GABA RECEPTOR GENE EXPRESSION DURING RAT LIVER CELL PROLIFERATION AND ITS FUNCTION IN HEPATOCYTE CULTURES

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BIJU M. P.

DEPARTMENT OF BIOTECHNOLOGY
COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY
COCHIN-682 022, KERALA, INDIA

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CERTIFICATE

This is to certify that the thesis entitled **"GABA RECEPTOR GENE EXPRESSION DURING RAT LIVER CELL PROLIFERATION AND ITS FUNCTION IN HEPATOCYTE CULTURES"** is a bonafide record of the research work carried out by **Mr BIJU.M.P.** under my guidance and supervision in the Department of Biotechnology, Cochin University of Science and Technology and that no part thereof has been presented for the award of any other degree.

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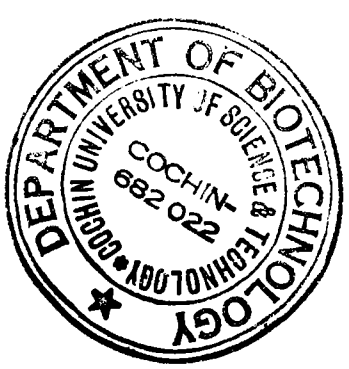


Dr.C.S.Paulose
Reader
Department of Biotechnology
Cochin University of Science & Technology

Dr. C.S. PAULOSE M.Sc., Ph.D., FIMSA.
READER, DEPT. OF BIOTECHNOLOGY
Cochin University of Science & Technology
COCHIN - 682 022, KERALA, INDIA

CONTENTS

ABBREVIATIONS USED IN THE TEXT

5-HT	5-hydroxytryptamine (serotonin)
6-OHDA	6-Hydroxydopamine
aFGF	Acidic fibroblast growth factor
AP-1	Activating protein 1
B_{max}	Maximal binding
C/EBP	CCAAT/enhancer binding protein
cAMP	Cyclic adenosine monophate
Cdk	Cyclin dependent kinase
CdkI	Cdk inhibitor
CREB	cAMP regulatory element binding protein
DAG	Diacyl glycerol
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
flg	FGF receptor-1
GABA	Gamma aminobutyric acid
GAD	Glutamic acid decarboxylase
HCC	Hepatocellular carcinoma
HGF	Hepatocyte growth factor
HPLC	High performance liquid chromatography
IFN	Interferon
IGF	Insulin like growth factor
IL	Interleukin
IP_3	Inositol triphosphate
IRS	Insulin receptor substrate
JNK	c-Jun amino-terminal kinase
K_d	Dissociation constant
K_m	Michaelis Menten constant
LN	Lead nitrate
LPA	Lysophosphatidic acid
LPR	Lysophosphatidic acid receptor

LRF	Liver regeneration factor
MAPK	Mitogen activated protein kinase
MEK	Mitogen-activated protein kinase kinase
NDEA	*N*-nitrosodiethylamine
NE	Norepinephrine
NF-κB	Nuclear factor kappa B
p	Level of significance
PH	Partial hepatectomy
PI-3K	Phosphatidyl-inositol-3 kinase
PIP_2	phosphatidylinositol-4,5-biphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PTX	Pertussis toxin
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcription polymerase chain reaction
S.E.M	Standard error of mean
SAPK	Stress activated protein kinase
SH	Src homology domain
STAT	Signal tranducer and activator of transcription
T_3	Triiodothyronine
TGF	Transforming growth factor
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
V_{max}	Maximal velocity

INTRODUCTION

INTRODUCTION

The fundamental characteristic of a cell is self-replication. The division of all cells must be carefully regulated and co-ordinated with both cell growth and DNA replication in order to ensure the formation of progeny cells containing intact genome. Defects in cell cycle regulation are a common cause for the abnormal cell proliferation leading to cancer. So, studies of the cell cycle and cancer have become closely interconnected. The adult mammalian liver is predominantly in a quiescent state with respect to cell division. The quiescent state changes dramatically, however, if the liver gets injured (Ponder.P.K, 1996), subjected to carcinogens (Futakuchi.M, 1999) or mitogens (Columbano.A & Shinozuka.H, 1996). Liver regeneration after 70% partial hepatectomy (PH) and hepatic proliferation after lead nitrate (LN) administration are models to study cell proliferation and transition from G_0 to G_1, S, G_2 and M phases of cell cycle. Progression through the phases of cell cycle is controlled by a conserved regulatory apparatus which not only co-ordinates the different events of the cell cycle but also links the cell cycle with extracellular signals that control cell proliferation. The deregulated hepatic proliferation after *N*-nitrosodiethylamine (NDEA) treatment in rats is a model system to identify and define the factors that are responsible for tumorigenesis (Futakuchi.M, 1999). Cells with a relatively long G_1 phase distinguish adult hepatocytes from other cell types with higher proliferative potential. Hence, G_1 events probably ensure strict control of hepatocyte proliferative activity (Hunter.T, 1993).

Liver regeneration after the loss of hepatic tissue is a fundamental parameter of natural response to injury. It is now defined as an orchestrated response induced by specific external stimuli involving sequential changes in gene expression, growth factor production and morphogenic structure. Knowledge about the events which regulate liver regeneration has come a long way since 1931, when two surgeons at the Mayo Clinic discovered that in rats resection of two thirds of the liver (partial hepatectomy) is followed by a regenerative response that restores the mass and function of the liver within little more than a week (Higgins.G.M & Anderso.R.M, 1931). Similar responses to liver resection have been noted in all species, including humans. More recent work has shown that liver

regeneration follows other types of liver injury, including damage inflicted by toxins or infections. Thus, regeneration appears to be a normal response of the liver whenever it is injured. The hepatocyte has a florid regenerative potential. In experimental partial resection in the rat, the remaining liver tissue starts to regenerate within few hours. Within 14-15 hr DNA replication is seen and within 20-21 hr mitoses appear. In 24-32 hr mitoses are at the peak (Grisham.J.W, 1962). In one week the remaining liver tissue has reached the original weight before resection. Cell division takes place in the periportal zone (Lee.V.M *et al.*, 1998). In pathological conditions, dead liver cells are replaced by proliferation of surviving liver cells. Hepatocytes, Kupffer, endothelial, and bile duct cells all proliferate (Widemann.J.J & Fahimi.H.D, 1975). However, unlike the brain or the heart, when the liver is injured, virtually all of the surviving hepatocytes leave their growth-arrested state and proliferate until the destroyed part of the liver is replaced. Amazingly, the liver is generally able to perform its usual functions even when large fractions of hepatocytes are actively replicating. Even more miraculous is its ability to recognise, when its functional mass has been normalised and to respond by terminating the compensatory growth response. In addition to providing new tools to cure liver diseases, clarifying how the liver accomplishes regeneration could open novel areas for exploration in the field of cancer biology, since cancer involves loss of specialised cellular functions during deregulated growth. The fact that liver regeneration is a normal, adult physiological response to injury provides evidence that regulated proliferation of highly specialised cells are not only possible, but occurs routinely in some organs.

There is increasing evidence that hepatocyte proliferation induced by some primary mitogen such as lead nitrate, is mediated by patterns of growth factor modulation and signal transduction different from those of compensatory regeneration (Columbano.A & Shinozuka.H, 1996). The difference in molecular events observed between liver regeneration and direct hyperplasia may affect differently the initiation step of chemical carcinogenesis (Columbano.A *et al.*, 1981). Definition of the factors that influencing and mechanisms by which primary mitogens stimulate liver cell proliferation may elucidate the nature of signals responsible for triggering the entry into cell cycle. Although liver regeneration still offers an attractive model for studying regulatory mechanisms that control

liver growth, the models of direct hyperplasia provide additional means to define how liver growth is controlled in body, as the mitogen acts on the liver without serious damage (Columbano.A & Shinozuka.H, 1996).

