutamate binding performed. In that particular experiment mere was a 75% increase in binding in GALN treated rats at the end of 36 hr and this change was statistically significant. In the CB of the GALN treated rats, the affinity of the receptors decreased when compared to that of normal animals whereas the Bmax values remained more or less unaltered.

In the present study, a relationship was demonstrated between cerebral ammonia levels (which were enhanced in GALN toxicity) and the changes in glutamate binding. It was suggested that ammonia might be responsible for the changes observed in glutamate binding in FHF. Presently, an effort was made to verify this tenet by studying the binding of both MK-801 and KA in rats treated with convulsive dose of ammonium acetate.

This group of animals also showed the same pattern of changes in the binding of MK-801 and KA to that of FHF (Fig. 2.17A-B). In ammonium acetate treated group of animals, the receptors were exposed to a very high concentration of ammonia. As the duration of hyperammonemia was < 10 min in these rats, the probability of upregulation of receptors was ruled out. Since, both the experimental group of animals (GALN and ammonium acetate treated) showed the similar pattern of [^{3}H]MK-801 binding, it might be suggested that ammonia, particularly at pathophysiological concentrations, might exert a direct action on the membranes *and/or* on the receptor-ion channel complex.

However, one more possibility for the change in glutamate binding has

to be considered before concluding the role of ammonium ions in this process. As was mentioned earlier, the animals stop feeding from 6-12 hr after GALN treatment. Therefore, it was felt appropriate to check whether the alterations observed in glutamate binding during GALN toxicity was due to starvation stress on the animal. In other words, stress due to starvation would be a factor in addition to the trauma of hepatic necrosis in GALN treated animals. This is important as stress was shown to affect the MK-801 binding in rats (Akinici and Johnston, 1993).

The pattern of change in glutamate binding during starved conditions was shown in Fig. 2.17C. Glutamate binding profiles during starvation were entirely different from that observed in GALN and ammonium acetate treatment. In the starved animals, binding of glutamate was decreased in the CC and BS which was unlike the above two experimental conditions. In CB, binding was not altered during starvation. These results, thus ruled out starvation as a causative factor for the changes observed in glutamate binding during FHF. This result further strengthened the role of ammonia in bringing about alteration in glutamate binding during FHF.

A delicate and dynamic balance between inhibitory and excitatory neurotransmission is a prerequisite for normal functioning of brain. Results obtained in the present study indicated mat the binding of glutamate, a major excitatory neurotransmitter, was affected in brain in conditions of FHF. Hence, it was felt appropriate to know whether changes also occur in the binding of inhibitory neurotransmitters, especially of GABA. The rationale behind selecting GABA is obvious - it is the major inhibitory neurotransmitter and is widely distributed in mammalian brain. It is known

to be released from neurons in a Ca^{2+} dependent manner, when they are depolarized and binds to the pre- and post-synaptic GABA receptors which results in the suppression of neuronal activity (DeLorey and Olsen, 1994).

In normal animals, GABA binding was higher in CB than in CC and BS (Fig. 2.18A). In CC and BS, identical values were obtained for GABA binding. In GALN treated rats, a decrease was observed in GABA binding to the membranes of CC and BS. The magnitude of decrease was less in CC (ranging from 20-30%) when compared to that of BS ($\cong$ 60%). Unlike the changes observed in these two regions, GABA binding in the CB of GALN treated rats was enhanced by two folds (Fig. 2.18B).

The profile of changes observed in the GABA binding under these conditions were exactly opposite to that of glutamate. In the regions where the glutamate binding decreased, there was an increase in GABA binding and vice versa. However, changes observed in glutamate binding, were time dependent, while those in GABA binding were not time dependent and the maximal change was observed even in early periods of GALN toxicity. They persisted more or less at the same level in subsequent time periods.

Changes observed, presently, in GABA binding were in concurrence with the earlier reports in other models of liver failure and hyperammonemic states. Enhanced binding to GABA receptors was reported earlier in HE induced by GALN (Baraldi and Zeneroli, 1982; 1984; Ferenci *et al*, 1984). Rao *et al*, (1991) reported an enhanced muscimol (GABA agonist) binding in the CB of rats treated with ammonium acetate.

Kinetics of GABA binding to its receptors were studied in the mem-

brane preparations of CC and BS of normal and GALN treated animals. Nonlinearity of the Scatchard plots indicated more than one binding site for GABA (Fig. 2.19-2.20). In normal animals there was no statistically significant difference in the Kd of high and low affinity binding sites in *CC* and CB. **Bmax** value of both the affinity systems were, however, higher in CC than CB. Significant changes were observed in the kinetic parameters of GABA binding sites in the brains of GALN treated rats when compared to normal animals (Table 2.5).

Scatchard analysis of binding sites indicated a significant decrease **(41%)** in the Bmax value of the high affinity binding site in CC while an increase was observed in CB. An opposite change was observed in the Kd values in these two regions. There was a **31%** decrease in the Bmax value of low affinity binding site in CC and in CB it was unaltered. Kd for GABA binding to the low affinity system showed an increase (95%) in CC while a significant decrease (26%) was observed in CB.

When the correlation coefficient for brain ammonia levels and GABA binding was calculated it was in the range of **0.81** to 0.98 (0.98 for CC; 0.9 for CB and 0.81 for BS; Fig. 2.1). Correlation coefficient for plasma ammonia levels and GABA binding was also in the range 0.69 to 0.79 (0.79 for CC; 0.69 for CB and 0.75 for BS; Fig. 2.22). These observations demonstrated the involvement of ammonia in altered GABA binding in conditions of FHF. Further evidence for this suggestion was obtained by studying GABA binding in animals treated with a convulsive dose of ammonium acetate. In these **animals,** the pattern of changes in GABA binding was similar to those observed in GALN treated rats (Fig. 2.23A).

To see if starvation had any effect on GABA binding, animals were subjected to 36 hr starvation and GABA binding was performed. The pattern of changes in binding in starved animals were more or less similar to that of GALN and ammonium acetate treated animals but the magnitude of change was different (more in CC and less in CB and BS) in this group (Fig 2.23B). There are previous reports stating that stress leads to alterations in GABAergic systems in the brains. Schwartz *et al.,* (1987) reported an enhanced GABAergic neurotransmission during acute swim stress, handling (Biggio *et al,* 1984) and saline injections (Maddison *et al,* 1987). It thus appears that the changes in GABA binding in CC, CB and BS in GALN treated rats might be due to ammonia *and or* stress (due to starvation) to which the animal was subjected to during this period.

In conclusion, GALN treatment resulted in the reduction of GABA binding and an increase in glutamate binding in CC and BS while a reverse pattern was observed in CB. These reciprocal changes might lead to a loss of inhibitory control on the neuronal activity in CC and BS and increased inhibition of the neuronal activity in CB. Such changes might play a key role in the loss of motor control and induction of convulsions in these rats. Moreover, BS is known to be the seat of reticular activating system which and involved in the control of quantity of consciousness. This system is known to exert an inhibitory control over CC, a modulator of the quality of consciousness. Loss of inhibitory control in this region might alter the state of consciousness and thus the rapport of animal with its environment.

With the reduction in GABA binding in these two regions, there would be prolonged excitation of the neurons. This might ultimately lead to

hyperpolarization and thus suppression of neuronal activity. Similar changes induced by ammonium acetate administration indicated that ammonia might be responsible for such changes in neurotransmitter binding. This study is important because alterations in the binding of these two neurotransmitters was induced in hyperammonemic states associated with and without liver failure. This indicates that ammonia might be directly responsible for these alterations.

GLUTAMATE UPTAKE

Neurotransmitter activity of glutamate released into the synaptic cleft would be terminated by its uptake into the pre- and post-synaptic neurons and into surrounding astrocytes. There are two types of uptake mechanisms - one with a high affinity and the other with a low affinity. The high affinity uptake system (HAUS) has a low Vmax and a low Km (high affinity) for glutamate and is supposed to internalize the amino acid when its extracellular concentration is low. This system requires the presence of sodium ions for its activity (Stallcup *et al*., 1979). The low affinity uptake system (LAUS) has high Vmax and high Km (low affinity) for glutamate and is supposed to internalize the amino acid when its extracellular concentration is very high. Several lines of evidence indicate that the HAUS is primarily involved in the termination of the action of the amino acid neurotransmitters (Erecinska and Silver, 1990).

As the results of the present investigation indicated changes in the release and binding of glutamate to its receptors, it was felt worthwhile to study the alterations in the reuptake of glutamate into synaptosomes in GALN treated animals.

Preliminary standardization studies in CC revealed that glutamate uptake into synaptosomes was linear up to 200 μg of protein (Fig. 2.24A). In the subsequent experiments 100 μg protein was used. Glutamate uptake into synaptosomes was found to be linear till 10 min of incubation time (Fig. 2.24B). In all the other experiments, incubation was performed for 5 min, so that the metabolism of glutamate could be minimized. Ouabain effects indicated that sustained transport requires the presence of sodium ions (Fig. 2.24C).

Kinetics of glutamate transport confirmed the earlier observations on the presence of two affinity systems (high and low affinity uptake systems) for glutamate transport in synaptosomes (Fig. 2.25A-2.25B). The affinity of the transport (as indicated by Km) of the LAUS was found to be 21 folds higher than that of HAUS (Table 2.6). Similarly, the rate of transport (as indicated by the Vmax values) of LAUS was about 2 folds higher than that of HAUS. Thus, the LAUS acts as low affinity and high capacity system for glutamate transport, while the opposite was true for HAUS (high affinity and low capacity system).

From twelve hours after the administration of GALN, marginal changes were observed in the Km of LAUS. It increased by 16%, which was statistically not significant. However, the Vmax of LAUS decreased in GALN treated to that of normal animals. At 12 hr of GALN treatment there was a marginal but statistically not significant increase (9%) in the Vmax. However, 24 hr after GALN administration, Vmax decreased by 24% and by the end of 36 hr, it decreased by 33%.

In the case of HAUS, Km remained unaltered till the end of 24 hr but at 36 hr of GALN treatment a 35% decrease in the Km values was noticed. A decrease in the Vmax of HAUS was observed with increasing time periods of GALN treatment. The magnitude of decrease was quite high (62%) by the end of 36 hr.

The decrease in the Km of HAUS at 36 hr indicated an early saturation of the transport system (due to increased affinity) at much lower glutamate concentrations in the extracellular fluid. The corresponding decrease in the Vmax of this above system could be due to decrease in the number of transporters or due to the increased affinity. Early saturation of the transport system occurring at a lower concentration of glutamate, suggested that the amount of glutamate transported might be less under these conditions. As a result, glutamate levels in extracellular space would be elevated. Similarly, the unaltered Km values and decreased Vmax values in LAUS in the experimental animals (especially the Vmax at 24 and 36 hr) might also result in prolonged presence of glutamate in the extracellular space, especially in synaptic cleft.

The overall decrease in the Vmax and Km of HAUS might be an indication of decreased uptake of glutamate into nerve terminals during GALN treatment. The decrease in high affinity transport might be due to a poor (decreased) energy status of the cell. Previous reports have indicated that cerebral ATP/ADP levels were decreased in the absence of a functional liver and in the presence of pathophysiological concentrations of ammonia (Hindfelt, 1983; Pulsinelli and Cooper, 1994). In this condition, the HAUS might transport glutamate from the cell into the extracellular fluid, thus

resulting in the release of glutamate, which was reported to be a Ca^{2+} independent process. It must also be mentioned here that an enhanced release of glutamate in a Ca^{2+} independent fashion in GALN treated rats (12 and 36 hr) has been observed in the present study (release experiments).

Rao and Murthy, (1991) reported that the Vmax value of LAUS in synaptosomes was unaltered in CB of hyperammonemic rats or by the addition of ammonium acetate (5 mM) to the preparations of normal animals. On the other hand, they observed an elevation in the Vmax of HAUS. Moreover, the Km of the HAUS was unaltered while that of LAUS was reduced. Differences in the results of Rao and Murthy (1991) to those of present study might be due to the differences in the regions used and the animal model. In the present study, CC was used, whereas Rao and Murthy used CB

Previous studies suggested mat at least two subtypes of sodium dependent high affinity glutamate transporters exist in mammalian CNS - one is expressed in forebrain and another in cerebellum (Ferkany and Coyle, 1986; Robinson *el al.*, 1991). Norenberg *et ai,* (1985) also reported that the exposure of primary cultures of astrocytes to 2 mM ammonia for 4 days suppressed the glutamate uptake into these cells.

Studies on the release, receptor binding and uptake indicated that neurotransmission mediated by glutamate and GABA might be altered during FHF. This could be due to increased levels of ammonia in the blood and brain.

Fig. 2.1: Release of preloaded [^{3}H]glutamate from synaptosomes from the CC of (A) normal (B) different time periods of GALN treated rats. Each value is Mean + SD of 5 separate experiments done in duplicates. * — P<0.01 when compared to normal. # = P<0.05 when compared to the nondepolarized condition. (▯) Normal; (▯) 12 hrs; (▯) 24 hrs; (▯) 36 hrs after the treatment of GALN.

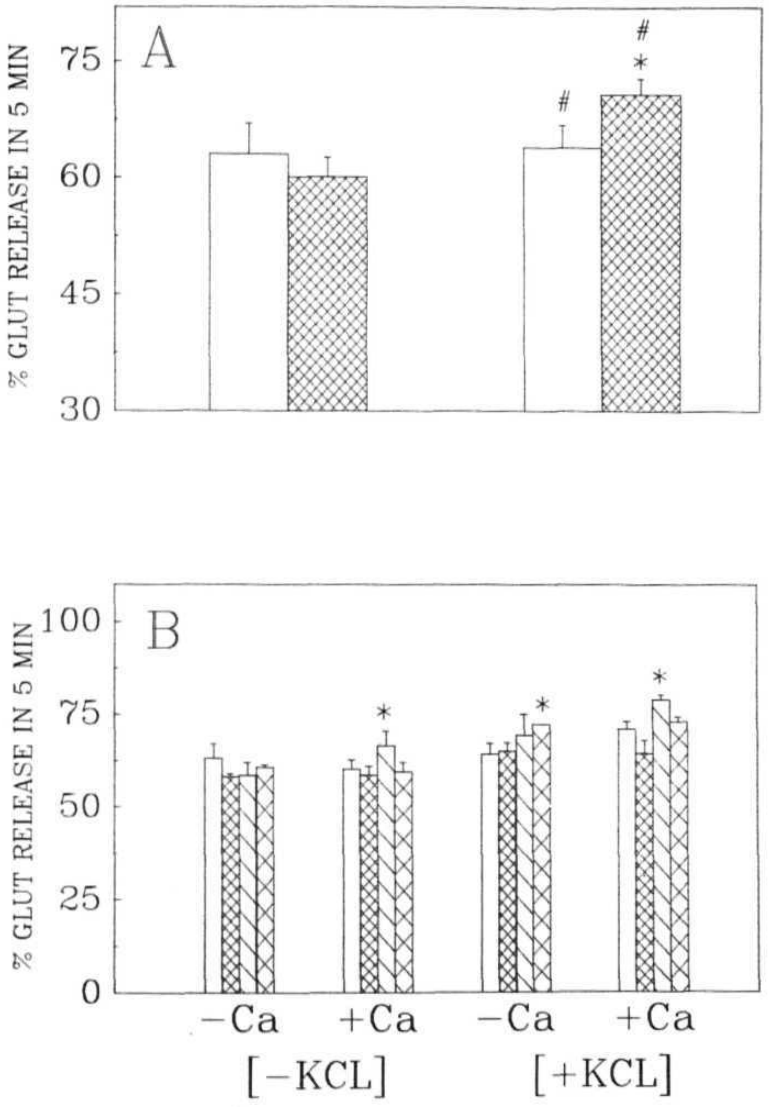

A
% GLUT RELEASE IN 5 MIN
75
60
45
30
#
*
#
B
% GLUT RELEASE IN 5 MIN
100
75
50
25
0
*
*
*
−Ca
+Ca
−Ca
+Ca
[−KCL]
[+KCL]

Fig. 2.2: Autoradiogram of [^{3}H]glutamate separated on Whatman No. 1 sheet (butanol : acetic acid : water; 65 : 25 : 15, ascending). Arrow indicates the pure glutamate spot

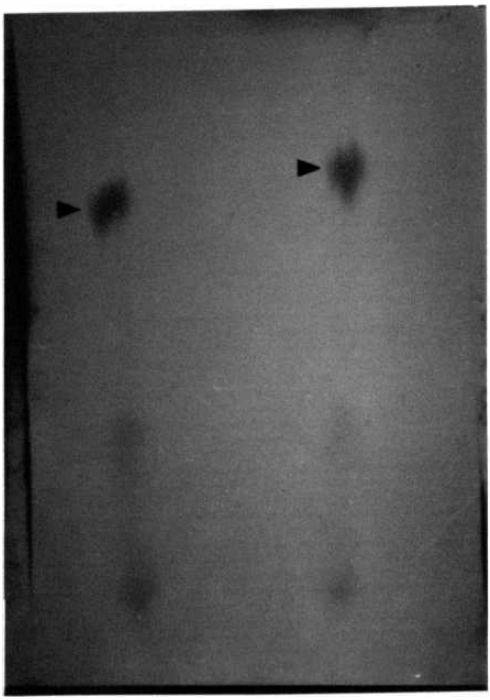


Fig. 2.3: (A) Purity of [^{3}H]glutamate separated by paper chromatography. 0 — 0 Amersham, △ — A ARC.
(B) Binding of [^{3}H]glutamate to the membranes isolated from the CC of normal rats as a function of time of incubation.
(C) Reversibility of [^{3}H]glutamate bound to the membranes isolated from the CC of normal rats. ↓ indicates the time point at which nonradioactive glutamate (1mM) was added. Each value is Mean ± SD of 3 separate experiments done in duplicates.