Carcinogenesis is not a single step event but a gradual developmental process, which may involve a series of sequential cellular alterations. A well characterised model of multistage carcinogenesis is that of hepatocarcinogenesis in rats. The histopathology as well as the cellular and molecular biology of initiation, promotion, and progression of the cells have been elucidated to varying degrees in this system. NDEA induced hepatocellular carcinoma is a good model for studying the mechanisms of metastasis. This model, with its essential similarities to malignant tumour behaviour in man, should find application not only for elucidation of the mechanisms underlying tumorigenesis and metastasis, but also in the development of anti-metastatic agents (Futakuchi.M, 1999).

Sera from patients with fulminant hepatic failure inhibit the growth of regenerating hepatocytes (Gove.C.D *et al.*, 1982). Some of the humoral factors responsible for this inhibitory effect have not yet been identified. Gamma aminobutyric acid (GABA) is a potent amino acid neurotransmitter that inhibits the growth of murine squamous cell carcinoma and HeLa cell lines (Boggust.W.A & Al-Nakib.T, 1986). GABA also plays an important role in terminating the growth of rapidly developing tissues *in utero*. In fulminant hepatic failure systemic serum GABA level increases 2-20 fold from normal values. Previous data indicate that at these concentrations certain parameters of hepatic regeneration including restitution of liver mass, protein synthesis rates, ornithine decarboxylase activity, and hepatic putrescine levels are significantly decreased following PH in GABA-treated rats compared with saline treated controls (Gilon.P *et al.*, 1987) (Seiler.N *et al.*, 1980). The mechanism whereby GABA exerts the inhibitory effects on regenerating liver remain to be elucidated.

Brain plays an important regulatory role in hepatic functions (Lautt.W.W, 1983). The liver is richly innervated (Rogers.R.C & Hermann.G.E, 1983) and autonomic nervous system has an important role in the process of hepatic cell proliferation (Tanaka.K *et al.*, 1987). Lateral lesions of hypothalamus cause an increase in DNA synthesis during liver regeneration and sympathectomy and vagotomy block this effect (Kiba.T *et al.*, 1994). There are several reports regarding the brain regulation of hepatic proliferation but the role

of central nervous system via neurotransmitters and receptors in mediating these effects are not well characterised. Central thyrotropin releasing hormone has been identified as one of the chemical messengers involved in brain regulation of hepatic proliferation (Yoneda.M *et al.*, 1997). GABA is the principal inhibitory neurotransmitter of the mammalian brain. Advanced liver disease of either acute or chronic nature can be associated with a significant impairment in hepatic regenerative activity and GABA metabolism (Eguchi.S *et al.*, 1997). The brain stem rostral nucleus ambigus as well as the dorsal motor nucleus of the vagus are the centres of autonomic nerves that innervate liver and is under the regulation of GABA (Coleman.M.J & Dampney.R.A, 1998). Autonomic regulation of GABA is reported to mediate through $GABA_A$ receptors (Bowery.N.G & Hudson.A.L, 1979). Also, elevated intra-cerebral concentrations of GABA significantly decreased ornithine decarboxylase activity in the liver (Lapinjoki.S.P *et al.*, 1983), which is an index for decreased hepatic proliferation.

Investigations to elucidate the mechanisms which regulate hepatic proliferation during liver diseases and liver cancers are to be conducted. This would save these patients from the suffering and premature death associated with these diseases and would also prevent them from developing cirrhosis, a complication of chronic liver injury for which there is, at present, no cure other than liver transplantation. It appears likely that mechanisms which regulate the regenerative response to liver injury may also be involved in wound healing responses in other tissues. Thus, the study of liver regeneration may give rise to entice regeneration in other organs (e.g., the heart and the brain) which are also highly specialised and normally do not regenerate after they are injured. These studies will have clinical significance in cardiologic/neurologic diseases.

The work that is presented here is an attempt to understand the role of GABA, $GABA_A$ and $GABA_B$ receptors in the regulation of liver cell proliferation using *in vivo* and *in vitro* models. The work also focuses on the brain GABAergic changes associated with normal and neoplastic cell growth in liver and to delineate its regulatory function. The investigation of mechanisms involving mitogenic models without cell necrosis may contribute our knowledge about both on cell growth, carcinogenesis, liver pathology and treatment.

OBJECTIVES OF THE PRESENT STUDY

1. To induce controlled liver cell proliferation by partial hepatectomy and lead nitrate administration and uncontrolled cell proliferation by *N*-nitrosodiethylamine treatment in male Wistar rats.
2. To study the DNA synthesis by [^{3}H]thymidine incorporation/thymidine kinase assay in regenerating, lead nitrate induced hyperplastic and NDEA induced neoplastic rat liver.
3. To study the changes in the content of GABA in various rat brain regions - brain stem, hypothalamus, cerebellum and cerebral cortex during controlled and uncontrolled liver cell proliferation using High Performance Liquid Chromatography (HPLC) integrated with an electrochemical detector.
4. To study the $GABA_A$ and $GABA_B$ receptor changes in brain stem, hypothalamus, cerebellum and cerebral cortex during the period of active DNA synthesis in liver of different experimental groups.
5. To study the changes in the levels of circulating GABA in the experimental groups using HPLC.
6. To study the $GABA_A$ and $GABA_B$ receptor changes during the active DNA-synthesis period in the liver of different experimental groups.
7. To study the effect of $GABA_A$ and $GABA_B$ receptors in DNA synthesis by using specific agonists in primary cultures of hepatocytes in combination with epidermal growth factor and/or transforming growth factor β1.
8. To study the gene expression alteration of $GABA_A$ subunit mRNA in the liver of the rats using reverse transcription polymerase chain reaction (RT-PCR) technique.

LITERATURE REVIEW

REVIEW OF LITERATURE

Cells traversing the cell cycle must follow a complex set of instructions and are equipped with important guidelines. Withdrawal from the cycling mode into G_0 and return from G_0 into the active G_1 phase occur in response to environmental signals provided by general growth condition or by growth factors (Michalopoulose.G.K & DeFrancis.M.C, 1997). The entry of G_0 cells into G_1 is a highly regulated process. Events that damage the cell's regulatory apparatus, triggering quiescent cells to enter into active growth cycle, are critical initiators of the inappropriate growth of cancer cell. Mammalian liver has tremendous regenerative capacity against tissue loss and also can proliferate in response to primary mitogens (Columbano.A & Shinozuka.H, 1996). Hepatocytes, the differentiated functional cells of liver, can re-enter into the cell cycle in response to appropriate environmental stimuli. This unique feature made liver an excellent *in vivo* model system to study the regulatory mechanisms of cell proliferation. It is likely that several different experimental model systems will be necessary to fully elucidate the mechanisms which regulate hepatocyte proliferation. Regeneration of liver after partial hepatectomy (PH), cell proliferation after the administration of primary mitogens, and deregulated cell proliferation after NDEA treatment in rats are the well established model systems to study the normal and abnormal cell replication (Futakuchi.M, 1999).