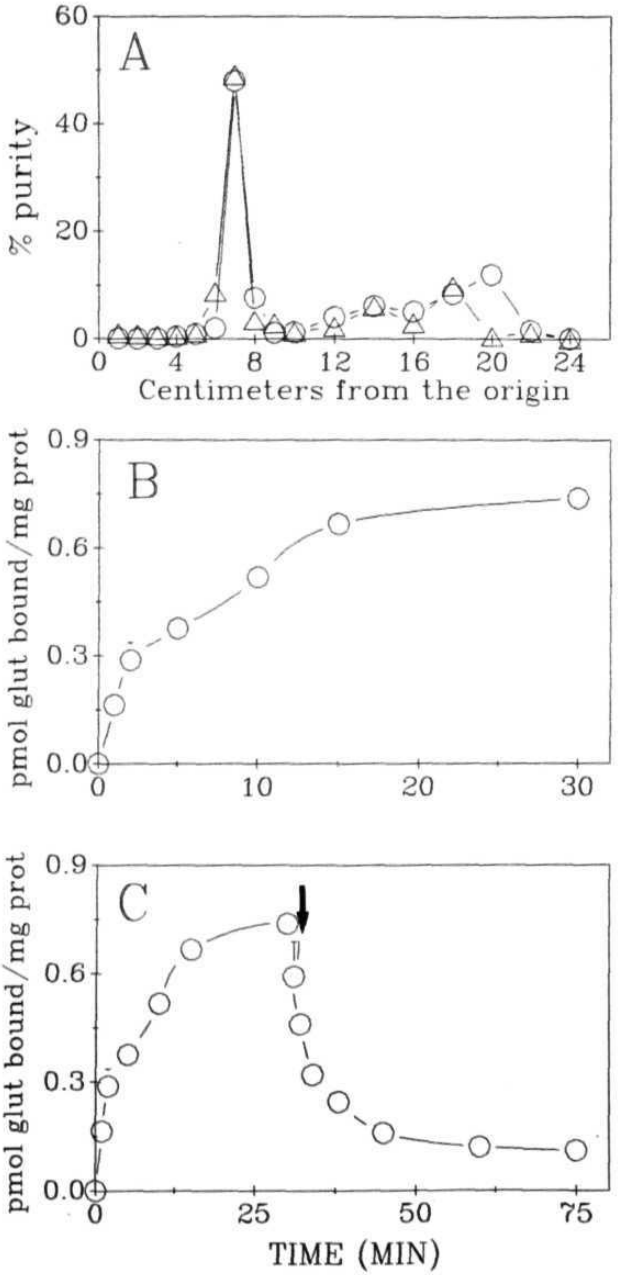

A
% purity
60
40
20
0
0
4
8
12
16
20
24
Centimeters from the origin
B
pmol glut bound/mg prot
0.9
0.6
0.3
0.0
0
10
20
30
C
pmol glut bound/mg prot
0.9
0.6
0.3
0.0
0
25
50
75
TIME (MIN)

Fig. 2.4: (A) Total (⊠), non-specific (▭) and specific (binding of [^{3}H]glutamate to the membranes from the CC of normal animals.
(B) Specific binding of [^{3}H]glutamate to the membranes of CC (⊠X CB (▭) and BS (▭) of normal rats.
(C) Specific binding of [^{3}H]glutamate to the membranes of CC, CB and BS of GALN treated rats. (▭) Normal, (⊠) 12 hrs, (▭) hrs, (▭) 36 hrs after GALN treatment. Each value is Mean ± SD of 10 separate experiments done in duplicates.

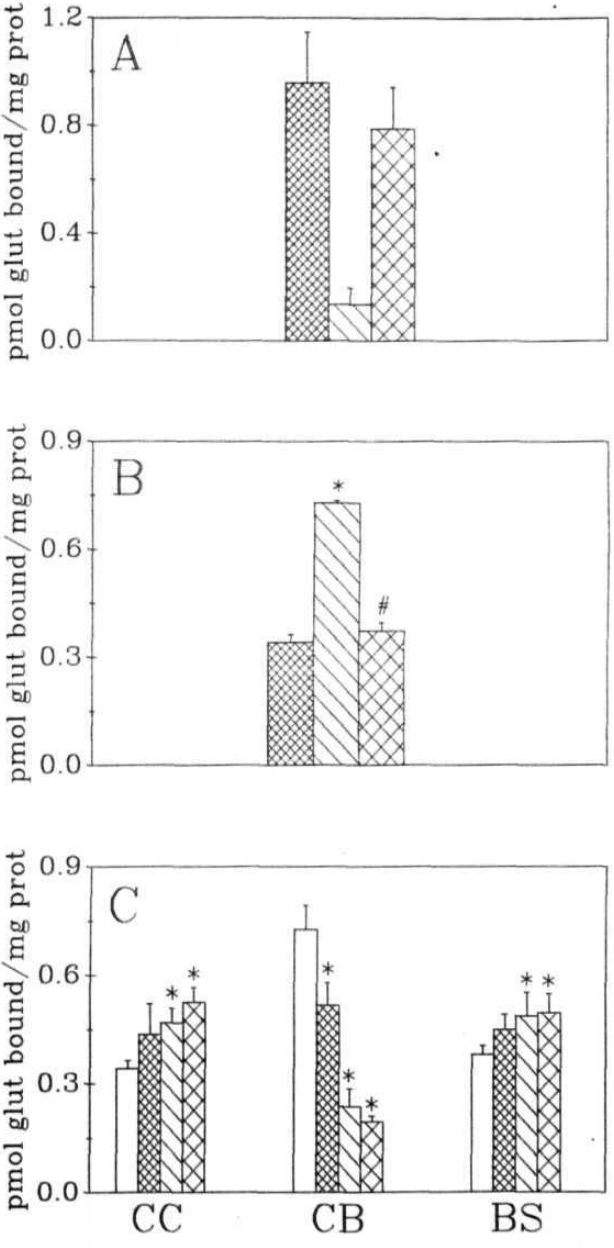
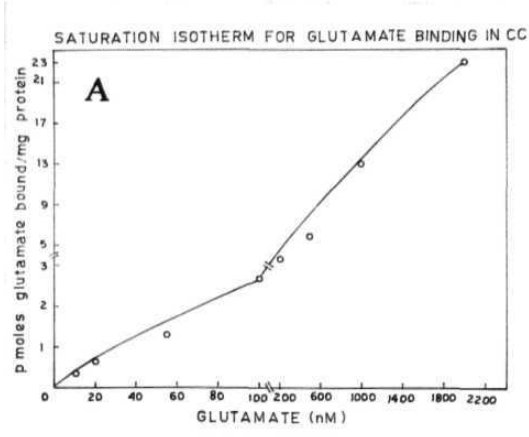
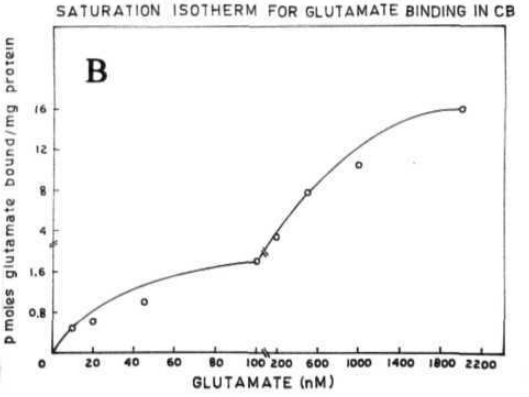

A
pmol glut bound/mg prot
1.2
0.8
0.4
0.0
B
pmol glut bound/mg prot
0.9
0.6
0.3
0.0
*
#
C
pmol glut bound/mg prot
0.9
0.6
0.3
0.0
*
CC
CB
BS

Fig. 2.5: Saturation isotherms of [^{3}H]glutamate binding to membranes isolated from the CC, CB and BS of normal animals. Each value is Mean ± SD of 4 separate experiments done in duplicates.


SATURATION ISOTHERM FOR GLUTAMATE BINDING IN CC
A
p moles glutamate bound/mg protein
23
21
17
13
9
5
3
2
1
0
20
40
60
80
100
200
600
1000
1400
1800
2200
GLUTAMATE (nM)


SATURATION ISOTHERM FOR GLUTAMATE BINDING IN CB
B
p moles glutamate bound/mg protein
16
12
8
4
1.6
0.8
0
20
40
60
80
100
200
600
1000
1400
1800
2200
GLUTAMATE (nM)

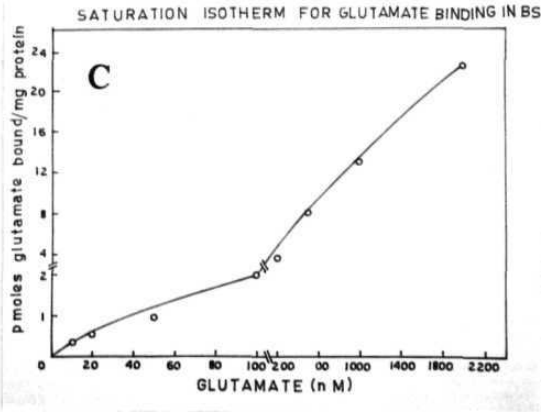

SATURATION ISOTHERM FOR GLUTAMATE BINDING IN BS
C
p moles glutamate bound/mg protein
24
20
16
12
8
4
2
1
0
20
40
60
80
100
200
00
1000
1400
1800
2200
GLUTAMATE (n M)

Fig. 2.6: Scatchard plots of [^{3}H]glutamate binding to the membranes from the CC, CB and BS of normal animals. Each value is Mean ± SD of 4 experiments done in duplicates. (○) High affinity, (●) Low affinity.

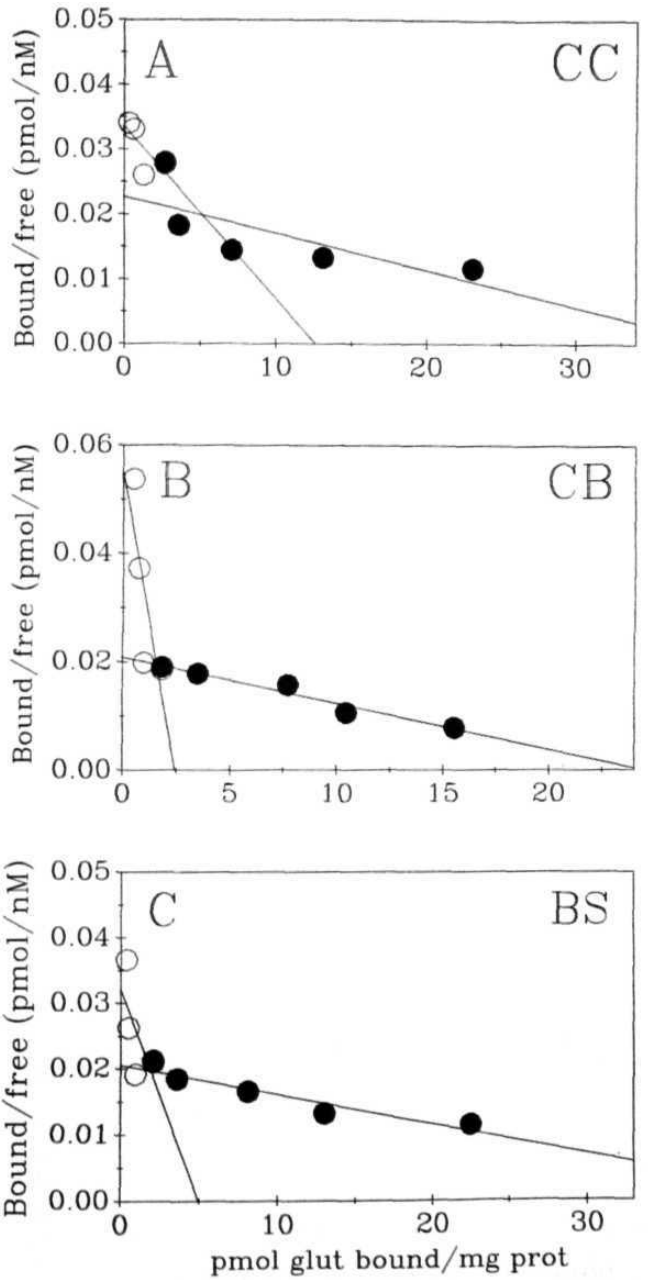

A
CC
B
CB
C
BS
Bound/free (pmol/nM)
pmol glut bound/mg prot
0.05
0.04
0.03
0.02
0.01
0.00
0.06
0
5
10
15
20
30

Fig. 2.7: Correlation coefficient between the percent changes in plasma ammonia levels and glutamate binding in CC, CB and BS of normal and different time periods of GALN treated rats.

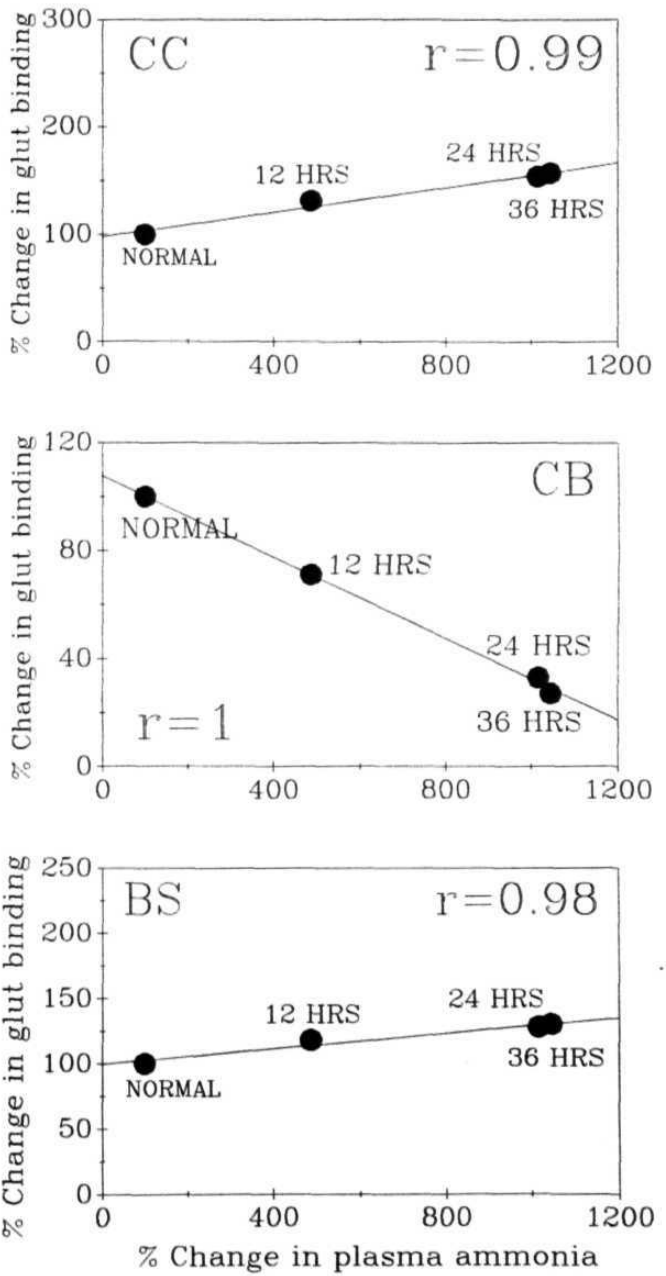

CC
r=0.99
12 HRS
24 HRS
36 HRS
NORMAL
% Change in glut binding
300
200
100
0
0
400
800
1200
CB
r=1
NORMAL
12 HRS
24 HRS
36 HRS
% Change in glut binding
120
80
40
0
0
400
800
1200
BS
r=0.98
12 HRS
24 HRS
36 HRS
NORMAL
% Change in glut binding
250
200
150
100
50
0
0
400
800
1200
% Change in plasma ammonia

Fig. 2.8: Correlation coefficient between the percent changes in brain ammonia levels and glutamate binding in CC, CB and BS of normal and different time periods of GALN treated rats.