The unique characteristic of liver to regenerate has been appreciated since ancient times, as evidenced by the Greek myth about Prometheus. Zeus punished his enemy by chaining Prometheus to a rock and ordering a bird of prey to devour his liver daily. Aware that the liver could regenerate, Zeus had devised a plan that would damn Prometheus to eternal torture without allowing him to escape by death. Unlike Prometheus, the rest of us are fortunate that the liver can regenerate after it is injured, since we have not yet developed any machine that can replace its vital functions. In some sense, it is odd that the liver possesses such tremendous regenerative ability since liver cells rarely proliferate in healthy adults. Rather, liver cells (hepatocytes) are usually pre-occupied with accomplishing a myriad of tasks which cannot be performed by any other tissue. In this regard, the liver is more like the brain or the heart (which are also largely composed of highly specialised, non-replicating cells) than the skin or the intestines (which contain

populations of cells that proliferate relatively actively to continuously renew the tissue). Morphological evidence of liver cell regeneration either normal or abnormal is given by the presence of mitoses, large polyploid nuclei, binucleation, multinucleation and hyperplastic changes. Thus, both immediate and long-term survival after any type of significant liver injury demands successful liver regeneration. In this sense, impaired liver regeneration contributes to the development of all types of acute and chronic liver diseases. Conversely, if strategies could be discovered to ensure that the liver would always regenerate optimally after injury, the need for other life-threatening and expensive treatments (e.g., liver transplantation) to salvage patients with advanced liver diseases would disappear.

Basic investigation of cellular proliferations often performed with isolated cells in simplified culture systems. However, it is becoming clear that, although this strategy provides a powerful tool to dissect individual responses, it ignores other important influences (e.g., cell-cell and cell-environment interactions) that regulate cellular proliferation and differentiation in living animals. The latter are better studied in models, which leave organ architecture intact. In addition, the latter models are necessary to identify responses of other liver components (including bile duct cells, blood vessels, the connective tissue) which are also involved in reconstitution of the liver after injury. Virtually nothing is known about the mechanisms, which regulate these important events during liver regeneration.

As suggested above, if we understood the processes which regulate the liver's regenerative response to injury, then we could manipulate factors in patients with active liver injury to ensure the normal regeneration. This would save these patients from the suffering and premature death associated with acute liver disease and would also prevent them from developing cirrhosis, a complication of chronic liver injury for which there is, at present, no cure other than liver transplantation.

We now know that liver injury causes the release of multiple factors which regulate cellular proliferative activity. These factors are produced both by the liver and by other tissues and, in other situations, their trophic actions are not limited to liver cells. However, unknown mechanisms restrict subsequent cellular proliferation to the injured liver, so that cellular proliferation does not increase in other injured organs. Reports from various laboratories have demonstrated that each of these different growth-regulatory factors

interacts with unique receptors on the surface of hepatocytes. This trigger a complex, yet orderly, cascade of events within the cell that, together, culminate in a "re-programming" of the hepatocyte's gene expression which, in turn, permits the cell to escape growth arrest and to do things that are necessary for it to replicate (Michalopoulose.G.K & DeFrancis.M.C, 1997). Elegant efforts by several groups have delineated a number of the intracellular signal transduction pathways which regulate the cell's proliferative activity (Diehl.A.M & Rai.R.M, 1996). However, emerging evidences indicate that these different signalling pathways often intersect and occasionally overlap, providing the cell ample opportunity to amplify or abort any given growth-regulatory signal (Taub.R, 1996). Much work will be required to diagram the cell's signalling network and to fully understand how it works. Also, mysterious is how the hepatocyte is able to accomplish its specialised functions while it is shouldering the additional burdens required for replication. Clarification of this aspect of hepatocyte proliferation will be particularly important because of the fact that mature hepatocytes proliferate refutes previously accepted dogma that only undifferentiated (i.e., non-specialised) cells can proliferate. PH leads to proliferation of all populations of cells within the liver, including hepatocytes, biliary epithelial cells and endothelial cells. DNA synthesis is initiated in these cells within 10 to 12 hr after surgery and essentially ceases in about 3 days. Cellular proliferation begins in the periportal region (i.e. around the portal triads) and proceeds toward the centres of lobules (Leevy.C.B, 1998). Proliferating hepatocytes initially form clumps, and clumps are soon transformed into classical plates. Similarly, proliferating endothelial cells develop into the type of fenestrated cells typical of those seen in sinusoids. It appears that hepatocytes have a practically unlimited capacity for proliferation, with full regeneration observed after as many as 12 sequential partial hepatectomies. Clearly the hepatocyte is not a terminally differentiated cell. Changes in gene expression associated with regeneration are observed within minutes of hepatic resection. An array of transcription factors (NF-kB, STAT3, Fos and Jun) are rapidly induced and probably participate in orchestrating expression of a group of hepatic mitogens. Proliferating hepatocytes appear to at least partially revert to a foetal phenotype and express markers such as alpha-foetoprotein. Despite what appears to be a massive commitment to proliferation, the regenerating hepatocytes continue to conduct their normal metabolic duties for the host such as support of glucose metabolism. Hepatic

regeneration is triggered by the appearance of circulating mitogenic factors. This conclusion was originally supported by experiments demonstrating that quiescent fragments of liver that had been transplanted to extrahepatic sites would begin to proliferate soon after PH and also, that hepatectomy in one of a pair of parabiotic rats led to hepatic proliferation in the other of the pair (Fisher.B *et al.*, 1971).

If the supportive reticular framework of liver is preserved, the lost cells are replaced and the regeneration is "*ad integrum*". If however, the reticulum is damaged, healing can be accomplished only by scar formation, "fibrosis", which may produce more damage by inducing rearrangement of the blood circulation that leads to cirrhosis (Takahiro.E *et al.*, 1998).

In addition, liver regeneration research will extend knowledge about general mechanisms that regulate the proliferation and specialised functioning of adult cells. This is likely to improve ongoing efforts to develop cures for cancer, a disease which is one of the leading causes of death.

Mechanisms which regulate the regenerative response to liver injury may also be involved in wound healing responses in other tissues. Thus, liver regeneration research may suggest novel strategies to entice regeneration in other organs (e.g., the heart and the brain) which are also highly specialised and normally do not regenerate after they are injured. If this could be accomplished, then we may be able to offer patients with incurable heart or neurologic diseases a new horizon of hope.

REGULATION OF DNA SYNTHESIS AND THE CELL CYCLE

Once the signal for growth stimulation reaches the nucleus, they are translated into cellular actions by a complex of proteins that mediate progression of cell through the various phases of cell cycle. The cell cycle is controlled by stage-specific activation of members of the cyclin-dependent kinase (Cdk) family, all serine/threonine kinases, and their interactions with various activating cyclins and suppressing Cdk inhibitor (CdkI) proteins. The Cdk-activating cyclins are categorised according to the cell cycle phase in which they are expressed (Menjo.M *et al.*, 1998) (Kitamura.T *et al.*, 1998).

Liver cell proliferation can also be induced by primary mitogens. Cell proliferation by direct hyperplasia in the absence of compensatory regeneration represents another

unique ability of hepatic cells to proliferate (Columbano.A & Shinozuka.H, 1996). This hepatocyte proliferation model does not necessarily require expression of certain immediate early genes or growth factors or even exhibits the same pattern of signal transduction associated with compensatory hyperplasia [Grassel-Kraupp.B, 1998]. The mechanisms involved in transition through the G_1 phase of cell cycle are quite different for the two types of liver cell proliferation. However, once the hepatocytes are committed to replication, progression through cell cycle appears to be the same for both types of growth. As soon as the mitogenic signals are withdrawn, liver mass and DNA content return to their original value, regressing through what appears to be the well-controlled process of apoptosis (Columbano.A *et al.*, 1985). The potency of the mitogenic stimulus and the peak of the S phase vary according to the nature of primary mitogens.

EARLY GROWTH RESPONSE GENES IN COMPENSATORY AND DIRECT HYPERPLASIA

Within minutes after PH, hepatocytes in the remnant liver undergo a transition from the quiescent G_0 state into G_1 phase of cell cycle. Even though precise mechanism (or mechanisms) responsible for triggering this transition is not known, the enhanced expression of genes occurring within 30 min to 2 hr after PH probably mediates G_0/G_1 transition. Many genes are identified and defined as immediate early genes including the *fos* and *jun* family, *egr-1*, *LFR-1*, *c-myc* and others (Goyette.M *et al.*, 1983) (Haber.B.A *et al.*, 1993). Liver cell proliferation induced by direct mitogens exhibits patterns of immediate early genes different from those seen after PH. For example, no significant increase of the expression of the immediate early gene *c-fos* could be detected after treatment with at least six different direct mitogens: the metal salt - lead nitrate, the chlorinated hydrocarbon - ethylene dibromide, the antiandrogen - cyproterone acetate and the peroxisome proliferators - nafenopin and Wy14643 (Goldsworthy.T.L *et al.*, 1994) (Koni.P *et al.*, 1993). Moreover, no changes in the hepatic levels of *c-jun* and *c-myc* mRNA were detected in liver cell proliferation induced by cyproterone acetate and nafenopin. These results thus show that *c-fos* is not required for liver cell proliferation induced in the rat liver by primary mitogen, and suggest that, depending on the nature of the primary mitogens used, *c-jun* and *c-myc* may also be irrelevant for this model of growth. It is possible that

activation of this set of genes reflects an "alert" pathway triggered when liver cells are exposed to a potentially dangerous damage rather than a "mitogenic" program. Transcription factor, nuclear factor kappa B (NF-κB), gets activated shortly after PH but activation of this transcription factor was not observed during hyperplasia induced by BR931 (Ohmura.T *et al.*, 1996). This suggests that different signal transduction pathways may be involved in triggering hepatocyte proliferation depending on 1) the nature of the proliferative stimulus (compensatory vs. direct hyperplasia) and 2) the type of the primary mitogen.

CELL PROLIFERATION AND HEPATOCARCINOGENESIS

Hepatic cell proliferation is considered to play an important role in the several steps of the carcinogenic process such as initiation, promotion, and progression (Pierce.G.B, 1998). Although the exact mechanism whereby cell proliferation plays a role in initiation is not known, its involvement in events such as fixation of miscoding lesion in newly made DNA has been entertained. In most studies aimed to determine the role of cell proliferation in the initiation step of chemical hepato-carcinogenesis, the proliferative stimulus has been achieved by compensatory regeneration. It is widely believed that abnormal production of polypeptide growth factors, together with other molecular alterations, play an important role in neoplastic development (Hu.Z *et al.*, 1996). In all hepatocellular carcinomas (HCC) examined, the transcripts of transforming growth factor-alpha (TGFα) and acidic fibroblast growth factor (aFGF) were highly expressed, while those of hepatocyte growth factor (HGF) were low (Tanno.S & Ogawa.K, 1994). With regard to the receptor expression in the tumors, EGF receptor was present at varying levels, c-Met (HGF receptor) was expressed at higher levels and FGF receptor-1 (*flg*) increased significantly, whereas *bek* remained at low levels. These data suggest that TGFα and aFGF are the major growth factors involved in the progression of HCC (Harada.K *et al.*, 1999), and that the signal of aFGF is mainly transduced by the receptor *flg* in HCC. Furthermore, HCC cells were phenotypically very similar to oval cells with regard to the gene expression of growth factor/receptor systems. EGF receptor and TGFα expression is very high in HCC cell lines (Hisaka.T *et al.*, 1999). HCC cells are positive for the oval cell antigen OV6, and that cytokeratin 19 is heavily expressed in both tumour and oval cells, strongly suggest that at

least some of the HCC induced by the Solt-Farber protocol may be derived from oval cells. In the HCCs examined, the transcripts of TGFα and aFGF were highly expressed, while those of HGF were low. TGFβ and its receptors are important in the development of HCC (Abou-Shady.M *et al.*, 1999) (Grasl-Kraupp.B *et al.*, 1998). The low expression of TGFβ1 receptor in hepatocyte is suggested to be one of the key event which promotes the HCC malignant growth by protecting them against the growth inhibition effect of active TGFβ (Liu.H *et al.*, 1999). One adenine deletion of poly A microsatellite tract within TGFβ1 receptor type II is frequently detected in patients with HCC, and the mutation may cause the abrogation of the function of TGFβ1 receptor type II gene (Furuta.K *et al.*, 1999).

N-NITROSO COMPOUNDS AND HEPATOCELLULAR CARCINOMA

NDEA is one of the potent carcinogenic dialkyl nitrosamine present in the tobacco smoke, water, cheddar cheese, different types of flesh, cured and fried meats and in a number of alcoholic beverages and is used as a solvent in the fibre industry as a softner for copolymers and as an additive in lubricants (Brown.J.L, 1999). The nitrosamines are 'direct' acting carcinogens. Bioactivation of nitrosamines, on the other hand occurs through an initial 2-hydroxylation, catalysed by cytochrome P-450 (Perantoni.A.O, 1998). Human exposure to *N*-nitroso compounds occurs through three main routes (i) exogenous level in foods, (ii) tobacco smoke and (iii) endogenous formation in the acidic environment of the stomach. The endogenous *N*-nitroso compounds are formed *in vivo* from the reaction of nitrosating agent with a number of dietary precursors in an environment. The nitrosating agent is commonly nitrous anhydride which in turn is formed from sodium nitrite added to a number of foods as a preservative and colour enhancer. The stomach has a favourable environment for this acid-catalysed reaction. The major dietary source of nitrate in the diet is vegetable whereas dietary nitrite comes primarily from cured meats. Gene mutation is a relatively early event in NDEA-induced hepatocarcinogenesis in rats (Yamada.Y *et al.*, 1999). There is sufficient evidence for the carcinogenicity of NDEA in experimental animals (Perantoni.A.O, 1998). When administered in the drinking water, NDEA induced liver tumours in guinea pigs, rabbits, dogs, and rats and nasal cavity tumours in rats. When administered in the feed or by gavage, NDEA induced liver tumours in rats, monkeys, mice,

and pigs, kidney tumours in rats, fore-stomach and lung tumours in mice and tumours of the oesophagus in mice and rats (Nakae.D *et al.*, 1997). When administered by inhalation, NDEA induced liver tumours in rats and tumours of the trachea, bronchi, and lungs in hamsters. When administered by subcutaneous injection, it increased the incidence of lung tumours in adult and newborn mice and induced respiratory tract tumours in Syrian golden hamsters. Subcutaneous administration or injection of NDEA induced tumours of the fore-stomach and oesophagus in chinese hamsters, liver tumours in birds, upper respiratory tract tumours in newborn hamsters and liver and respiratory tract tumours in gerbils, guinea pigs, and hedgehogs. When administered subcutaneously to pregnant mothers, the compound induced pulmonary adenomas and liver, oesophagus, and fore-stomach tumours in mouse offspring; kidney and mammary tumours in rat offspring; and tracheal and other respiratory tract tumours in hamster offspring. Intraperitoneal injection of the compound induced liver tumours in adult and newborn monkeys, mice, rats, and hamsters; lung tumours in mice; and respiratory tract tumours in hamsters. When administered by intravenous injection, NDEA induced kidney tumours in rats and nasal cavity tumours in gerbils. When administered by intra-rectal injection, the compound induced HCC in rats. When administered intra-dermally, NDEA induced nasal cavity papillomas in hamsters. When administered by gavage followed by subcutaneous injections, the compound induced a large leiomyosarcoma of the liver. When administered by immersion, NDEA induced liver tumours in fish. *N*-nitroso compounds are a broad class of compounds formed from the nitrosation of substituted amide, ureas, carbamates and guanidines.