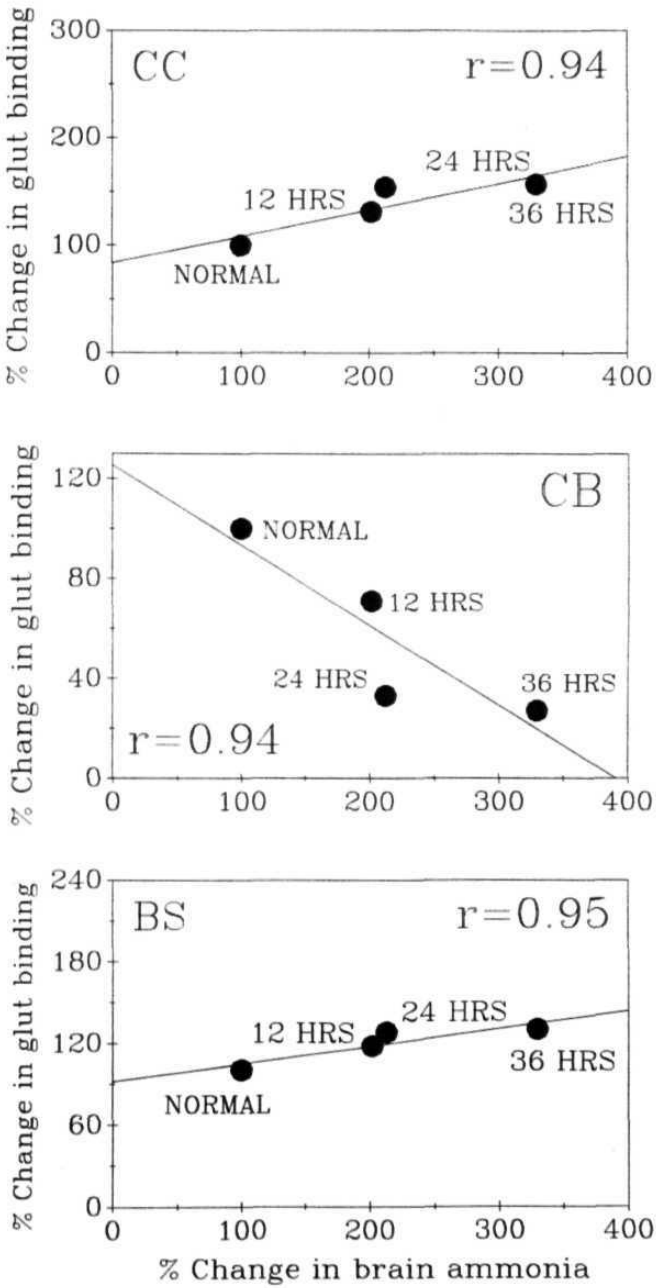
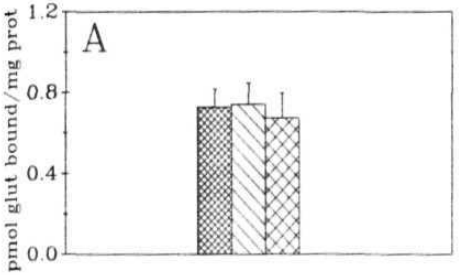
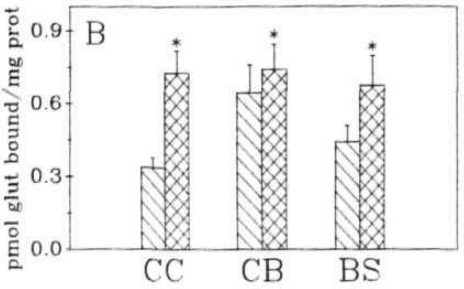
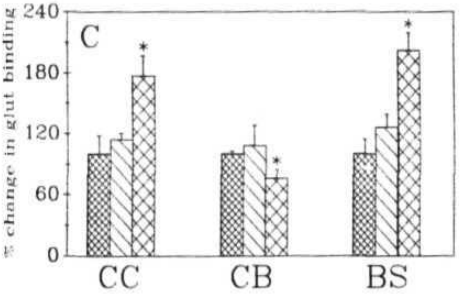

CC
r=0.94
24 HRS
12 HRS
36 HRS
NORMAL
% Change in glut binding
300
200
100
0
0
100
200
300
400
CB
r=0.94
NORMAL
12 HRS
24 HRS
36 HRS
% Change in glut binding
120
80
40
0
0
100
200
300
400
BS
r=0.95
12 HRS
24 HRS
36 HRS
NORMAL
% Change in glut binding
240
180
120
60
0
0
100
200
300
400
% Change in brain ammonia

Fig. 2.9: (A) Specific binding of [^{3}H]glutamate to the membranes from CC ( ), CB ( ) and BS ( ) of normal rats.
(B) Specific binding of [^{3}H]glutamate to the membranes from CC, CB and BS of male ( ) and female ( ) rats.
(C) Specific binding of [^{3}H] glutamate to the membranes from CC, CB and BS of normal ( ), nonconvulsive ( ) and convulsive ( ) group of animals
(D) *In vitro* effects of ammonium acetate on [^{3}H]glutamate binding to the membranes of CC, CB and BS of normal animals. Normal ( ), 1 mM ( ) and 5mM ( ) ammonium acetate. Each value is Mean ± SD of 5 separate experiments done in duplicates. * = P<0.01 when compared to normals.


A
pmol glut bound/mg prot
1.2
0.8
0.4
0.0


B
pmol glut bound/mg prot
0.9
0.6
0.3
0.0
CC
CB
BS


C
% change in glut binding
240
180
120
60
0
CC
CB
BS

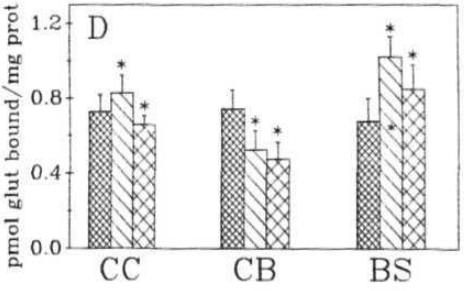

D
pmol glut bound/mg prot
1.2
0.8
0.4
0.0
CC
CB
BS

Fig. **2.10:** Specific binding of [^{3}H]glutamate to subtypes of glutamate receptors to the membranes from CC, CB and BS of normal animals. Total ( ), NMDA ( ) and KA ( ) Each value is Mean ± SD of 10 separate experiments done in duplicates.

pmol glut bound/mg prot
0.5
0.4
0.3
0.2
0.1
0.0
CC

pmol glut bound/mg prot
0.9
0.6
0.3
0.0
CB

pmol glut bound/mg prot
0.5
0.4
0.3
0.2
0.1
0.0
BS

Fig. 2.11: Specific binding of [^{3}H]glutamate to total, NMDA and KA glutamate receptors to the membranes of (A) CC, (B) CB and (C) BS of normal and GALN treated animals. (▭) Normal, (▨) 12 hrs, (▧) 24 hrs, (▩) 36 hrs of GALN treatment. Each value is Mean ± SD of 5 separate experiments done in duplicates. • = P<0.01 when compared to normals.

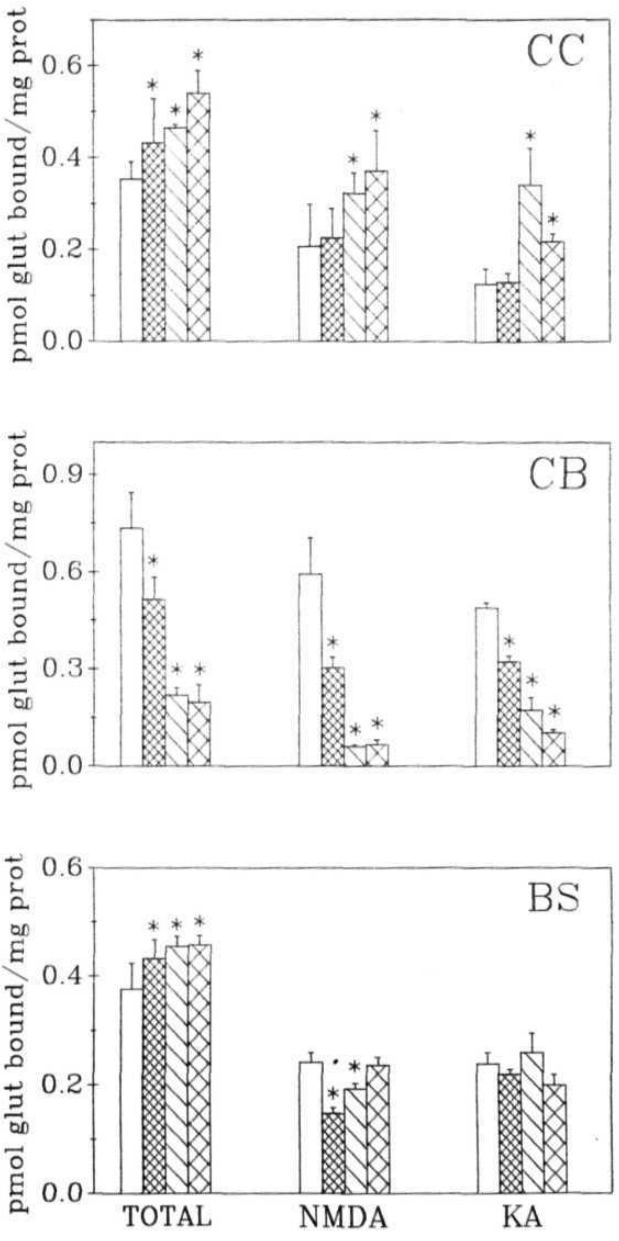

CC
CB
BS
pmol glut bound/mg prot
0.0
0.2
0.4
0.6
0.9
0.3
TOTAL
NMDA
KA

Fig. 2.12: Specific binding of [^{3}H]MK-801 to the membranes from the CC, CB and BS of normal and GALN treated rats. (▨) Normal, (▨) 36 hrs of GALN treatment. Each value is Mean ± SD of 3 separate experiments done in duplicates. • = $P<0.01$ when compared to normals.

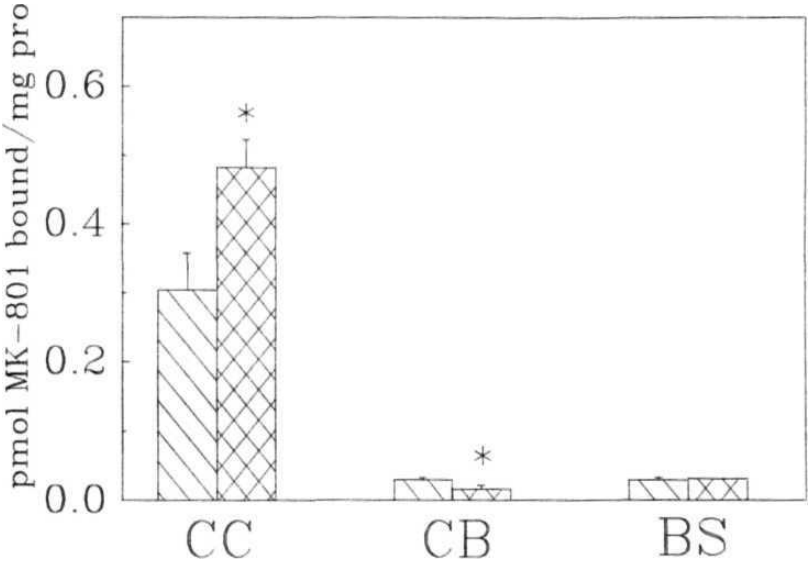
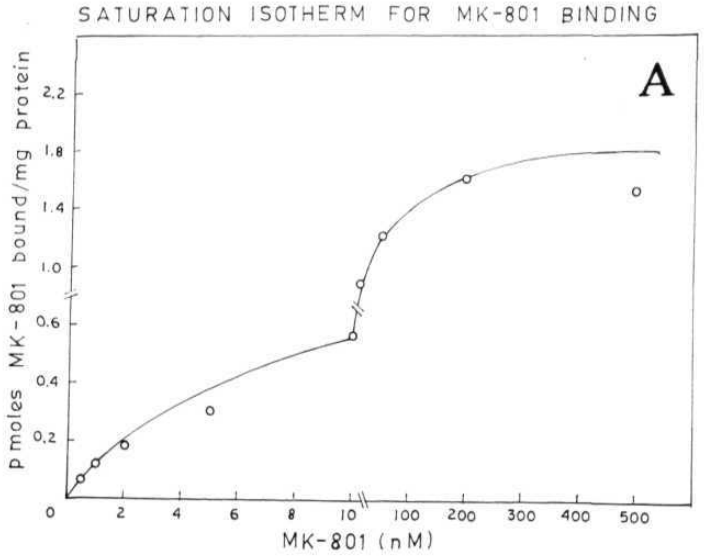

pmol MK-801 bound/mg pro
0.6
0.4
0.2
0.0
*
*
CC
CB
BS

Fig. 2.13: (A) Saturation isotherm of [^{3}H]MK-801 binding to themembranes isolated from the CC of normal animals.
(B) Scatchard plots of [^{3}H]MK-801 binding to the membranes isolated from the CC of normal animals. (○) High affinity, (●) Low affinity. Each value is Mean ± SD of 3 separate experiments done in duplicates.


SATURATION ISOTHERM FOR MK-801 BINDING
A
pmoles MK-801 bound/mg protein
2.2
1.8
1.4
1.0
0.6
0.4
0.2
0
2
4
6
8
10
100
200
300
400
500
MK-801 (nM)

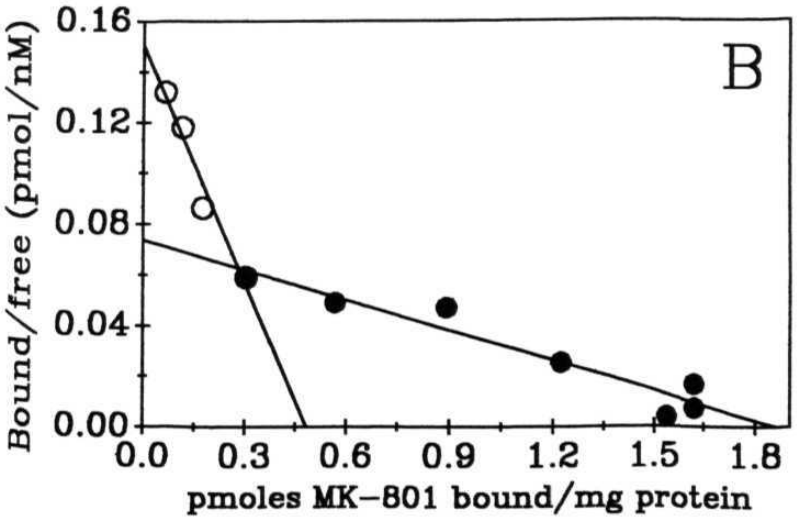

B
Bound/free (pmol/nM)
0.16
0.12
0.08
0.04
0.00
0.0
0.3
0.6
0.9
1.2
1.5
1.8
pmoles MK-801 bound/mg protein

Fig. 2.14: Specific binding of [^{3}H]KA to the membranes from the CC and CB of normal (☐) and 36 hrs GALN (☒) treated rats. Each value is Mean ± SD of 3 separate experiments done in duplicates.

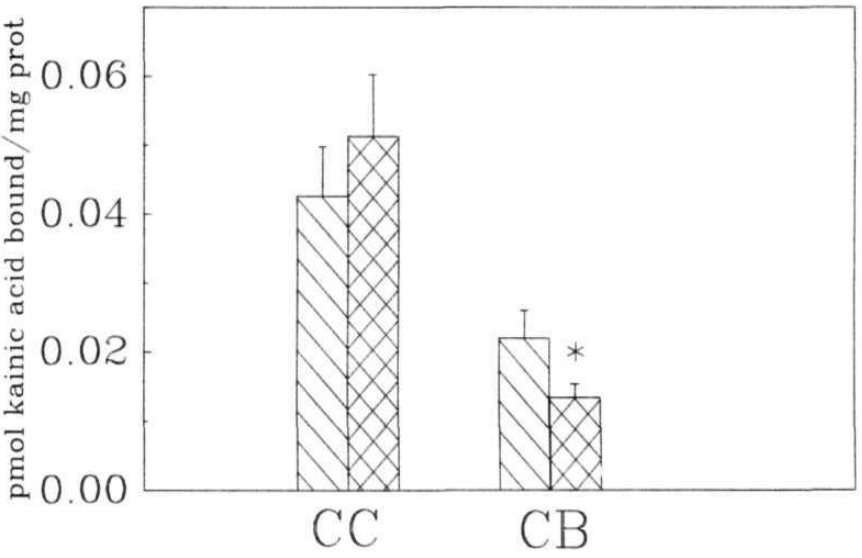

pmol kainic acid bound/mg prot
0.06
0.04
0.02
0.00
*
CC
CB

Fig. 2.15: Saturation isotherm of [^{3}H]KA binding to the membranes from the CC and CB of normal animals. Each value is Mean ± SD of 3 separate experiments done in duplicates.

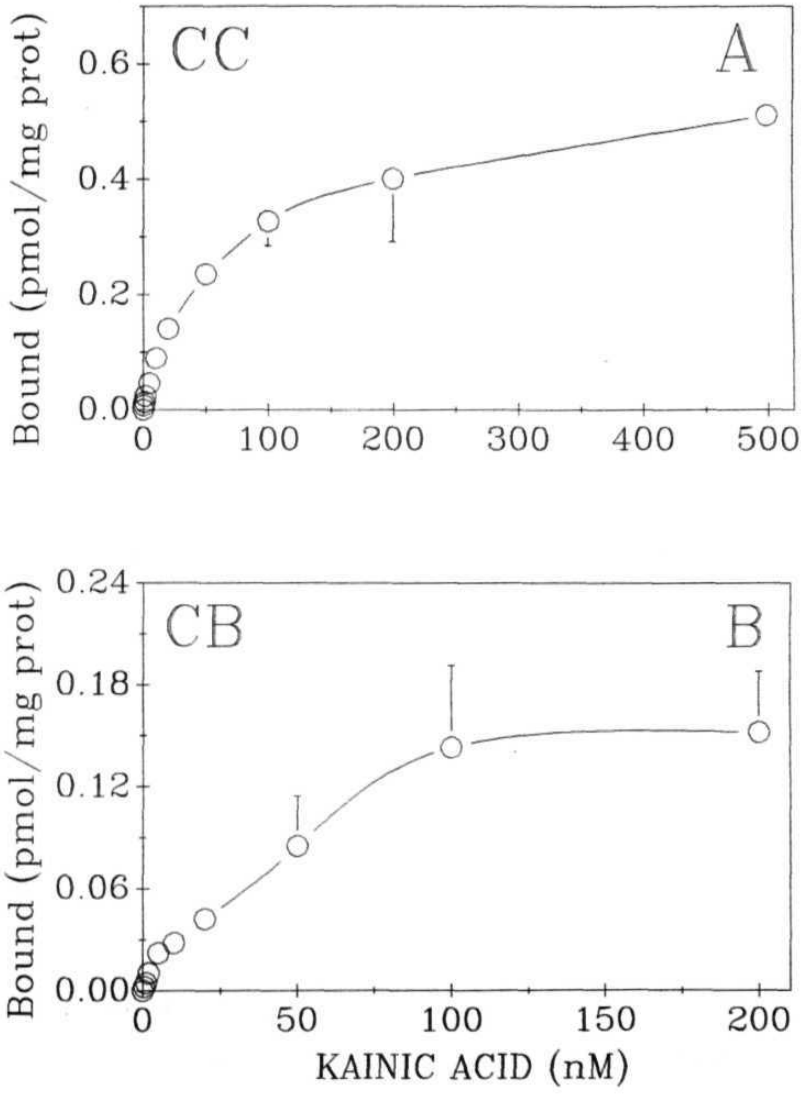

CC
A
Bound (pmol/mg prot)
0.6
0.4
0.2
0.0
0
100
200
300
400
500
CB
B
Bound (pmol/mg prot)
0.24
0.18
0.12
0.06
0.00
0
50
100
150
200
KAINIC ACID (nM)

Fig. 2.16: Scatchard plots of [^{3}H]KA binding to membranes from the CC and CB of normal animals. Each value is Mean ± SD of 3 separate experiments done in duplicates.

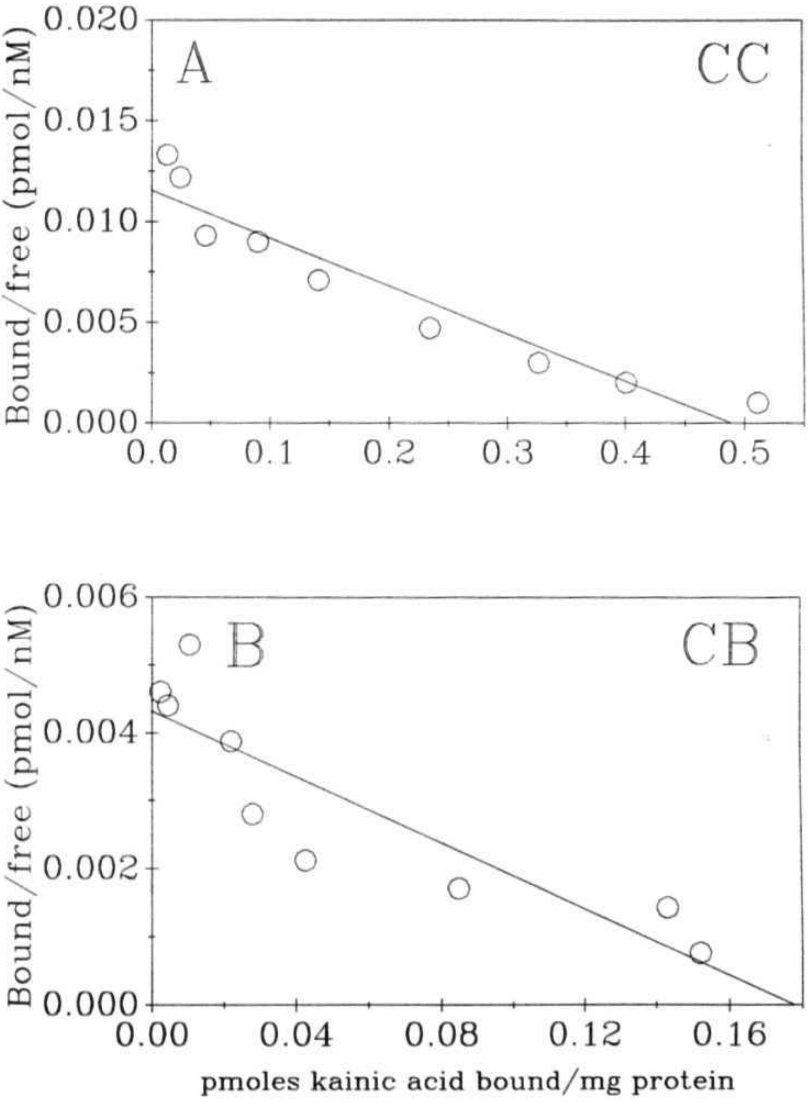

A
CC
Bound/free (pmol/nM)
0.020
0.015
0.010
0.005
0.000
0.0
0.1
0.2
0.3
0.4
0.5
B
CB
Bound/free (pmol/nM)
0.006
0.004
0.002
0.000
0.00
0.04
0.08
0.12
0.16
pmoles kainic acid bound/mg protein

Fig. 2.17: (A) Specific binding of [^{3}H]MK-801 to the membranes from the CC, CB and BS of normal (▭) and convulsive dose (▨) of ammonium acetate treated rats.

(B) Specific binding of [^{3}H]KA to the membranes of CC, CB and BS of normal (▭) and convulsive dose (▭) of ammonium acetate treated rats. Each value is Mean ± SD of 3 separate experiments done in duplicates. • = P<0.02 when compared to normal values.

(C) Specific binding of [^{3}H]glutamate to the membranes from the CC, CB and BS of normal (▭) and 36 hrs starved (▭) rats Each value is Mean ± SD of 5 separate experiments done in duplicates. • = P<0.01 when compared to normal animals.

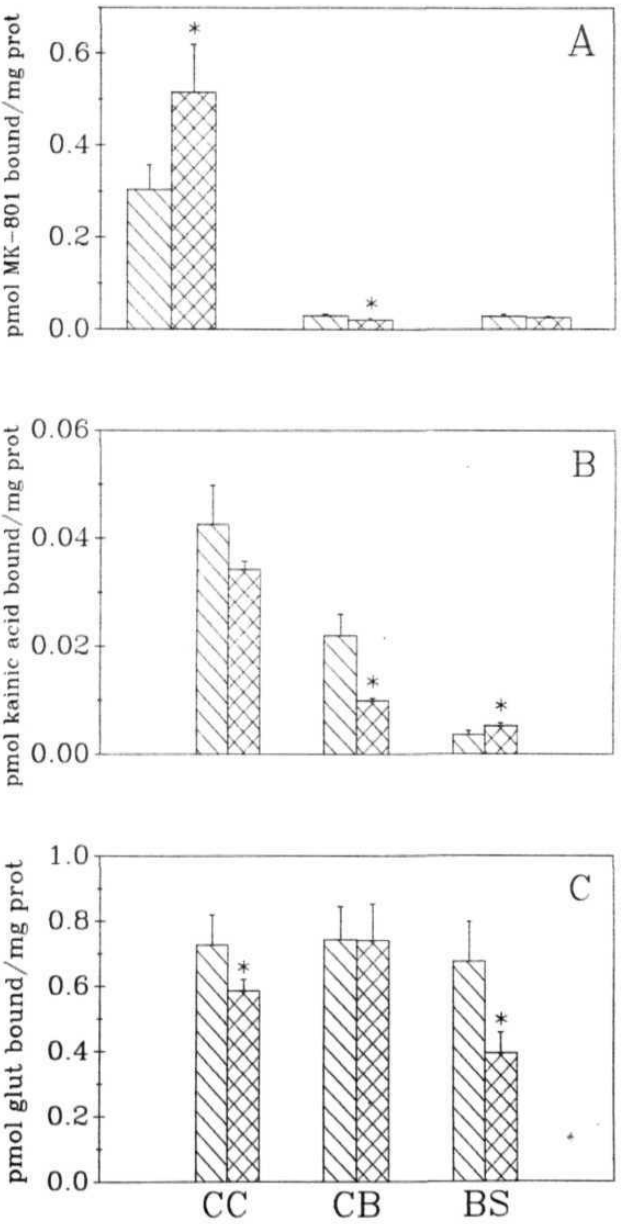

A
pmol MK-801 bound/mg prot
0.0
0.2
0.4
0.6
*
*
B
pmol kainic acid bound/mg prot
0.00
0.02
0.04
0.06
*
*
C
pmol glut bound/mg prot
0.0
0.2
0.4
0.6
0.8
1.0
*
*
CC
CB
BS

Fig. 2.18: (A) Specific binding of [3H]GABA to the membranes from the ( ) CC, ( ) CB, ( ) BS of normal animals (B) Specific binding of [3H]GABA to membranes isolated from the CC, CB and BS of GALN treated rats. ( ) Normal, ( ) 12 hrs, ( ) hrs, ( ) 36 hrs of GALN treatment. Each value is Mean ± SD of 5 separate experiments done in duplicates. • = P<0.01 when compared to normals.

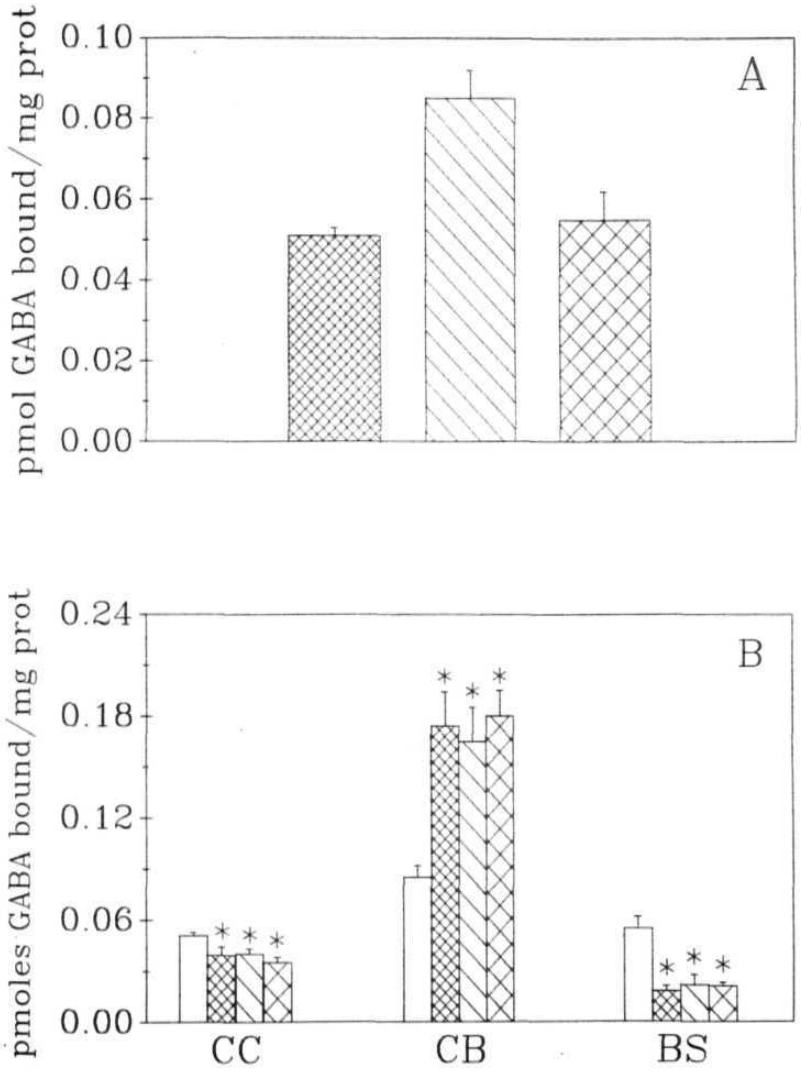
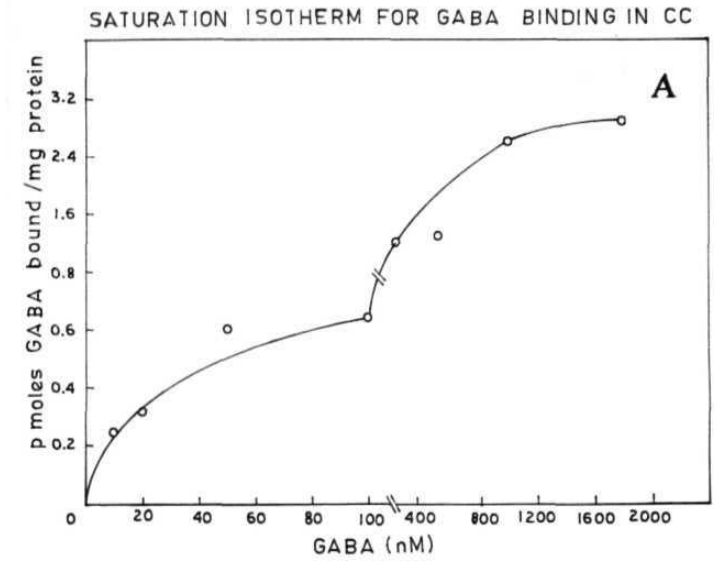

pmol GABA bound/mg prot
0.10
0.08
0.06
0.04
0.02
0.00
A
pmoles GABA bound/mg prot
0.24
0.18
0.12
0.06
0.00
B
CC
CB
BS

Fig. 2.19: Saturation isotherms of [^{3}H]GABA binding to membranes isolated from the CC and CB of normal rats. Each value is Mean ± SD of 4 separate experiments done in duplicates.


SATURATION ISOTHERM FOR GABA BINDING IN CC
A
p moles GABA bound /mg protein
3.2
2.4
1.6
0.8
0.6
0.4
0.2
0
20
40
60
80
100
400
800
1200
1600
2000
GABA (nM)

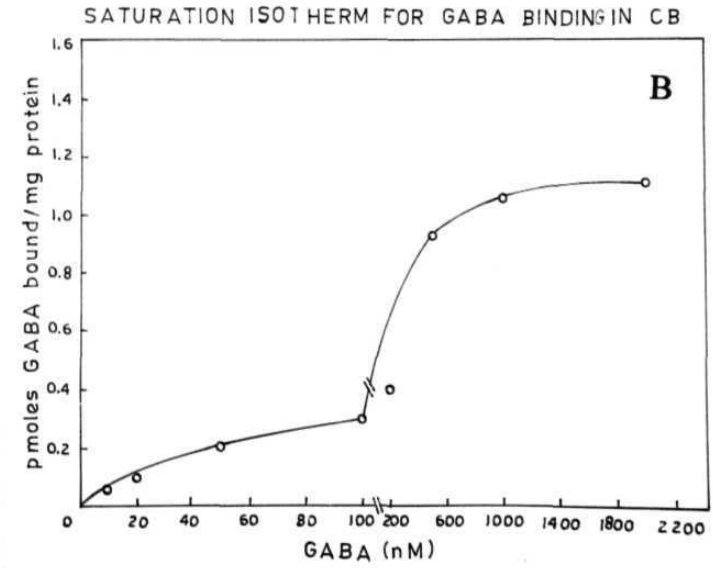

SATURATION ISOTHERM FOR GABA BINDING IN CB
B
pmoles GABA bound/mg protein
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
20
40
60
80
100
200
600
1000
1400
1800
2200
GABA (nM)

Fig. 2.20: Scatchard plots of [^{3}H]GABA binding to membranes from the CC and CB of normal animals. Each value is Mean ± SD of 4 separate experiments done in duplicates.

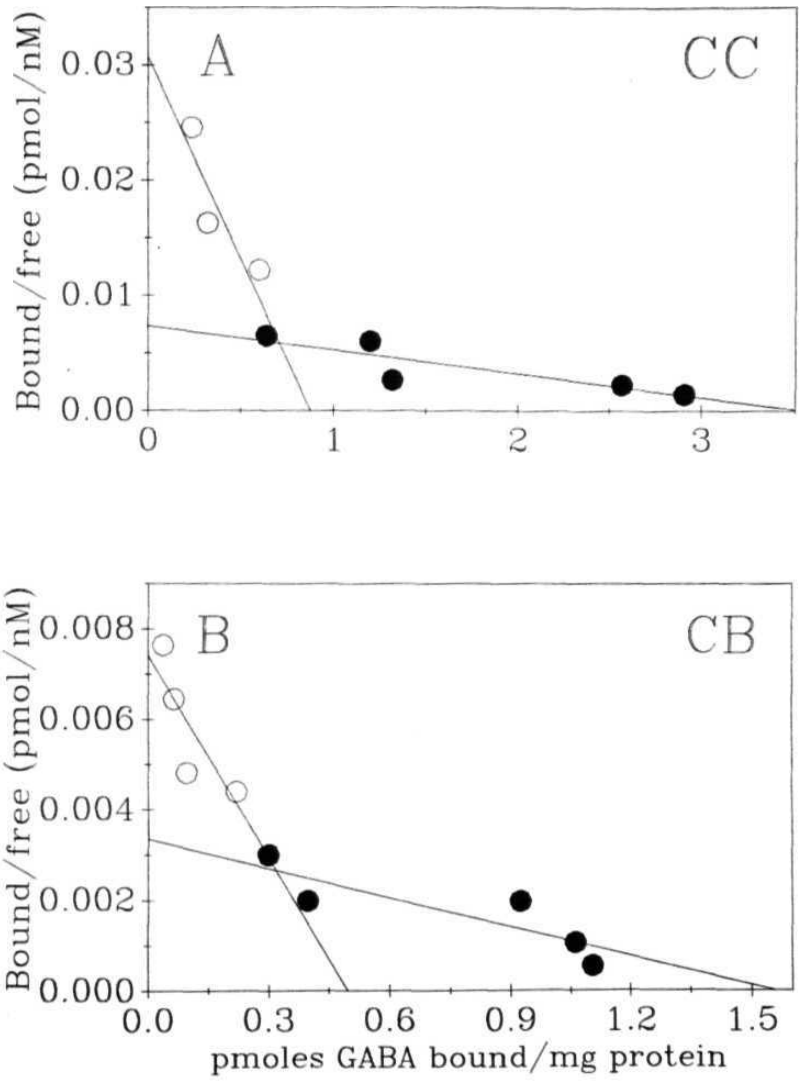

A
CC
Bound/free (pmol/nM)
0.03
0.02
0.01
0.00
0
1
2
3
B
CB
Bound/free (pmol/nM)
0.008
0.006
0.004
0.002
0.000
0.0
0.3
0.6
0.9
1.2
1.5
pmoles GABA bound/mg protein

Fig. 2.21: Correlation coefficient between percent changes in brain ammonia levels and GABA binding in CC, CB and BS of normal and different time periods of GALN treated rats.