REGULATORY SIGNALS OF LIVER CELL PROLIFERATION

The molecular signals controlling liver cell proliferation came out from the studies of liver regeneration after PH and serum free cultures of hepatocytes. Using these two models several growth regulatory factors of hepatic cell proliferation have been identified. The factors identified can be separated into three categories: 1) Complete hepatocyte mitogens, 2) growth inhibitors and 3) growth triggers (co-mitogenic substances or incomplete mitogens) (Michalopoulose.G.K, 1990).

COMPLETE HEPATOCYTE MITOGENS

Complete mitogens are substances that are able by themselves, in chemically defined media and in the absence of serum, to stimulate hepatocyte DNA synthesis and mitosis in otherwise quiescent hepatocyte populations. HGF, EGF, TGFα and heparin binding growth factor -1 have been defined as complete mitogens for hepatocytes and implicated in the control of liver growth (Michalopoulose.G.K & DeFrancis.M.C, 1997).

Hepatocyte growth Factor (HGF)

HGF is a polypeptide with mitogenic and morphogenic effects on different cell types including hepatocytes (Bell.A *et al.*, 1999) (Fausto.N *et al.*, 1995). HGF is expressed as two biologically active isotypes resulting from alternative RNA splicing. HGF and its receptor c-Met are key factors for liver growth and function. HGF-c-Met system may play an important role in the regeneration of hepatocytes as well as in the development of hepatocellular carcinoma in paracrine or autocrine mechanisms (Nakayama.N *et al.*, 1996) (Hu.Z *et al.*, 1996). HGF has been demonstrated to be essential for liver regeneration after PH (Uemura.T *et al.*, 2000). HGF rises to over 20 fold within one hour after PH (Lindroos.P.M *et al.*, 1991). HGF concentrations decline slowly during the first 24 hr but remain elevated for more than 72 hr, eventually returning to basal level. HGF-related signal transduction cascades which contribute to hepatocyte proliferation are initiated within one min after PH (Stolz.D.B *et al.*, 1999). Studies have shown that plasma concentrations of HGF rise substantially in humans when hepatic mass is decreased (Higaki.I *et al.*, 1999). Rapid rise of HGF in the plasma is the mitogenic stimulus leading hepatocytes into DNA synthesis. HGF induces expression of some immediate early genes, liver regeneration factor-1 and insulin like growth factor binding protein-1, suggesting that HGF may be one of the stimuli leading to the rapid changes in gene expression after PH (Stolz.D.B *et al.*, 1999). HGF is a potent mitogen for hepatocytes in culture. Expression of HGF mRNA increases in hepatic Ito cells 3-6 hr after PH and lasts for 24 hr (Kren.B.T *et al.*, 1997). The studies with constructs from promoters of the genes of both HGF and its receptor, c-Met, suggest that interleukin-1 (IL-1) and interleukin-6 (IL-6) may be involved (Otte.J.M *et al.*, 2000).

Tumour necrosis factor-α and Interleukin-6

Recent studies have established that TNFα and IL-6 are important components of the early signalling pathway leading to regeneration. TNF production from Kupffer cells primes the hepatocyte DNA synthesis in liver of rat (Shinozuka.H *et al.*, 1996). Compounds paradoxically increase TNFα mRNA in Kupffer cells enhanced the induction of IL-6 as well as *c-jun*. CAAT enhancer binding proteinβ (C/EBPβ) treatment with antibodies to TNFα before PH resulted in decreased DNA synthesis and abrogation of increases in jun kinase, *c-jun* mRNA, and nuclear activating protein (AP-1) activity (Diehl.A.M & Rai.R.M, 1996). Signalling through tumour necrosis factor receptor type 1 (TNFR-1) using a pathway that involves NF-κB, IL-6, and signal transducer and activator of transcription 3 (STAT3) is required for the initiation of liver regeneration (Webber.E.M *et al.*, 1998).

IL-6 is secreted by Kupffer cells and this secretion is stimulated by TNFα. Plasma IL-6 concentrations increase after PH reaching high levels by 24 hr (Michalopoulose.G.K & DeFrancis.M.C, 1997). IL-6 is a mitogen in cultures of bile duct epithelial cells. Hepatocyte DNA synthesis was found to be suppressed in mice carrying a homozygous deletion of the IL-6 gene where STAT3 activation was markedly reduced (Taub.R *et al.*, 1999). Changes both in DNA synthesis and cell cycle gene expression were corrected by injection of IL-6. The above studies with TNFα and IL-6 clearly document that the early signalling mechanisms that trigger liver regeneration do not proceed normally without these cytokines.

Epidermal Growth Factor and Transforming Growth Factor-α

Both EGF and TGFα are primary mitogens for hepatocytes in culture (Fausto.N *et al.*, 1995). In rats sialadenectomy, which cause a major reduction in plasma EGF, also decrease the hepatic regenerative response. EGF plays a mitogenic role in liver regeneration by abruptly becoming more available to hepatocytes after PH. Rapid tyrosine phosphorylation and down regulation of the EGF receptor occur shortly after PH (Stolz.D.B *et al.*, 1999), suggesting that EGF plays an early mitogenic role. DNA synthesis in culture by EGF is preceded by changes in the expression of cell cycle associated genes.

Within 15 minutes after PH, EGF mRNA levels increase over 10 fold in the remnant liver and diminish below basal levels prior to the first wave of regenerative cell division. This rapid increase in the EGF mRNA levels in the immediate early phase of liver regeneration points to EGF as an autocrine factor in the pre-replicative hepatic growth programme (Mullhaupt.B *et al.*, 1994). EGF has been shown to activate the STAT3, which is a transcription factor complex that pre-exists in the liver (Ruff-Jamison.S *et al.*, 1993). The targets of STAT3 are *c-myc*, *c-fos* and *c-jun* which are immediate early genes required for liver regeneration (Taub.R, 1996). Thus, STAT3 activation may be one of the mechanisms of EGF mitogenesis in liver regeneration. EGF receptor regulate the mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase cascade activation which is the key signalling pathway involved in the regulation of G_1 phase progression in proliferating hepatocytes (Talarmin.H *et al.*, 1999).

TGFα is thought to be involved in liver regeneration, cellular proliferation, and hepato-carcinogenesis. Though EGF may be involved at the early stages of liver regeneration, TGFα appears to play a role at later times. TGFα expression in regenerating liver is strongly correlated with hepatocyte mitosis (Scotte.M *et al.*, 1997). In well-differentiated HCC, nodules are strongly positive for TGFα, while specimens positive for EGF receptors were found mainly in poorly differentiated HCC. This shows the expression of TGFα and EGF receptor might be related to the pattern of histologic differentiation of HCC (Kira.S *et al.*, 1997). TGFα mRNA is induced in hepatocytes within 2 to 3 hr after PH., rises to a peak between 12 and 24 hr and remain elevated for at least 48 hr after PH (Fausto.N *et al.*, 1995). Recent studies have shown that TGFα levels in liver and blood more closely correlate with hepatocyte mitogenesis than HGF levels (Tomiya.T *et al.*, 1998).