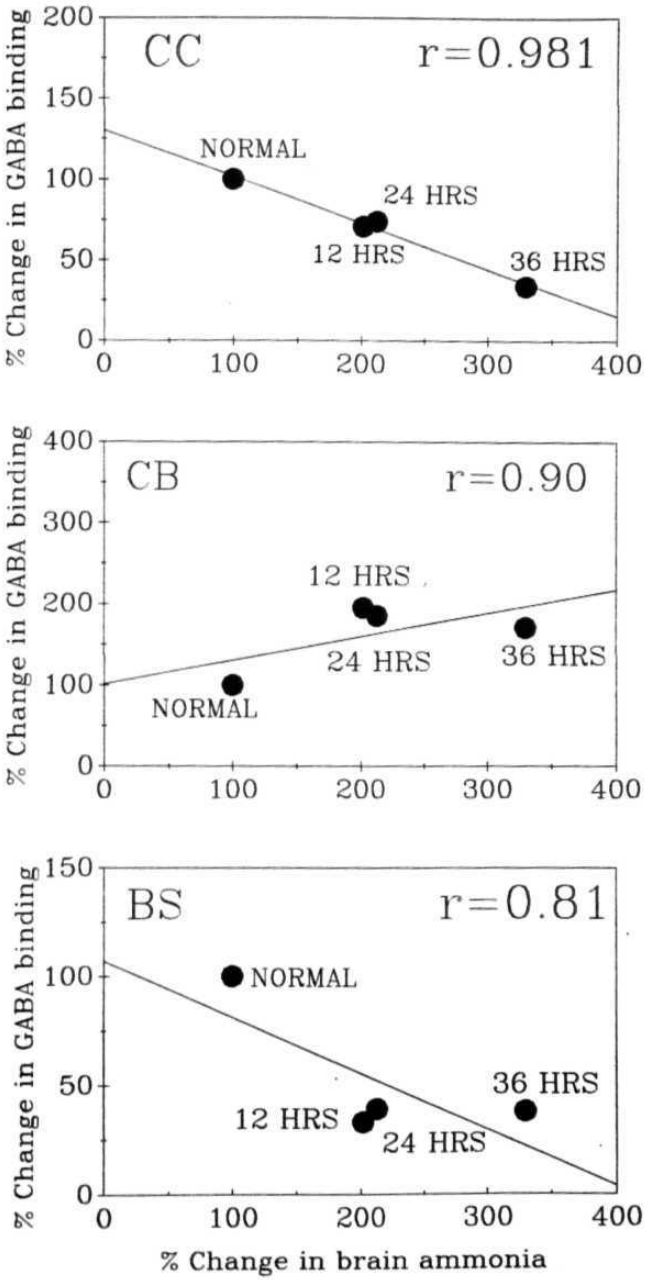

CC
r=0.981
NORMAL
24 HRS
12 HRS
36 HRS
% Change in GABA binding
CB
r=0.90
12 HRS
24 HRS
36 HRS
NORMAL
% Change in GABA binding
BS
r=0.81
NORMAL
36 HRS
12 HRS
24 HRS
% Change in GABA binding
% Change in brain ammonia

Fig. 2.22: Correlation coefficient between percent changes in plasma ammonia levels and GABA binding in CC, CB and BS of normal and different time periods of GALN treated rats.

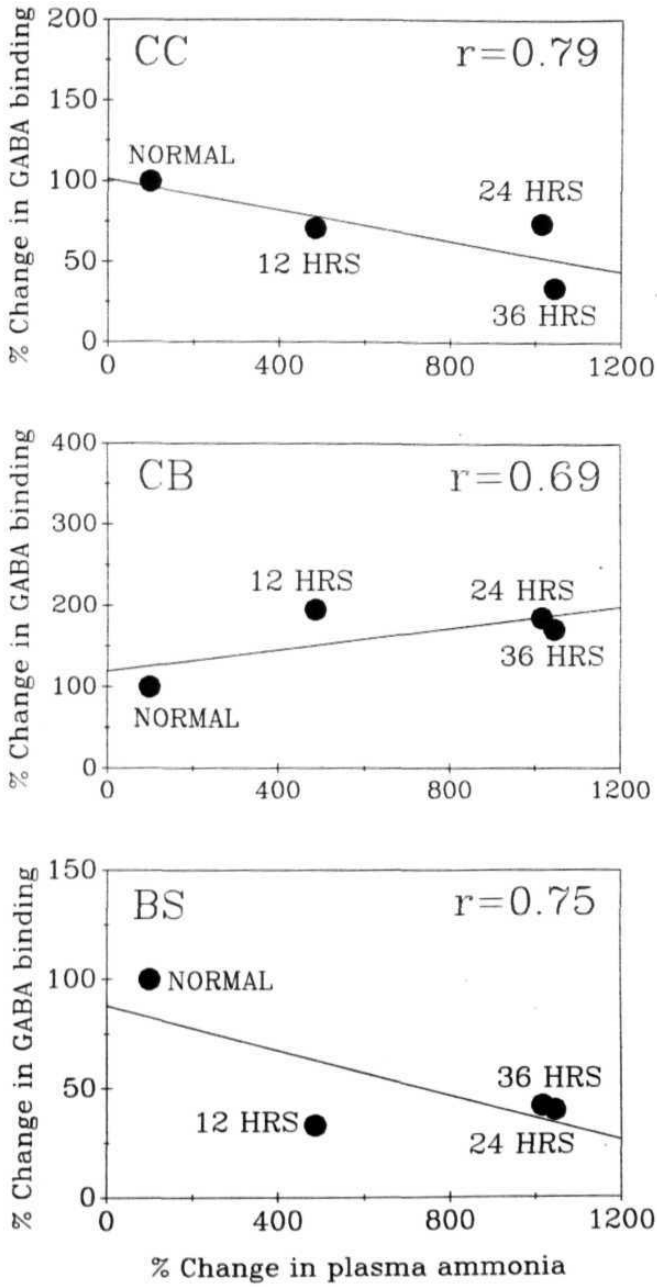
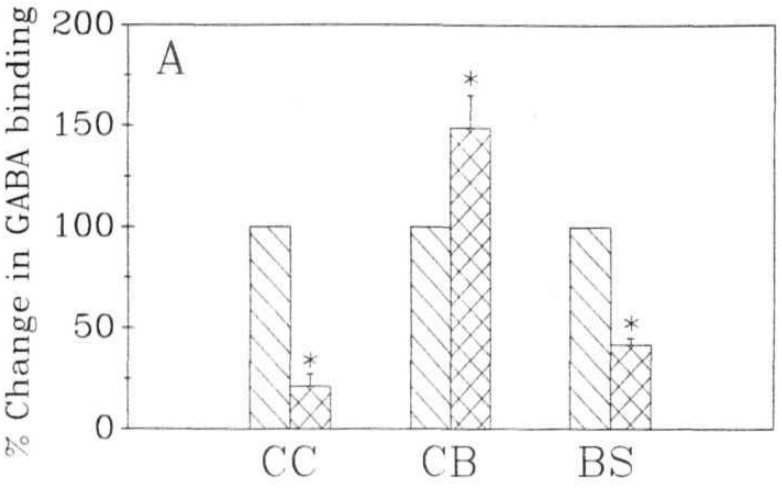

CC
r=0.79
NORMAL
12 HRS
24 HRS
36 HRS
% Change in GABA binding
200
150
100
50
0
0
400
800
1200
CB
r=0.69
12 HRS
24 HRS
36 HRS
NORMAL
% Change in GABA binding
400
300
200
100
0
0
400
800
1200
BS
r=0.75
NORMAL
36 HRS
12 HRS
24 HRS
% Change in GABA binding
150
100
50
0
0
400
800
1200
% Change in plasma ammonia

Fig. 2.23: (A) Specific binding of [^{3}H]GABA to the membranes isolated from the CC, CB and BS of normal ( ), nonconvulsive ( ) and convulsive ( ) group of rats. (B) Specific binding of [^{3}H]GABA to the membranes isolated from the CC, CB and BS of normal ( ) and 36 hrs starved ( ) rats. Each value is Mean ± SD of 3 separate experiments done in duplicates. • = $P<0.01$ when compared to normals.


A
% Change in GABA binding
200
150
100
50
0
CC
CB
BS

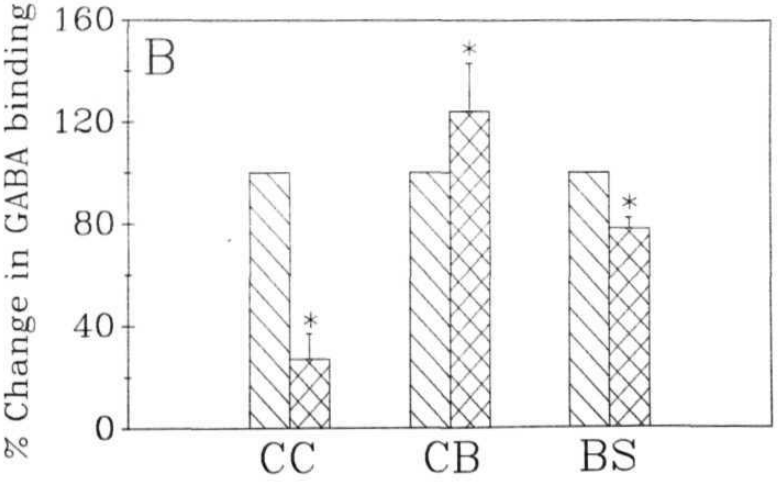

B
% Change in GABA binding
160
120
80
40
0
CC
CB
BS

Fig. 2.24: (A) Glutamate uptake into synaptosomes prepared from the CC of normal rats as a function of protein concentration.
(B) Glutamate uptake into synaptosomes prepared from the CC of normal rats as a function of time of incubation.
(C) Efffect of ouabain on the uptake of glutamate into synaptosomes prepared from the CC of normal rats. Each value is Mean ± SD of 3 separate experiments done in duplicates.

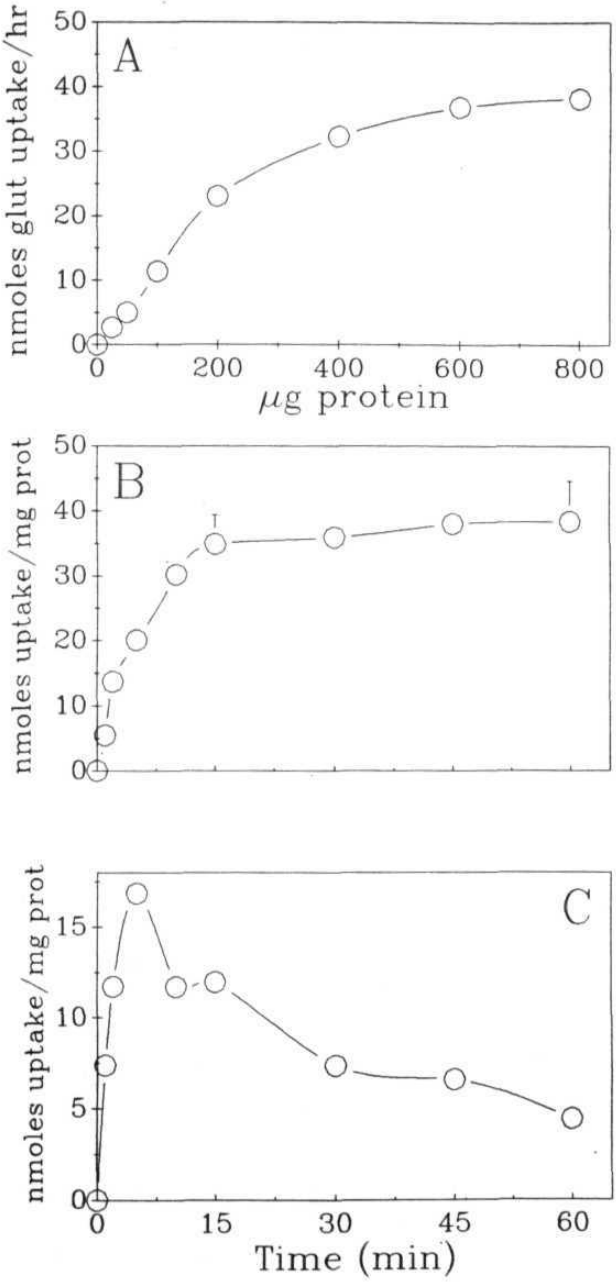
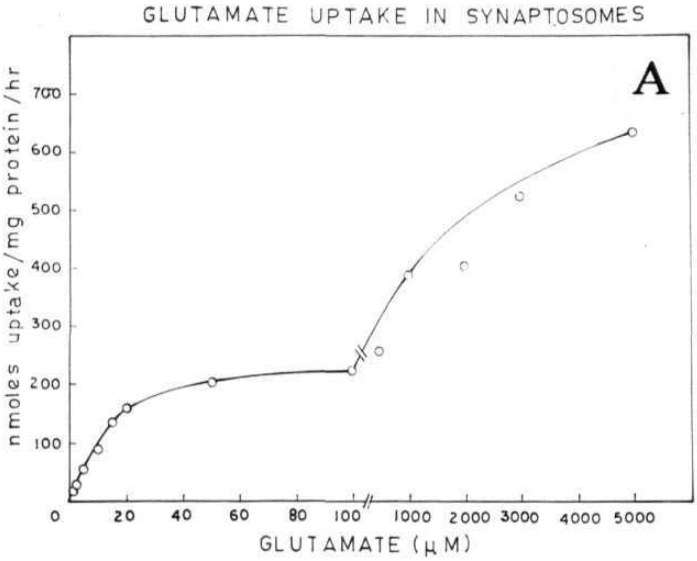

A
nmoles glut uptake/hr
50
40
30
20
10
0
0
200
400
600
800
μg protein
B
nmoles uptake/mg prot
50
40
30
20
10
0
C
nmoles uptake/mg prot
15
10
5
0
0
15
30
45
60
Time (min)

Fig. **2.25:** (A) Saturation isotherm of glutamate uptake into **synaptosomes** isolated from the CC of normal rats.
(B) Scatchard plots of glutamate uptake into synaptosomes prepared from the CC of normal rats. (○) **High** affinity, (●) Low affinity. Each value is Mean ± SD of 3 separate experiments done in duplicates.


GLUTAMATE UPTAKE IN SYNAPTOSOMES
A
nmoles uptake/mg protein/hr
700
600
500
400
300
200
100
0
20
40
60
80
100
1000
2000
3000
4000
5000
GLUTAMATE (μM)

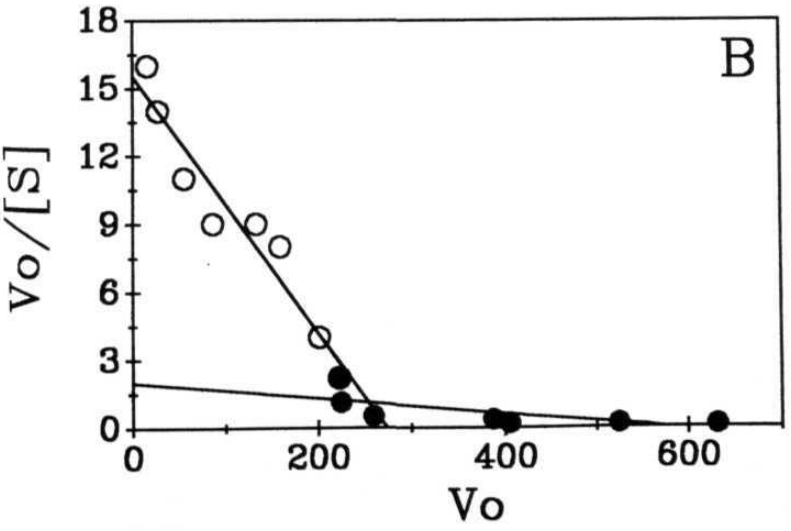

B
Vo/[S]
18
15
12
9
6
3
0
0
200
400
600
Vo

TABLE 2.1

KINETIC CONSTANTS OF HIGH AND LOW AFFINITY BINDING OF [^{3}H]GLUTAMATE IN MEMBRANES FROM CEREBRAL CORTEX, CEREBELLUM AND BRAIN STEM OF NORMAL AND 36 HRS GALACTOSAMINE TREATED RATS

		HABS		LABS	
		Kd (nM)	Bmax (pmoles/mg protein)	Kd (μM)	Bmax (pmoles/ mg protein)
CC	NORMAL	278 ± 13	9.44 ±0.7	1.87 ±0.54	41.9±8.3
	36 HRS	370 ± 50*	13.5 ± 2.6•	1.54 ±0.17	41.9±8.4
CB	NORMAL	54 ±3	2.60 ± 0.07	1.68 ±0.40	25.9 ±5.9
	36 HRS	56 ±10	1.84 ± 0.04•	2.04 ±0.35	31.5 + 5.8
BS	NORMAL	173 ±59	5.45 ±1.7	2.41 ±0.39	48.7 ±5.5
	36 HRS	239 ±34*	9.29 ±3.3*	2.00 ±0.32	45.0 ±7.9

Values are Mean ± S.D. of 4 experiments done in duplicates Kinetic constants (Kd and Bmax) were calculated from Scatchard plots • = P<0.05, • = P<0.1. HABS: High affinity binding system LABS: Low affinity binding system

TABLE 2.2

BRAIN AMMONIA LEVELS IN NORMAL AND GALACTOSAMINE TREATED RATS

	BRAIN
NORMAL	A. 0.23 ± 0.04 (5) B. 0.31 ±0.05 (5)
12 HRS	A. 0.47 ± 0.06 (3)* B. 0.63 ± 0.08 (3)*
24 HRS	A. 0.49 ± 0.05 (3)* B. 0.65 ± 0.07 (3)*
36 HRS	A. 0.77 ± 0.03 (3)* B. 1.03 ±0.04 (3)*

UNITS: Serum: mM. Brain: A: μmoles/g wet wt. B: mM. • - P<0.01. Each value is Mean ± S.D. Number in parenthesis indicates the number of experiments. Water content of the brain was assumed to be 75%, while converting the brain values to mM.

TABLE 2.3

KINETIC CONSTANTS OF [^{3}H]MK-801 BINDING IN MEMBRANES FROM CEREBRAL CORTEX OF NORMAL AND GALACTOSAMINE TREATED RATS

	HIGH AFFINITY		LOW AFFINITY	
	Kd (nM)	Bmax (pmoles bound/mg protein)	Kd (nM)	Bmax (pmoles bound/mg protein)
NORMAL	4.40 ± 0.69	0.48 ± 0.11	24.30 ± 2	1.85 ±0.05
36 HRS	5.41 ± 0.47•	0.74 ±0.16	82 .18 ± 14*	2.48 ± 0.17*

Rats were killed 36 hr after galactosamine treatment. Values are Mean ± S. D. of 4 different experiments in duplicates. Kinetic constants (Kd and Bmax) were calculated from Eadie-Scatchard plots. * = P<0.005, • = P<0.1.