GROWTH INHIBITORS

These substances have also been defined in primary culture based on their capability to inhibit EGF mitogenesis.

Transforming Growth factor β1

TGFβ1 is an inhibitor of hepatocyte proliferation in cultures (Michalopoulose.G.K & DeFrancis.M.C, 1997). TGFβ1 is low in normal livers but its expression increase during liver regeneration (Fausto.N *et al.*, 1995). TGFβ1 mRNA increases within 3-4 hours after PH, reaching plateau at 48 to 72 hr (Kren.B.T *et al.*, 1997). Little is known about the determinants governing the termination of the proliferation phase during liver regeneration, although TGFβ1 has been implicated as an important inhibitor of hepatocyte replication in this model. TGFβ1 has been implicated as an inhibitor of cell proliferation and a potent inducer of apoptosis *in vitro* and *in vivo* after the administration of high doses (Fan.G *et al.*, 1998). During liver regeneration there is a pronounced up regulation of expression of TGFβ1. It appears that apoptosis is induced via altered local concentration of TGFβ1, in a paracrine and/or autocrine way (Grasl-Kraupp.B *et al.*, 1998). Hepatocytes were more sensitive to inhibition of DNA synthesis, when the TGFβ1 protein was added at later times in culture, corresponding to the presence of increased TGFβ receptors. When TGFβ1 protein was added to hepatocyte cultures for a short period, 6-24 hr, after cell attachment, it inhibited DNA synthesis more effectively in hepatocytes from regenerating compared with resting livers (Nishikawa.Y *et al.*, 1998). The kinetics of TGFβ1 induction during carcinogenesis were quite different from that of regeneration after PH. HCC initiated with NDEA alone induced TGFβ1 expression for 24 days, and subsequent stimulation by PH on the 14th day after NDEA initiation super-induced TGFβ1 mRNA (50 times that of the control level), as opposed to a transient expression for less than 5 days by PH alone (Simpson.K.J *et al.*, 1997).

Interleukin 1β

Interleukin 1β (IL-1β) is reported to inhibit hepatocyte proliferation. IL-1β is the major inhibitor of mitogen induced hepatic proliferation and IL-1 receptor antagonist abrogated the inhibition. Expression of IL-1β mRNA in whole rat liver following PH was down regulated at 10 hr in the pre-replicative phase of liver regeneration and up regulated at 24 hr and 48 hr during active proliferation (Taub.R *et al.*, 1999). Rat hepatocytes isolated from liver 24 hr after PH showed increased sensitivity to the inhibitory action of

IL-1. Exogenous IL-1β, administered to a group of rats at zero and 12 hr after PH significantly reduced the incorporation of the thymidine analogue, bromodeoxyuridine (BrdU), into hepatocytes at 18 hr (Boulton.R *et al.*, 1997).

Hepatocyte proliferation inhibitor, a protein of molecular weight 15 kDa, inhibited hepatocyte proliferation in cultures (Huggett.A.C *et al.*, 1987). A trypsin-sensitive relatively heat-stable 14 to 17 kDa protein released from non-parenchymal cells also demonstrated to have powerful mito-inhibitory effect on hepatocyte proliferation (Woodman.A.C *et al.*, 1992).

GROWTH TRIGGERS (CO-MITOGENS)

These are substances that affect hepatocyte growth in a positive direction but in an indirect manner. They enhance the mitogenic effect of complete mitogens and decrease the inhibitory effect of growth inhibitors. Apart from this they do not have the direct mitogenic effects of their own in serum-free cultures. The plasticity of growth responses seen during liver cell proliferation is governed by complete mitogen as well as by co-mitogenic substances such as hormones and neurotransmitters.

Hormones

Thyroid Hormones

Studies on the role of thyroid hormones have shown that triiodothyronine (T_3) can induce hepatic proliferation responses after subcutaneous administration in the intact liver (Francavilla.A *et al.*, 1994) (Tessy.T.M *et al.*, 1997). The regenerative response of intact liver after subcutaneous T_3 administration is shown to mimic the DNA synthesis pattern induced by 40% hepatic resection (Francavilla.A *et al.*, 1994). Liver cell proliferation can be induced by primary or direct mitogens, without preceding cell loss and the process is defined as direct (Columbano.A & Shinozuka.H, 1996). Thus, T_3 can act as a primary mitogen inducing direct hyperplasia. Results from our laboratory indicate that thyroid hormones can influence DNA synthesis during liver regeneration by regulating the activity of thymidine kinase, a key enzyme for DNA synthesis (Tessy.T.M *et al.*, 1997). Hypothyroid hepatectomised animals showed significantly lower level of DNA synthesis

than euthyroid counterparts. T_3 treatment of hypothyroid hepatectomised animals caused an additive effect of DNA synthesis. The growth associated genes which are expressed during liver regeneration are also expressed due to T_3 administration (Francavilla.A *et al.*, 1994). Thyroid hormone has been shown to decrease the expression of EGF (Kesavan.P *et al.*, 1991). This may be a possible mechanism of mitogenicity of thyroid hormones in the liver as down regulation of EGF receptors are important for the regenerative response.

Insulin and Glucagon

Intravenous infusion of insulin and glucagon into normal adult rats triggered small but significant DNA synthesis in hepatocytes. Previous evisceration including pancreatic resection largely suppressed liver DNA synthesis 24 hr after PH in untreated rats. This DNA synthesis suppression was not observed in animals that received peripheral injections of insulin and glucagon (Bucher.N.L.R *et al.*, 1978). Rat hepatocytes could be stimulated to synthesize DNA by EGF in combination with insulin and glucagon *in vivo* and *in vitro* (McGowan.J.A & Butcher.N.L.R, 1981) (Hashimoto.M *et al.*, 1998). Insulin and glucagon have been suggested to act synergistically as major regulators of hepatic regeneration (Sato.Y *et al.*, 1989). Peripheral infusion of insulin antiserum substantially blocked hepatic DNA synthesis 24 hr after PH in rats (Bucher.N.L.R *et al.*, 1978). Cultured hepatocytes degenerate and die in the absence of insulin (Michalopoulose.G.K, 1990). Tyrosyl phosphorylation of insulin receptor substrate-1 (IRS-1), a specific target molecule for insulin β-subunit kinase was strikingly enhanced prior to major wave of DNA synthesis after PH. Phosphatidyl-inositol-3-kinase which is involved in proliferative pathway was seen to be associated with IRS-1 following tyrosyl phosphorylation *in vivo* (Sasaki.Y *et al.*, 1993). The number of insulin binding sites was significantly increased and the ratio of insulin to glucagon binding was markedly increased after PH in rats. This can lead to increased uptake of insulin resulting in hepatic proliferation (Gerber.M.A *et al.*, 1983). Previous report from our laboratory shows that the activity of thymidine kinase is regulated by insulin. Streptozotocin-diabetes caused an increase in the maximal velocity (V_{max}) of the enzyme (Waliuala.M.P. *et al.*, 1996) after PH. DNA synthesis was also significantly higher in the regenerating liver of diabetic rats. The low levels of insulin in the diabetic conditions are sufficient to promote proliferation responses of the hepatocyte after PH.

The diabetic state which does not represent a zero level but a relative deficiency of plasma insulin was reported to promote proliferative response of the liver cell following PH in the early hours of liver regeneration (Nakata.R *et al.*, 1986). Probably, the low levels of insulin sensitise the insulin receptor for its ligand resulting in active hepatic extraction of insulin, thereby promoting DNA synthesis. Suppression of hepatic DNA synthesis after PH in rats by exogenous insulin infusions suggests that high plasma levels of insulin are inhibitory for liver regeneration. This correlates well with the observation that plasma insulin levels decline after PH (Knopp.J *et al.*, 1997) (Johnston.D.G *et al.*, 1986). This led to the hypothesis that hypoinsulinemia and hyperglucagonemia are characteristics of enhanced proliferation potential.

Vasopressin, angiotensin II and angiotensin III

Vasopressin, angiotensin II and angiotensin III act through receptors that enhance phosphatidylinositol-4,5-biphosphate (PIP_2) turnover, like norepinephrine (NE). NE is more potent than these substances in enhancing EGF mitogenesis as well as in decreasing TGFβ-mediated DNA synthesis inhibition. Liver regeneration is impaired in rat strains which are congenitally deficient in production of vasopressin (Russell.W.E & Butcher.N.L.R, 1983). Vasopressin synergistically stimulates DNA synthesis in normal and regenerating rat liver cells in presence of hepatocyte growth factor (Metcalfe.A.M *et al.*, 1997). Vasopressin is secreted in the synapses of the sympathetic nerves of the liver along with NE, thus forming a part of the sympathetic control of liver regeneration (Francavilla.A *et al.*, 1989).

Oestrogen

Oestrogens rise after 2/3 PH, reaching a peak at 24-48 hr and testosterone levels decrease. Tamoxifen, an oestrogen receptor antagonist, given after 2/3 PH blocks hepatic DNA synthesis (Francavilla.A *et al.*, 1989) (Francavilla.A *et al.*, 1986). Oestrogen added to primary cultures with serum or EGF induced mitogenesis (Shi.Y.E & Yager.J.E, 1989).

Neurotransmitters

Neurotransmitter receptors linked to second messengers mediate growth regulation in neuronal and non-neuronal cells. Stimulation of proliferation is most often associated with activation of G-proteins negatively coupled to adenylate cyclase (G_i), or positively coupled to phospholipase C (G_q) or to pertussis toxin (PTX) sensitive pathways (G_o, G_i) (Lauder.J.H, 1993).

Norepinephrine

NE is reported to amplify the mitogenic signals of both EGF and HGF by acting on the α1 adrenergic receptor. Serum NE levels reported to be increased in plasma of rat immediately after PH (Knopp.J *et al.*, 1999). It induces the production of EGF and HGF at distal sites and also enhance the response to HGF at target tissues (Broten.J *et al.*, 1999). NE rises rapidly in the plasma within one hour after PH (Knopp.J *et al.*, 1999). NE also offsets the mito-inhibitory effects of TGFβ1 on cultured hepatocytes isolated from the early stages of regeneration (Michalopoulose.G.K & DeFrancis.M.C, 1997). Prazosin, a specific antagonist of α1 adrenergic receptor, as well as sympathetic denervation greatly decrease DNA synthesis at 24 hr after PH (Cruise.J.L *et al.*, 1987). Addition of NE to hepatocyte cultures stimulates Ca^{2+} mobilisation or phosphatidylinositol turnover and either or both of these processes was proposed to be involved in the mitogenicity of NE (Exton.J.H, 1981) (Exton.J.H, 1988) (Nagano.T *et al.*, 1999). Rat hepatomas lacked the α1a and α1b mRNA and receptor binding, while in the human HCC cell line, HepG2,their expression is high but they lack receptor binding (Kost.D.P *et al.*, 1992). Hepatic neoplasms are characterised by an increase in α2 and β-adrenergic receptors and a concomitant decline in α1 receptors (Sanae.F *et al.*, 1989).

Serotonin

Serotonin (5-hydroxytryptamine) caused a dose dependent increase in DNA synthesis in primary cultures of rat hepatocytes in presence of EGF and insulin. The serotonin S_2 receptor antagonists, ketanserin and spiperone, blocked the stimulation of DNA synthesis by serotonin. Binding studies in the membranes of control and regenerating

liver tissues showed an increased involvement of S_2 receptors of serotonin in the regenerating liver during the DNA-synthetic phase. Moreover, serotonin enhanced the phosphorylation of a 40 kDa substrate protein of protein kinase C in the regenerating rat liver (Sudha.B & Paulose.C.S, 1997).

Gamma aminobutyric acid

There are three major GABA receptors, termed $GABA_A$, $GABA_B$ and $GABA_C$ receptors. $GABA_A$ and $GABA_C$ receptors are members of a super-family of transmitter-gated ion channels that include nicotinic acetylcholine, strychnine-sensitive glycine and 5-HT_3 receptors. $GABA_B$ receptors are seven transmembrane receptors that are coupled to G-proteins and activate second messenger systems and Ca^{2+} and K^+ ion channels (Kaupmann.K *et al.*, 1997).

$GABA_A$ receptor

These receptors are composed of five subunits that can belong to eight different subunit classes. $GABA_A$ receptors are hetero-oligomeric Cl^- channels that are selectively blocked by the alkaloid bicuculline and modulated by steroids, barbiturates and benzodiazepines (Sieghart.W, 1995). To date, 16 human $GABA_A$ receptor cDNA have been cloned. Family of $GABA_A$ receptor subtypes exists, generated through the co-assembly of polypeptides selected from $\alpha 1$ to $\alpha 6$, $\beta 1$ to $\beta 3$, $\gamma 1$ to $\gamma 3$, δ, ε and π to form what is most likely a pentameric macromolecule. The gene transcripts, and indeed the polypeptides, show distinct patterns of temporal and spatial expression, such that the $GABA_A$ receptor subtypes have a defined localisation that presumably reflects their physiological role (Whiting.P.J *et al.*, 1999).

$GABA_B$ receptor

To date, three $GABA_B$ receptor proteins have been cloned and these resemble metabotropic glutamate receptors (Kaupmann.K *et al.*, 1997). $GABA_B$ receptors are hetero-oligomeric receptors made up of a mixture of a combination of the subunits. These receptors are selectively activated by (-)-baclofen and CCGP27492 and are blocked by phaclofen, the phosphonic acid analogue of baclofen (Chebib.M & Johnston.G.A, 1999). It

has been found that expression of a fully functional $GABA_B$ receptor requires coupling between two separate and distinct gene products: $GABA_B$ R1 and $GABA_B$ R2. Besides being the first example of a functional heterodimeric metabotropic receptor, the components and molecular configuration of the $GABA_B$ receptor suggest novel mechanisms for producing pharmacologically distinct subtypes of G-protein coupled receptors (Bowery.N.G & Enna.S.J, 2000).

$GABA_C$ receptor

$GABA_C$ receptors represent a relatively simple form of transmitter-gated Cl^- channel made up of a single type of protein subunit. Two human $GABA_C$ receptor cDNA have been cloned. These receptors are not blocked by bicuculline nor are they modulated by steroids, barbiturates or benzodiazepines. Instead, $GABA_C$ receptors are selectively activated by the conformationally restricted analogues of GABA in the folded conformation cis-4-aminocrotonic acid and (1s,2R)-2-(aminomethyl)-1-carboxycyclopropane. (1,2,5,6-Tetrahydropyridine-4-yl)methylphosphinic acid, a methylphosphinic acid analogue of GABA in a partially folded conformation, is a selective antagonist at $GABA_C$ receptors. (Chebib.M & Johnston.G.A, 1999).