TABLE 2.4

KINETIC CONSTANTS OF [^{3}H]KAINIC ACID BINDING IN MEMBRANES FROM CEREBRAL CORTEX, CEREBELLUM AND BRAIN STEM OF NORMAL AND GALACTOSAMINE TREATED RATS

		Kd (nM)	Bmax (pmole/mg protein)
CC	NORMAL	41.01 ±8.14	0.51 ±0.07
	36 HRS	49.85 ± 7.6	0.63 ± 0.03*
CB	NORMAL	39.41 ±3.71	0.17 ±0.04
	36 HRS	82.20 ± 23.0*	0.17 ±0.04

Rats were killed 36 hr after galactosamine treatment. Values are Mean ± S.D. of 3 different experiments done in duplicates. Kinetic constants (Kd and Bmax) were calculated from Eadie-Scatchard plots • = P<0.05, • = P<0.1

TABLE 2.5

KINETIC CONSTANTS OF HIGH AND LOW AFFINITY BINDING OF [^{3}H]GABA IN MEMBRANES FROM CEREBRAL CORTEX AND CEREBELLUM OF NORMAL AND GALACTOSAMINE TREATED RATS

		HABS		LABS	
		Kd (nM)	Bmax (pmoles/mg protein)	Kd (nM)	Bmax (pmoles/mg protein)
CC	NORMAL	29.6 ±8.72	0 96 ±0 19	460 ± 68	3.54 ±0 45
	36 HRS	107 ± 13.9*	0.57 ± 0.08•	861 ± 102*	2.43 ± 0.4•
CB	NORMAL	55.4 ± 14 5	0.44 ±0.06	587 ±75	1.75 ±0 13
	36 HRS	51 8± 107	0.53 ±0 04•	432 ± 57•	1.78 ±0.32

Rats were killed 36 hr after galactosamine treatment. Values are Mean ± S D of 4 experiments done in duplicates Kinetic constants (Kd and Bmax) were calculated from Scatchard plots * = P<0 005, • = P<0 05 HA : High affinity, LA : Low affinity

TABLE 2.6

KINETIC CONSTANTS OF HIGH AND LOW AFFINITY UPTAKE OF [^{3}H]GLUTAMATE INTO SYNAPTOSOMES FROM CEREBRAL CORTEX OF NORMAL AND GALACTOSAMINE TREATED RATS

	HIGH AFFINITY		LOW AFFINITY	
	Km (μM)	Vmax (nmoles/mg protein/hr)	Km (μM)	Vmax (nmoles/mg protein/hr)
NORMAL	17±1	283 ± 40	360 ± 37	612 ±47
12 HRS	18±4	257 ± 28	385 ±41	669 ±80
24 HRS	18 ±3	194 ± 9♦	340 ± 36	465 ± 22*
36 HRS	11 ± 2♦	108 + 13*	334 ±41	409 ± 32*

Values are Mean ± S.D. of 4 experiments done in duplicates. Kinetic constants (Km and Vmax) were calculated from Eadie-Scatchard plots. •• P<0.01; • = P<0.05.

CHAPTER 5

PHYSICOCHEMICAL CHANGES IN MEMBRANES

Major neurotransmitter functions are (i) release of the neurotransmitter from pre-synaptic terminal (ii) binding of the neurotransmitter to the receptor and induction of appropriate post-synaptic events and (iii) termination of post-synaptic activity by a reuptake process. All these functions are mediated by proteins. These are integral proteins of plasma membranes and span the entire lipid bilayer. Lipid-protein interactions are responsible for the proper orientation and conformation of these proteins. Therefore, any alteration in lipid composition of the membrane would be reflected in the functioning of these proteins.

Results of the present study, indicated an increased release, altered glutamate and GAB A binding and a decreased transport of glutamate. It was suggested that ammonia might be involved in bringing about the changes in neurotransmitter functions. Precise mechanism involved in these changes is not understood. Altered synthesis and degradation of the concerned protein might be responsible for these changes. This seems to be a remote possibility at least in animals which were injected with ammonium acetate. Both the receptors and transporters are integral proteins of the membranes and usually such proteins have longer half-life and their turn over is usually slow. This is because of the fact that these proteins are to be synthesized in the neuronal perikarya, processed and transported down the axon to the nerve terminal where they are inserted into the membranes.

Though not much information is available on the rates of synthesis and membrane insertion, the protein that are transported down the axon were identified as fast and slow axonal transport proteins. Glutamate receptors and transporters does not belong to the class of proteins which are

transported at a faster rate. To further verify this tenet, protein composition of the membranes isolated from normal and GALN treated rats was analyzed by SDS-PAGE.

Region specific changes in the membrane protein composition were observed in the GALN treated animals. In the CC of GALN treated rats, there was an increase in the staining intensity of a band at 35 and 42 kDa at 12 and 24 hr. However, the intensity of the former protein band decreased at 36 hr (Fig. 3.1A). Intensity of a 60 and 220 kDa proteins in CC decreased with increasing time periods of GALN treatment and by 36 hr this protein completely disappeared. In CB, new proteins appeared at 36 hr in the low molecular weight region (Fig. 3.1B). The intensity of 43 and 46 kDa proteins increased in this brain region with increasing time periods of GALN treatment. Not many changes in the protein profiles were observed in BS in both normal and experimental conditions.

Though some changes were observed in the membrane protein profile in GALN treated animals, these changes were not in the region expected for glutamate and GAB A receptor subunits and transporters (Eaton *et ai,* 1990 Amara and Kuhar, 1993). Moreover, except for the region specific changes there were not much differences in the membrane protein profiles in different regions of the brain in rats treated with ammonium acetate (Fig. 3.2). Only in CC there was a slight increase in a 68 kDa protein in non-convulsive and convulsive groups compared to the normal animals. Thus, it appears that altered rates of synthesis and degradation might not be the mechanism involved in the changes in the activities (functions) of receptor and transporter proteins.

Alternatively, another possibility may be suggested to account for the changes in the activities of receptors and transporter proteins. This could be due to changes in major post-translational modification like phosphorylation and dephosphorylation of proteins.

Phosphorylation of proteins is a common pathway of fundamental importance in biological regulation (Krebs and Fischer, 1956; Girault, 1993). The view that protein phosphorylation is the molecular currency through which protein function is regulated in response to extracellular stimuli has been supported by over a generation of research (Nestler and Greengard, 1984) Phosphorylation alters the charge over a protein since this process imparts negative charge. This would alter conformation of the protein and ultimately its functional efficacy.

Functions of several neuronal proteins are known to be regulated by phosphorylation. Protein phosphorylation has been reported to affect neurotransmitter biosynthesis, neurotransmitter release, generation of post-synaptic potentials, ion channel conductance etc. (Hunter and Sefton, 1991; Huganir and Greengard, 1990). Several of the cellular second messenger systems like Ca^{2+}, cAMP, cGMP, nitric oxide etc., have been reported to mediate their effects through protein phosphorylation. It is well known that several neurotransmitters promote the formation of second messengers through protein phosphorylation. Acetylcholine acting through muscarinic receptors, dopamine through D1 and D5 receptors, noradrenalin through (3-adrenergic receptors etc., are known to stimulate the production of second messengers and bring about protein phosphorylation.

It has been reported that glutamate receptor function can be directly modulated by protein phosphorylation (Wang *et al.*, 1993). It was reported that this is mediated through NMDA receptors. It was shown that glutamate binding to NMDA receptors stimulates protein phosphorylation through Ca^{2+} dependent protein kinases.

Keeping this in view, protein phosphorylation was studied in membrane samples from all the three regions of GALN and ammonium acetate treated rats. Since, most of the neurotransmitter functions are mediated through Ca^{2+}-calmodulin dependent protein kinases, it was felt appropriate to carry out phosphorylation studies in presence and absence of Ca^{2+}.

Silver stained gel and the corresponding autoradiograms were shown in Figs. 3.3 A&B. In normal animals, when phosphorylation studies were performed in the absence of Ca^{2+}, the extent of protein phosphorylation was highest in CC followed by CB (Fig. 3.3B). In BS, the extent of protein phosphorylation was almost negligible. Region specific differences were observed in the proteins which were phosphorylated. In CC, 220, 200 and 150 kDa proteins were phosphorylated and these proteins were absent in CB and BS. Phosphorylation of a 50 kDa protein in CC was high compared to other two regions. Similarly, in CB, the extent of phosphorylation of a 35 kDa protein was more than that in CC and BS.

In the CC of GALN treated rats, a 43 kDa protein was phosphorylated more man the corresponding one in normal animals. In the CB of the rats with FHF, phosphorylation of this protein was less when compared to normal animals. Similarly, phosphorylation of a doublet protein of 50 kDa

was decreased in CC of GALN treated animals compared to normal ones. In CC of GALN treated rats, phosphorylation of a 40 kDa protein was decreased when compared to normal animals. A similar result was observed for CB. A 35 kDa protein was not phosphorylated in the CB of GALN treated rats which was present in normal animals.

There was an overall decrease in the extent of phosphorylation of almost all the proteins in CB of GALN treated animals. In BS the overall phosphorylation remained more or less unaltered in the experimental condition compared to the normal animals except for a 40 kDa protein where the phosphorylation rate was lower during GALN treatment.

Neuronal activity might lead to marked increase in the concentration of cytosolic Ca^{2+} which functions as a second messenger and mediates a wide range of cellular responses. When phosphorylation was carried out in the presence of Ca^{2+}, there was an overall increase in phosphorylation when compared to that observed in the absence of Ca^{2+} (Fig. 3.4B; Fig. 3.4A is the corresponding silver stained gel). There were regional differences in phosphorylation. Phosphorylation was highest in CC compared to CB and BS. Most of the high molecular weight proteins were heavily phosphorylated when Ca^{2+} was present in the medium. Phosphorylation of 43, 45, 48 and 50 kDa proteins were higher in CC than CB and BS of normal animals.

Not many differences were observed in the protein phosphorylation pattern in CC of normal and GALN treated animals except that the magnitude of phosphorylation decreased in GALN treated animals. In CB, phosphorylation of several proteins was unaltered excepting a decrease in the

phosphorylation of a 45 kDa protein in the experimental animals. While in BS, extent of protein phosphorylarion, especially of a 37-38 and a 40 kDa protein, was enhanced in GALN treated rats. Phosphorylarion of several of the high molecular weight proteins was amplified.

Results of the above experiments indicated that (a) there were regional difference in protein phosphorylation both in the presence and absence of Ca^{2+} (b) protein phosphorylation was stimulated by Ca^{2+} (c) types of proteins that were phosphorylated in the presence of Ca^{2+} were different from those in the absence of Ca^{2+} (d) there was an overall decrease in the phosphorylation of membrane proteins in GALN treated rats (e) regional differences and Ca^{2+} dependency persisted even in conditions of FHF.

As was suggested earlier, that elevated cerebral ammonia levels in might be responsible for the changes observed in neurotransmitter functions in GALN treatment. Hence, phosphorylation studies were also carried out in membranes isolated from rats injected with an acute dose of ammonium acetate (Fig. 3.3B).

In the ammonium acetate treated animals, not many changes were observed in the phosphorylation of proteins in the absence of Ca^{2+} in CC except for increased phosphorylation of 200 and 206 kDa proteins. In the CB of these animals, a 35 kDa protein was not phosphorylated while it was phosphorylated in normal animals. The extent of phosphorylation of a 40 kDa protein was increased in this brain region following ammonium acetate treatment. Some high molecular weight proteins (206 kDa) in the cerebellum were phosphorylated more than the corresponding ones in normal

animals. In the BS of ammonium acetate treated animals, except for the decreased phosphorylation of 40 kDa protein, overall pattern remained the same as that of normal animals.

Protein phosphorylation pattern in the membranes of CC in presence of Ca^{2+} in ammonium acetate treated animals was more or less similar to that of normal animals (Fig. 3.4B). Whereas in CB, in general, the extent of phosphorylation was decreased during ammonium acetate treatment compared to normal ones. In the BS, the phosphorylation of proteins was increased in membranes from ammonium acetate treated animals compared to normal ones. The changes observed in BS were more or less similar to that observed in GALN toxicity.

Results of the present study indicated an increased release and a decreased uptake of glutamate. Under such conditions, glutamate might persist in the synaptic cleft for a longer period of time. In other words, cells would be exposed to higher concentrations of glutamate for a longer duration under these conditions - a situation congenial for glutamate to exert its excitotoxic effects. Such effects were reported to be mediated by the activation of post-synaptic AMPA and NMDA receptors (Dingledine and McBain, 1994; Collingridge and Bliss, 1995). An increase in the intracellular concentrations of Ca^{2+} under these conditions was reported to be due to the entry of Ca^{2+} through the NMDA receptor complex and voltage sensitive Ca^{2+} channels (Ghosh and Greenberg, 1995). Elevation of intracellular Ca^{2+} would then trigger a cascade of second-messenger systems, many of which remain activated for a long time even after the removal of stimulus. The inability of the cells to maintain a resting membrane

potential would precipitate a positive feedback, which will lead to neuronal cell injury and death.

Such a situation might exist in GALN treated rats due to increased life time of glutamate in the extracellular environment of brain. Hence, protein phosphorylation was carried out in the membranes of both normal and GALN treated rats in the presence of excitotoxic concentration of glutamate. As the excitotoxic effects of glutamate were reported to be mediated through NMDA receptors (Ghosh and Greenberg, 1995), effects of NMDA and its antagonist, MK-801 were also studied. In these experiments, membranes were preincubated with glutamate (100 μM) or NMDA (50 μM), a concentration which is reported to be excitotoxic (Eboli *et al*., 1994) .

When the membranes from the CC and CB of normal, GALN treated or ammonium acetate treated rats were preincubated with glutamate or NMDA, overall phosphorylation of membrane proteins was decreased when compared to the membranes which were not pre-treated (Fig. 3.5B, 3.6B, 3.7B). Fig. 3.5A, 3.6A and 3.7A are the corresponding silver stained gels for Figs. 3.5B, 3.6B and 3.7B, respectively. Interestingly, pre-treatment with 10 μM of MK-801 prevented the changes in the membrane protein phosphorylation induced by glutamate or NMDA.

These results indicated that glutamate and NMDA, at excitotoxic concentrations, affected membrane protein phosphorylation and MK-801 offered protection from the excitotoxic effects of glutamate and NMDA. Presently, only 10 μM MK-801 was used. Probably, at higher concentrations, MK-801 might offer a complete protection against the

excitotoxic effect of glutamate or NMDA.

Alternatively, another possibility might be suggested to account for the changes in the activities of receptors and transporter proteins. These proteins being integral proteins of plasma membrane, their activity would also be influenced by changes in the physicochemical properties of membranes. Any alteration in the membrane microenvironment would affect the structural organization, thus the function of these proteins. If this possibility was true, then the activities of other membrane bound proteins should also be altered in GALN treated rats. To verify this, activities of two membrane bound enzymes, Na^+,K^+-ATPase, and γ-glutamyl transpeptidase were determined in the membranes isolated from the CC, CB and BS of normal and GALN treated rats.

Na^+, K^+-ATPase was shown to be essential for the active transport of Na^+ and K^+ across the cell membrane and plays a key role in the maintenance of ionic gradients which are essential for neuronal function. (Stahl, 1986; Wu, 1986; Georgina Rodriguez de Lores Arnaiz, 1993). Na^+, K^+-ATP-ase activity was enhanced in the CC and BS in the initial stages of GALN toxicity and then declined at later stages (Fig. 3.8A). However, in the CB of these rats, there was a progressive decrease with time in the activity of this enzyme. In the present study there was increased release of glutamate in CC during GALN induced FHF. Earlier reports have shown that Na^+, K^+-ATPases are also involved in neurotransmitter release and deactivation (Vizi, 1977; Meyer and Cooper, 1984). Therefore, presently observed increase in Na^+, K^+-ATPase activity in CC might be involved in the increased release of glutamate. Pattern of changes observed in Na^+, K^+-

ATPase activity during GALN treatment in CC, CB and BS was similar to that of changes observed in glutamate binding.

Alterations in the activities of another membrane bound enzyme, GGTP, was also studied in normal and GALN treated rats. GGTP was suggested to be involved in specific functions of the brain, e.g., in the transport of amino acids across the BBB or in the inactivation of excitatory neurotransmitters through their removal from the synaptic cleft during neural transmission (Lisy *et al,* 1979; Varga *et al.,* 1985; Reichelt and Poulsen, 1992; Dvorakova *et al.,* 1994).

Changes were also observed in the activity levels of cerebral GGTP in GALN treated rats (Fig. 3.8B). In the CC of these rats, activity of this enzyme decreased up to 24 hr and remained at this level till 36 hr. Changes observed in GGTP activity in BS under these conditions was opposite to that observed in CC. However, in the CB of these rats there was an initial increase (at 12 hr) followed by a decrease at later time periods in the activity of this enzyme.

Results of the above studies indicated that more than one membrane functions were affected in GALN treated animals. Such a change suggests an overall change in the membrane organization and architecture of GALN treated rats. Hence, attention was focused on this aspect.

Cell membrane consists of a fluid phospholipid bilayer with embedded macromolecules (Singer and Nicholson, 1972). These macromolecules include receptors for hormones and neurotransmitters, enzymes and the carriers of ion and nutrient (glucose, amino acids etc.) transport.

Macromolecular function, especially of proteins, is strongly influenced by the composition and consequently the physical state and the fluidity of the membrane lipids. Shinitzky and Henkant (1979) have reported that mobility of membrane bound proteins is directly modulated by lipid fluidity. Membrane fluidity influences the molecular order and relative motion of the protein and is a major determinant of many membrane associated functions like receptor binding, neurotransmitter release and uptake, protein phosphorylation and membrane bound enzymatic activity (Ghosh *et a!.*, 1993). Membrane fluidity (thus viscosity) depends on the lipid composition, especially phospholipids and cholesterol and the ratio of these two lipids (C/P ratio). Hence, changes in the content of these two lipids were studied in the cerebral membranes of GALN treated rats.

Region specific and time dependent changes were observed in the phospholipid content of membranes of CC, CB and BS of GALN treated rats (Fig. 3.9A). A progressive increase in the phospholipid content, with a maximal increase at 36 hr, was observed in the membranes of CC after the administration of GALN. In CB, there was a marginal decrease in the phospholipid content of the membranes at 12 and 24 hr after the administration of GALN. However, its content increased at 36 hr time period. In BS of these rats, the magnitude of initial decline in the membrane phospholipid content was higher than that of CB during 24 and 36 hr. Thereafter, there was an increase in the phospholipid content of this region. This result, though suggested a change in the content of phospholipid, the nature of change can not be clearly envisaged.

Changes observed in the cholesterol content were different from those of phospholipids. In the CC and CB of GALN treated rats, there was a progressive decrease in the membrane cholesterol content (Fig. 3.9B). The magnitude of decrease was greater in CB than in CC. In the BS of these rats, there was a progressive increase in the membrane cholesterol content.

As a consequence of changes in the membrane phospholipid and cholesterol content in the GALN treated rats, the ratio of these two lipids was also altered. Ratio of cholesterol to phospholipid (inversely proportional to fluidity of membranes) in the membranes of BS increased while those of CC and CB decreased consistently with time in GALN treated rats. This suggested membrane fulidity decreased in BS (i.e., membrane becomes more viscous) while that of CC and CB increased (hence the membrane being less viscous) in the GALN treated rats (Fig. 3.9C). Results obtained in the above experiments provided indirect evidence of changes in the fluidity of the cerebral membranes in GALN toxicity.

Changes in C/P ratio fail to provide information on the site where these changes occurring in the membrane i.e., whether the changes in the membrane fluidity was in the core of the membrane or whether this change was on the surface of the membrane. To substantiate these results, direct measurements of membrane fluidity were made, by monitoring fluorescence changes of the incorporated probes, 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-[4-(trimethylammonio)phenyl]6-phenyl-1,3,5-hexatriene (TMA-DPH). These probes monitor the fluidity of two different regions of membrane bilayer. DPH, being lipophilic, is known to partition into the hydro-

phobic core of the lipid bilayer, whereas the amphipathic, TMA-DPH gets oriented in the membrane bilayer with its positive charge located at the lipid/water interface, and its DPH moiety sensing the inerfacial and head group regions of the membrane (Revathi *et al*., 1994).

Results of fluorescent polarization studies using DPH, indicated regional heterogeneity in the fluidity of the brain membranes in normal animals. Membranes from CC exhibited more fluidity compared to CB and BS (Fig. 3.10A-3.10B) at the core. Membranes of BS exhibited lesser fluidity when compared to the other two regions.

Results from GALN treated animals indicated changes in the fluidity of the membranes isolated from three different regions of the brain (Fig. 3.11). A marginal and statistically significant increase in fluidity of the membranes of CC and CB was observed while there was a statistically significant decrease in fluidity of the membranes of BS under these conditions (Table 3.1). Observed changes in membrane fluidity in the three different regions at the membrane core (using DPH) was similar to the results obtained by calculating the cholesterol/phospholipid ratio.

In contrast to the results with DPH, studies with TMA-DPH indicated that the membranes of CC and CB less fluid compared to BS (Table 3.2). In other words, the membrane lipid core was more fluid while the surface of the membrane is less fluid in CC and CB while the membranes of BS had an exactly opposite architecture.

Fluorescent polarization results for membrane surface (using TMA-DPH) from GALN treated rats were exactly opposite to that observed with DPH. The surface fluidity of the membrane of CC and CB in GALN treated animals was decreased when compared to that of normal animals. In the BS of these rats, the surface fluidity of the membranes was increased.

When membrane fluorescent polarization values were studied using DPH for membrane from ammonium acetate treated animals no statistically significant changes observed (Table 3.3).

These results indicated that membranes prepared from various brain regions differ in their fluidity. Differences in the neurotransmitter functions such as release, binding of neurotransmitters (to agonists and antagonists) and reuptake in these regions of brain might be due to the differences in membrane **lipid** fluidity.

Changes in membrane lipid fluidity in GALN treated animals might influence the membrane functions such as release of neurotransmitters, transport of several substances (ions, nutrients and neurotransmitters), and neurotransmitter receptor mediated **functions**. Results of the present study also demonstrated that elevated ammonia levels in brain in FHF might be responsible for changes in membrane **functions** mentioned above. Such changes might ultimately play a role in the aetiology of the disease process.

Fig. 3.1: (A) SDS-PAGE of membrane proteins from CC of normal, 12, 24 and 36 hrs of GALN treated rats.
(B) SDS-PAGE of membrane proteins from CB and BS of normal, 12, 24 and 36 hrs of GALN treated rats. 10 μg protein/lane was loaded. Proteins were separated on a 10% gel and silver staining done.

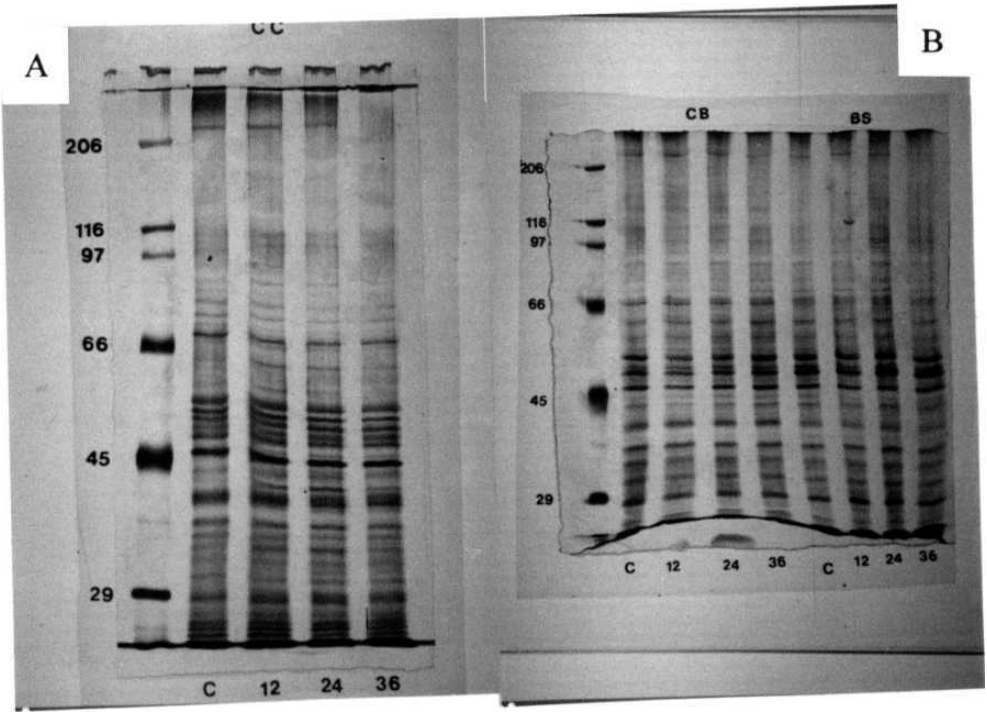

A
CC
206
116
97
66
45
29
C
12
24
36
B
CB
BS
206
116
97
66
45
29
C
12
24
36
C
12
24
36

Fig. 3.2: SDS-PAGE of membrane proteins from CC, CB and BS of normal, nonconvulsive and convulsive dose of ammonium acetate treated rats.

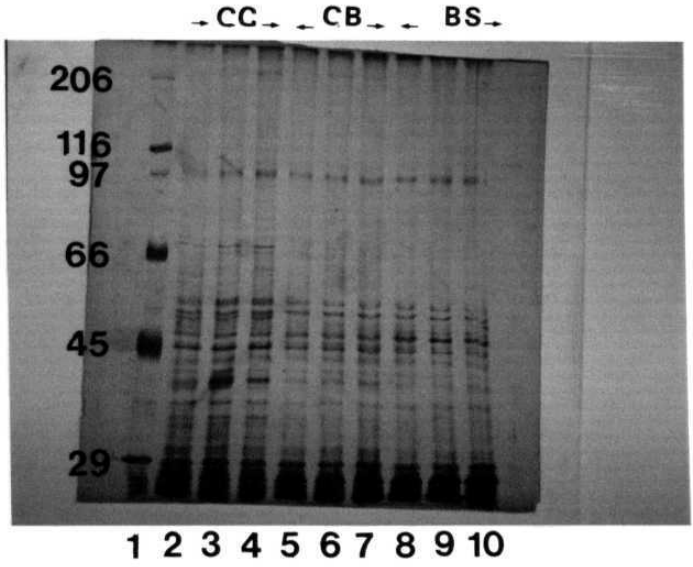

CC
CB
BS
206
116
97
66
45
29
1 2 3 4 5 6 7 8 9 10

Fig. 3.3: (A) SDS-PAGE of membrane proteins from CC, CB and BS of normal, 36 hrs GALN and convulsive dose of ammonium acetate treated rats.

(B) Autoradiograph showing Ca^{2+} independent phosphorylation of membrane proteins from the CC, CB and BS of normal, 36 hrs GALN and convulsive dose of ammonium acetate treated rats. 10 μg protein/lane were separated on a 10% gel. Fig. A represents the corresponding gel. C : control; G : GALN; A : ammonium acetate.

Fig. 3.4: (A) SDS-PAGE of membrane proteins from CC, CB and BS of normal, 36 hrs GALN and convulsive dose of ammonium acetate treated rats.

(B) Autoradiograph showing Ca^{2+} dependent phosphorylation of membrane proteins from the CC, CB and BS of normal, 36 hrs GALN and convulsive dose of ammonium acetate treated rats. 10 μg protein/lane were separated on a 10% gel. Fig. A represents the corresponding gel. C : control; G : GALN; A : ammonium acetate.

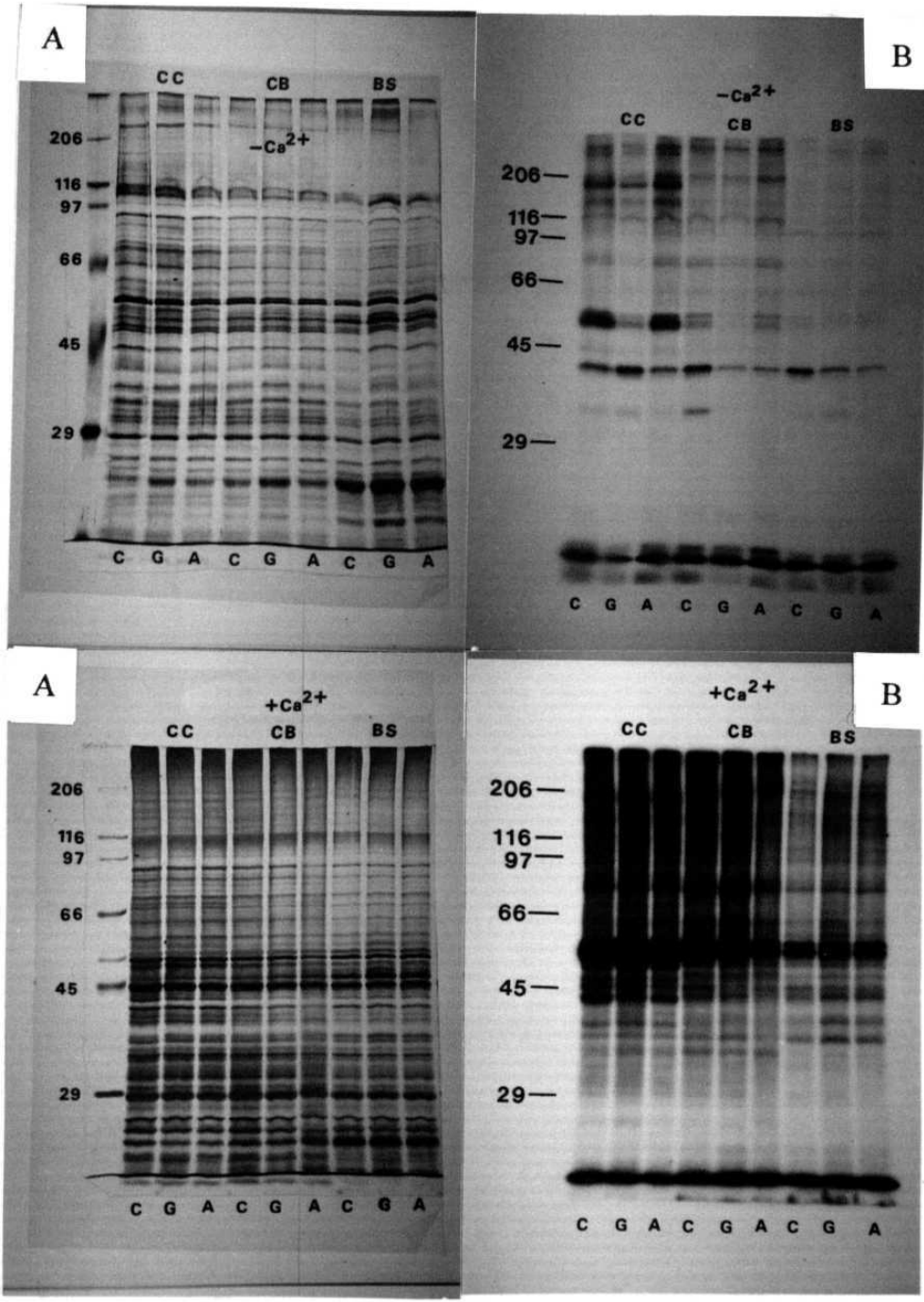

A
CC
CB
BS
−Ca2+
206
116
97
66
45
29
C G A C G A C G A
B
−Ca2+
CC
CB
BS
206
116
97
66
45
29
C G A C G A C G A
A
+Ca2+
CC
CB
BS
206
116
97
66
45
29
C G A C G A C G A
B
+Ca2+
CC
CB
BS
206
116
97
66
45
29
C G A C G A C G A

Fig. 3.5: Effect of glutamate (100 μM), NMDA (50 μM) and MK-801 on phosphorylation of membrane proteins from the CC of normal and 36 hrs GALN treated rats. Fig. A represents the corresponding silver stained gel. Fig. B represents corresponding autoradiogram. Lane 1: control, lane 2: + glutamate, lane 3: +NMDA, lane 4: +MK-801 and NMDA. 10 μg protein was loaded per lane on a 10% gel.

Fig. 3.6: Effect of glutamate (100 μM), NMDA (50 μM) and MK-801 on phosphorylation of membrane proteins from the CB of normal and 36 hrs GALN treated rats. Fig. A represents the corresponding silver stained gel. Fig. B represents corresponding autoradiogram. Lane 1: control, lane 2: + glutamate, lane 3: +NMDA, lane 4: +MK-801 and NMDA. 10 μg protein was loaded per lane on a 10% gel.

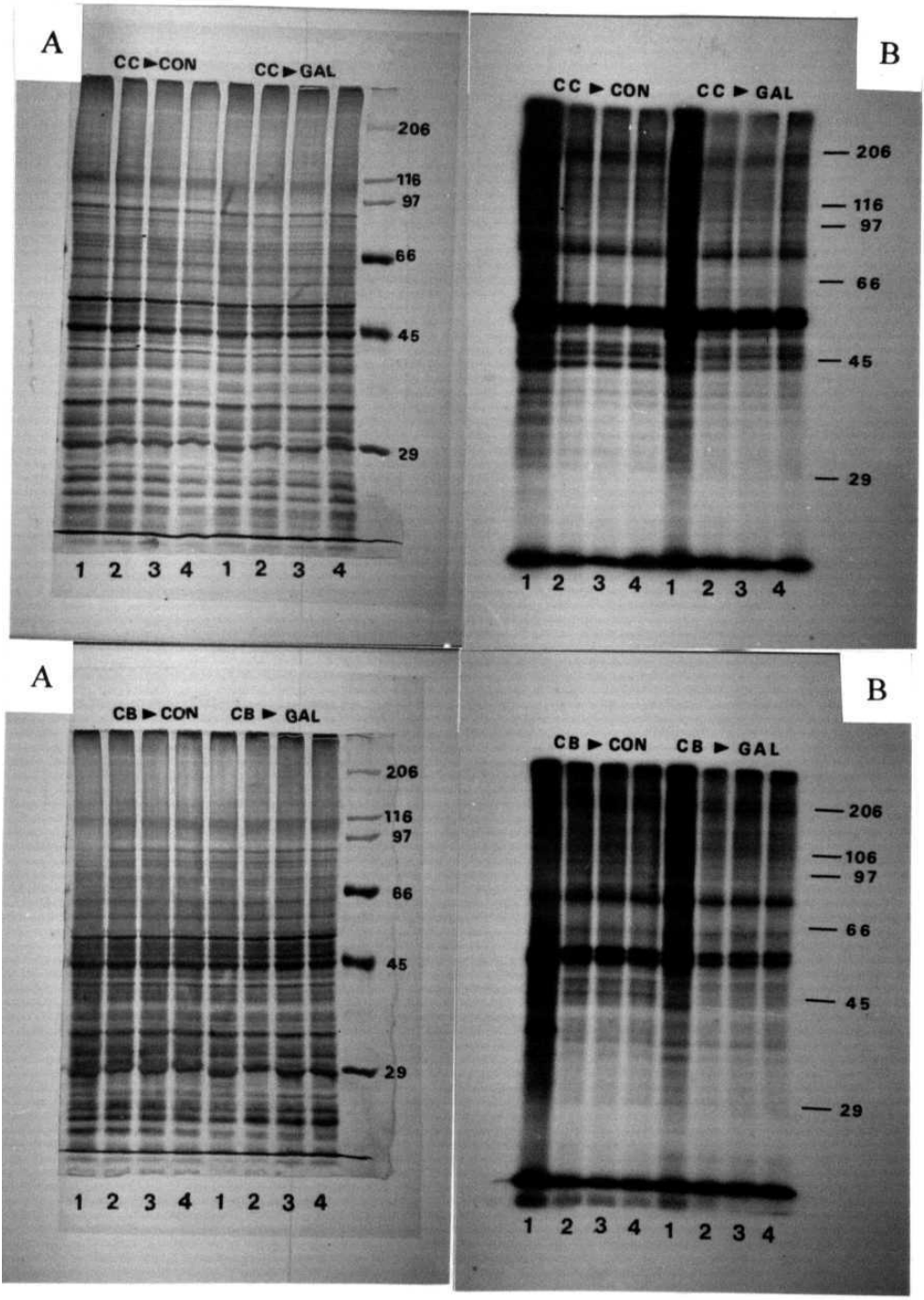
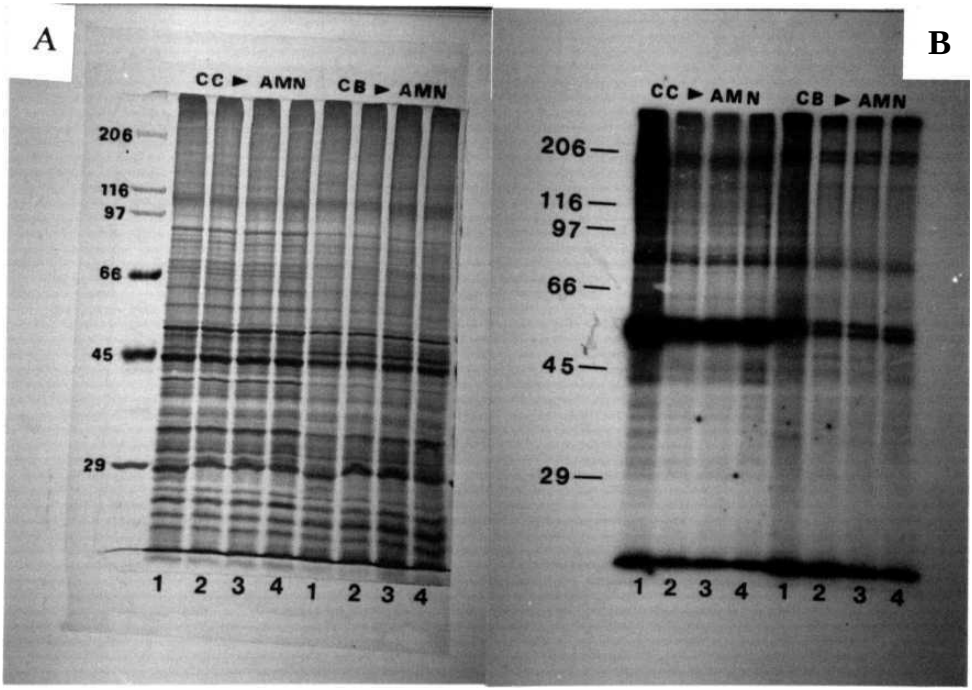

A
CC►CON
CC►GAL
206
116
97
66
45
29
1 2 3 4 1 2 3 4
B
CC ► CON
CC ► GAL
206
116
97
66
45
29
1 2 3 4 1 2 3 4
A
CB ► CON
CB ► GAL
206
116
97
66
45
29
1 2 3 4 1 2 3 4
B
CB ► CON
CB ► GAL
206
106
97
66
45
29
1 2 3 4 1 2 3 4

Fig. 3.7: Effect of glutamate (100 μM), NMDA (50 μM) and MK-801 on phosphorylation of membrane proteins from the CC and CB of convulsive dose of ammonium acetate treated rats. Fig. A represents the corresponding silver stained gel. Fig. B represents corresponding autoradiogram. 10 μg protein was loaded per lane on a 10% gel. Lane 1: control, lane 2: + glutamate, lane 3: +NMDA, lane 4: +MK-801 and NMDA..


A
CC ► AMN
CB ► AMN
206
116
97
66
45
29
1 2 3 4 1 2 3 4
B
CC ► AMN
CB ► AMN
206
116
97
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45
29
1 2 3 4 1 2 3 4

Fig. 3.8: (A) Activities of Na^+, K^+-ATPase in the membranes from *CC* (○), CB (●), BS(△) of normal and different time periods of GALN treated rats.
(B) Activities of GGTP in the membrane preparations from CC(○), CB(●)BS( ) of normal and different time periods of GALN treated rats.

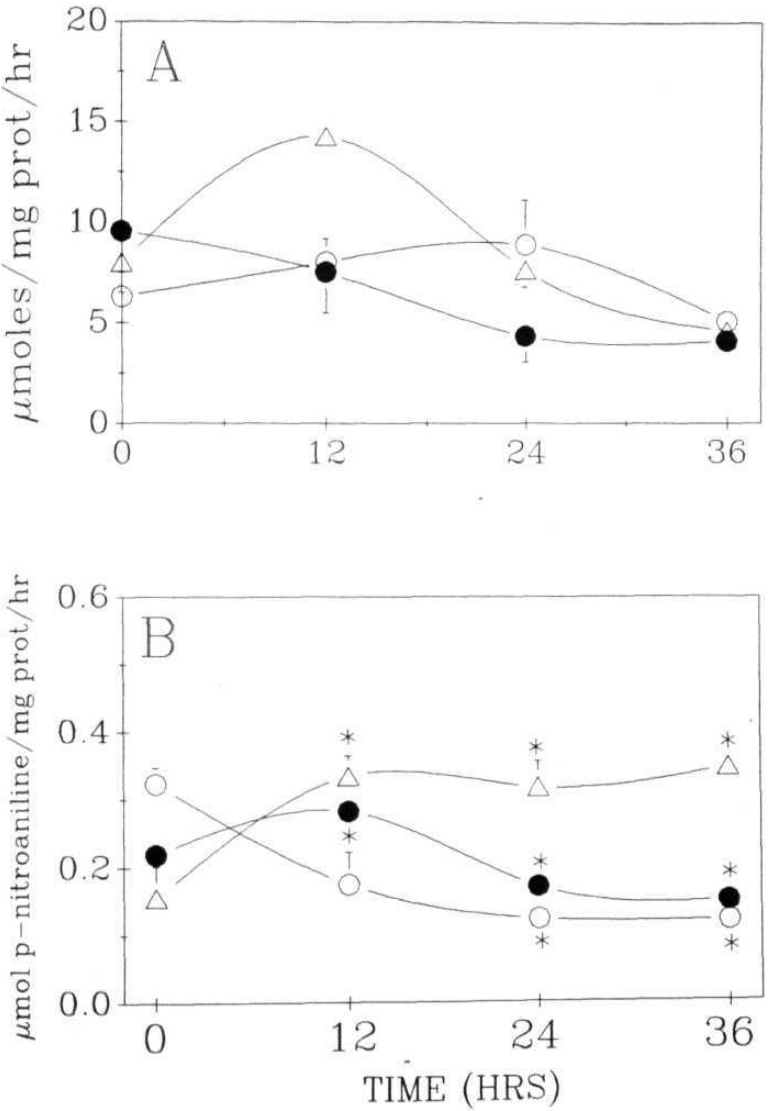
A
B
μmoles/mg prot/hr
μmol p-nitroaniline/mg prot/hr
TIME (HRS)
0
12
24
36
0.0
0.2
0.4
0.6
5
10
15
20

Fig. 3.9: (A) Phospholipid content in membrane preparations from the CC, CB, BS of normal and different time periods of GALN treated rats.

(B) Cholesterol content in the membrane preparation from CC, CB, BS of normal and GALN treated rats.

(C) Ratio of C/P in the membrane preparation from CC, CB, BS of normal and different time periods of GALN treated rats.

(○)CC,(●)CB,(△)BS.

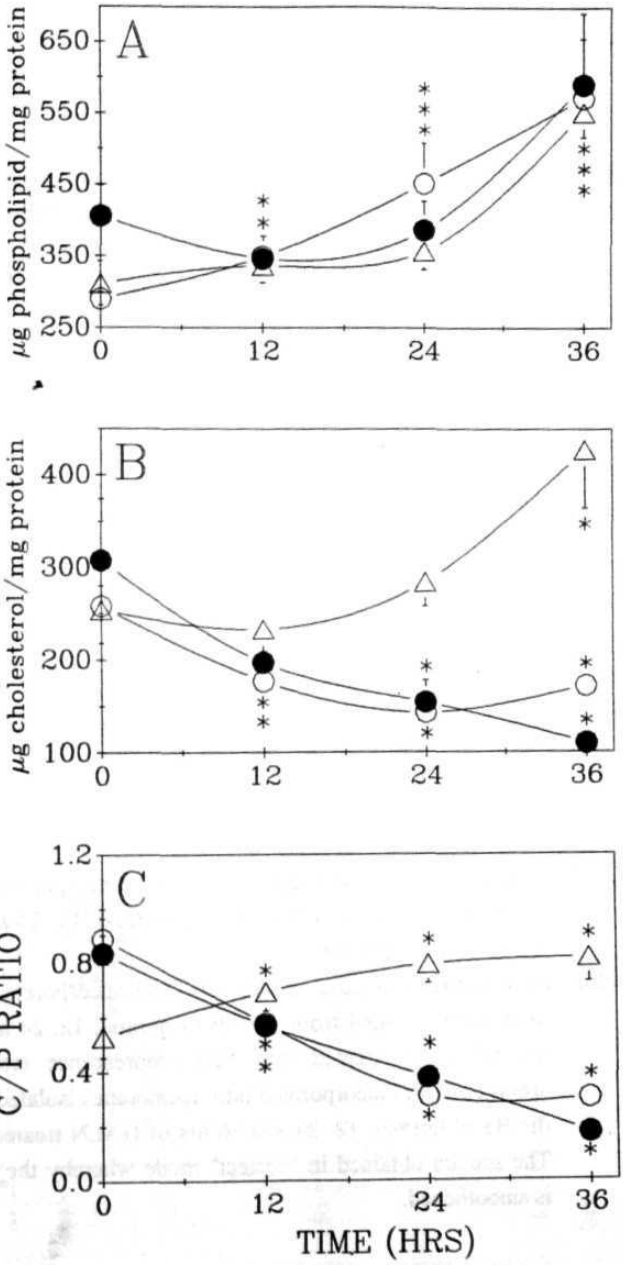
A
μg phospholipid/mg protein
650
550
450
350
250
0
12
24
36
B
μg cholesterol/mg protein
400
300
200
100
0
12
24
36
C
C/P RATIO
1.2
0.8
0.4
0.0
0
12
24
36
TIME (HRS)

Fig. 3.10: (A) Corrected fluorescence emission spectra of DPH incorporated into membranes isolated from the CC, CB and BS of normal rats.

(B) Corrected fluorescence emission spectra of TMA-DPH incorporated into membranes isolated from the CC, CB and BS of normal rats.

Fig. **3.11:** (A) Fluorescence emission spectra of DPH incorporated into membranes isolated from the CC of normal, 12, 24 and 36 hrs of GALN treated rats.

(B) Fluorescence emission spectra of DPH incorporated into membranes isolated from the CB of **normal**, 12, 24 and 36 hrs of GALN treated rats. (C) Fluorescence emission spectra of DPH incorporated into membranes isolated from the BS of normal, 12, 24 and 36 hrs of GALN treated rats. The spectra obtained in "correct" mode whereby the curve is smoothened.

1484
DPH
CC
CB
BS
0
400
428
500
12
TMA–DPH
BS
CB
CC
0
400
428
500

320
CC
DPH
24
12
36
CON
428
312
CB
DPH
12
CON
24
36
428
632
BS
DPH
36
24
12
CON
428

TABLE 3.1

FLUORESCENT POLARIZATION (P) VALUES OF DPH INCORPORATED INTO MEMBRANES FROM DIFFERENT REGIONS OF NORMAL AND GALACTOSAMINE TREATED OF RAT BRAIN

REGION	NORMAL	12 HRS	24 HRS	36 HRS
CC	0.314 ±0.017	0.302 ± 0.011 (Δ P 0.012)	0.297 ± 0.006 (Δ P 0.017)	0.298 ±0.010 (Δ P 0.016)
CB	0.327 ± 0.014	0.322 ± 0.024 (A P 0.005)	0.323 ±0.016 (A P 0.004)	0.307 ±0.016 (A P 0.02)
BS	0.33 ± 0.004	0.336 ±0.006 (A P 0.006)	0.337 ±0.006 (A P 0.007)	0.345 ± 0.005 (Δ P 0.015)*

= P<0.05 (Neuman Kuel's multiple range)

TABLE 3.2

FLUORESCENT POLARIZATION (P) VALUES OF TMA-DPH INCORPORATED INTO MEMBRANES FROM DIFFERENT REGIONS OF NORMAL AND 36 HRS GALACTOSAMINE TREATED RAT BRAIN

REGION	NORMAL	36 HRS GALN
CC	0.330 ± 0.011	0.368 ± 0.019 (A P 0.038)*
CB	0.339 ± 0.023	0.353 ±0.014 (A P 0.014)
BS	0.374 ± 0.020	0.346 ±0.012 (ΔP 0.028)*

• = P <0.01 (Students 't' test)

TABLE 3.3

FLUORESCENT POLARIZATION (P) VALUES OF DPH INCORPORATED INTO MEMBRANES FROM DIFFERENT REGIONS OF NORMAL, CONVULSIVE AND NON-CONVULSIVE AMMONIUM ACETATE TREATED RAT BRAIN

REGION	NORMAL	NON-CONVUL	CONVULSIVE
CC	0.314 ±0.017	0.324 ± 0.01 (Δ P 0.010)	0.31 ± 0.016 (A P 0.004)
CB	0.327 ± 0.014	0.324 ± 0.007 (A P 0.003)	0.32710.007
BS	0.33 + 0.004	0.328 ± 0.003 (A P 0.005)	0.324 ± 0.021 (A P 0.009)

CONCLUSIONS

> 1. Animal model for FHF was developed. GALN induced FHF was found to mimic human disease conditions in both morphological and functional features.

(i) Plasma and liver ammonia levels were increased with increasing time period of GALN treatment with that of normal animals.

(ii) There was an increase in plasma urea content in GALN treated rats. In liver there was an initial increase in urea and from 6 hr onwards it remained more or less stable.

(iii) Increase in the GS level in the liver of GALN treated rats indicated that perivenous hepatocytes were affected and the periportal cells were spared by galactosamine.

(iv) Liver and plasma glucose levels are decreased in GALN treated rats.

(v) There was significant decrease in the liver and plasma protein content during GALN treatment.

(vi) Activity levels of some liver marker enzymes - GGTP, AAT, AlAT and SODH was decreased in the hver and a significant increase were observed in the activities of these enzymes in the plasma of GALN treated rats compared to normal animals.

(vii) Histopathological changes in the liver of GALN treated rats by light microscopy revealed widespread necrosis, inflammation, variation in the size of cells and acidophillic degeneration which confirmed liver degeneration during the drug treatment.

(viii) Levels of glycolytic and citric acid cycle intermediates in brain of normal and GALN treated rats were altered. These results with the glucose metabolites levels of 36 hr starved rats were found to be different from mat of GALN treated rats. The results suggested that changes observed in GALN treatment were not due to starvation.

➢2. Neurotransmitter functions (release, binding to receptors and uptake) of glutamate and GABA were altered during FHF.

(i) Release of glutamate from nerve terminals **(synaptosomes)** was increased in GALN induced FHF.

(ii) Binding of glutamate to its receptors was increased in CC and BS of GALN treated rats while a decrease was observed in CB . There was an increase in the **Kd** and **Bmax** value of the high affinity binding site in CC and BS whereas in CB there was a decrease in Bmax value without a change in the Kd.

(iii) Brain ammonia levels were increased with increasing time periods of GALN treatment.

(iv) A close correlation was observed between the changes in plasma and brain ammonia levels to that of glutamate binding suggesting that elevated ammonia levels might be responsible for the changes observed in glutamate binding in GALN induced FHF.

(v) To confirm the above tenet, glutamate binding was performed in rats injected with nonconvulsive and convulsive dose of ammonium acetate and glutamate binding was studied. Changes observed in glutamate binding in these animals were similar to mat of GALN treatment. These results confirmed the above tenet that increase in cerebral ammonia levels might be responsible for the changes in glutamate binding.

(vi) This was further confirmed by studying the effects of added ammonium acetate (1 and S mM) to the membranes of normal animals. Results obtained were similar to that of GALN and *in vivo* ammonium acetate experiments.

(vii) As there are multiple types of glutamate **receptors,** glutamate binding to NMDA and KA subtype of receptors was studied in GALN induced FHF and in ammonium acetate treated animals.

(viii) Two methods were adopted for studying glutamate receptor subtypes - receptor masking studies and specific ligand binding. Results obtained by both these methods were complimentary to each other.

(ix) Changes observed in the kinetics of NMDA and KA receptors in these two animal models were similar to those described above.

(x) As the food consumption stops in animals with FHF, changes in glutamate binding was studied in animals starved for 36 hr. These were observed to be entirely different from those seen during FHF. This suggested that changes observed during FHF were not due to starvation stress thus strengthened the role of ammonium ion in bringing about alteration in glutamate binding during FHF.

(xi) Changes observed in GABA binding were exactly opposite to that of glutamate binding. There was a decrease in the Bmax of the high affinity binding system in the CC and an increase was observed in the CB of GALN treated rats.

(xii) A close correlation was observed between the increase in plasma and brain ammonia levels and changes in GABA binding.

(xiii) Pattern of changes observed in GABA binding in rats treated with ammonium acetate were similar to those observed in GALN treatment.

(xiv) Changes were observed in GABA binding when animals were starved for 36 hr. The changes were more or less similar to that of GALN and ammonium acetate treated rats but the magnitude of change was different.

(xv) Uptake of glutamate into cortical synaptosomes decrease in rats at different time periods of GALN treatment.

As the release, receptor binding and uptake are membrane associated functions, physico-chemical changes in membranes were studied.

(i) SDS-PAGE analysis indicated minor changes in the protein profiles of membrane proteins.

(ii) Membrane protein phosphorylation was decreased during GALN and ammonium acetate treatment in CC and CB whereas in BS the phosphorylation was increased in these two experimental conditions compared to the normal ones. The extent of phosphorylation was high in all the three regions in presence of Ca^{2+}.

(iii) Glutamate and NMDA at excitotoxic concentrations had adverse effects on membrane protein phosphorylation. MK-801 (antagonist) partially prevents the adverse effects of glutamate and NMDA.

(iv) Altered contents of phospholipids, cholesterol and the ratio between these membrane components indicated changes in membrane fluidity in GALN treated animals. This was further confirmed by fluorescence polarization studies using fluidity monitoring probes, DPH and TMA-DPH. Surface fluidity of the membranes of CC and CB decreased while the membrane core fluidity increased in GALN toxicity. Changes observed in BS were opposite to the above.

➢ **4.** Results of the present study suggested that the **neurotransmitter** functions of glutamate and GABA are altered in conditions of fulminant hepatic failure. These changes may be due to alterations in the physico-chemical properties of the cerebral membranes. Ammonia might be the chief culprit in bringing these changes.

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