GABA AND LIVER

Although originally identified as the principal inhibitory neurotransmitter in the mammalian brain, GABA has since been demonstrated to be present and physiologically active in different tissues throughout the body (Erdo.S.L & Wolff.J.R, 1990). Liver possesses sodium-independent, bicuculline-sensitive $GABA_A$ receptor sites which when innervated cause marked hyperpolarisation of the hepatocyte transmembrane potential (Minuk.G.Y *et al.*, 1987). Liver also contains a sodium-dependent, bicuculline-insensitive GABA transport system (Minuk.G.Y *et al.*, 1984). Hepatic GABA appears to be derived from two sources: extrahepatic GABA synthesis (associated with efficient GABA uptake mechanisms) and *in situ* GABA production through specific hepatic GABA synthetic pathways (Minuk.G.Y, 1986). Detectable amounts of GABA have been reported in the livers of all animal species (Minuk.G.Y, 1993). During the process of identifying and characterising the GABA transport system in liver it was noted that bicuculline, a GABA

receptor antagonist, caused a significant decrease in the specific binding of GABA to isolated rat hepatocytes (Minuk.G.Y *et al.*, 1984). The specific binding of GABA to hepatocytes does occur under receptor-binding conditions, i.e., in the absence of sodium and blocked by the presence of bicuculline. Moreover, the physiological effect of activating this receptor system was identified when hepatocyte transmembrane potential was shown to increase by approximately 5 mV when GABA was added to a suspension of isolated hepatocytes. The hyperpolarising effect was even more striking when muscimol, a stable $GABA_A$ receptor agonist, caused a 22 mV increase in hepatocyte transmembrane potential (Minuk.G.Y *et al.*, 1987).

CLINICAL IMPLICATIONS OF HEPATIC GABA RECEPTOR ACTIVITY IN LIVER

The precise role of the GABA receptor system in the liver remains unclear. The following neurotransmitter-mediated events have been studied in relation to possible GABAergic activity: (1) Alterations in hepatic blood flow - intravenous infusion of GABA in anaesthetised dogs were found to cause a slight decrease in portal venous flow but no change in hepatic arterial flow (Minuk.G.Y & MacCannell.K.L, 1988). (2) Regulation of hepatic bile flow - GABA infusions in rats were weakly choleretic but only at systemic concentrations well beyond physiologic levels (Minuk.G.Y & Sargeant.E.J, 1984). Hepatic venous glucose concentrations increased following portal venous infusions of GABA in rats but the increase never exceed 15% of baseline values (Minuk.G.Y, 1986). The possibility that GABA might play a role in regulating hepatic regenerative activity is intriguing for a number of reasons. Firstly, it had previously been shown that GABA concentrations increase as the development of tissues *in utero* approach maturation (Gilon.P *et al.*, 1987) (Gilon.P *et al.*, 1987). This had been interpreted as indicating that GABA was responsible for or at least contributed to the suppression of rapid growth by these tissues. Secondly, exogenous GABA has been reported to completely inhibit the growth of murine squamous cell carcinoma and HeLa cell lines (Boggust.W.A & Al-Nakib.T, 1986). Increasing malignancy of gliomas correlates with a decrease of GABA binding sites (Jussofie.A *et al.*, 1994). Gliomas with highest malignancy grade IV according to WHO classification and with high proliferation rate, lack expression of functional GABA receptors (Labrakakis.C *et al.*, 1998). Increased GABAergic activity inhibits the growth of the liver following partial

hepatectomy and during recovery from ethanol, galactosamine and carbon tetrachloride forms of hepatic injury (Minuk.G.Y *et al.*, 1995) (Zhang.M *et al.*, 1996) (Kaita.K.D.E *et al.*, 1998). These data further supported the concept that GABA possesses growth-regulatory properties. Thirdly, GABA is a derivative of putrescine, a compound that is essential for hepatic regeneration (Luk.G.D, 1986). Thus, a negative feedback loop might exist where by excess GABA concentrations serve to inhibit further putrescine production. Exogenous GABA significantly decreased the amount of restituted liver mass and rate of protein synthesis by the liver following PH (Minuk.G.Y & Gauthier.T, 1993). The effect of GABA on hepatic polyamine concentrations provided some insight as to how GABA achieved the inhibitory effect on liver growth. Specifically, GABA attenuated the rise in hepatic putrescine levels by 64-100% (Minuk.G.Y *et al.*, 1991). Combination of ethanol plus GABA has a greater inhibitory effect on hepatic DNA synthesis following PH than ethanol alone (Zhang.M *et al.*, 1998). Recently it has been shown that increased GABAergic inhibition rather than decreased putrescine stimulation is more likely to play a role in ethanol-induced inhibition of hepatic regeneration (Lou.G *et al.*, 1999).

NERVOUS SYSTEM AND LIVER REGENERATION

Although abundant anatomical, physiological and pharmacological evidences suggest that the autonomic nervous system plays an important role in regulating hepatic functions (Lautt.W.W, 1983) (Shimazu.T, 1983), the transmitters in central nervous system mediating these effects are not well characterised. The liver is richly innervated and the autonomic nervous system has an important role in the process of hepatic proliferation after PH and experimental liver necrosis (Cruise.J.L *et al.*, 1987) (Tanaka.K *et al.*, 1987). Vagotomy inhibits and delays DNA synthesis and proliferation of liver cells after PH, suggesting an involvement of the parasympathetic nervous system (Kato.H & Shimazu.T, 1983). Lesions of the ventromedial hypothalamic nucleus facilitates liver regeneration after PH and this effect was inhibited by vagotomy (Kiba.T *et al.*, 1994). The hepatic sympathetic nervous system has been implicated to be important in DNA synthesis during liver regeneration (Morley.C.G.E & Royse.V.L, 1981). It was also reported that the increase of DNA synthesis after lateral lesions of the hypothalamus blocked by hepatic sympathectomy and vagotomy (Kiba.T *et al.*, 1995). Chemical sympathectomy had varying

influences on rat liver regeneration. Reserpine, which depletes catecholamine stores, inhibited incorporation of [^{3}H]thymidine into liver DNA at 24 hr post-hepatectomy (Ashirf.S *et al.*, 1974) (Cihak.A & Vaptzarova.K.I, 1973). 6-Hydroxydopamine (6-OHDA) administration destroys adrenergic nerve terminals and its acute administration has been reported to enhance DNA synthesis in the regenerating liver (Ashirf.S *et al.*, 1974). This may be explained by false neurotransmitter effects of 6-OHDA or by leaking of NE from damage terminals. More long term treatment with this compound has been reported to decrease activity in the regenerating liver (Morley.C.G.E & Royse.V.L, 1981). Guanethidine which blocks sympathetic neuro-effector functions (Johnson.E.M & Manning.P.T, 1984) has been shown to depress DNA synthesis in the regenerating liver (Ashirf.S *et al.*, 1974). Thus, *in vivo* studies suggest a role for the sympathetic nervous system in liver regeneration. The hypothalamus is crucial for co-ordinating neurohormonal responses (Oomura.Y & Yoshimatsu.M, 1984). The autonomic nervous system links the hepatic parenchyma to the autonomic centres in the hypothalamus (Nobin.A *et al.*, 1978). Hence, the hypothalamus and other brain regions may play a crucial role in governing the process of liver regeneration by direct innervation.

SIGNAL TRANSDUCTION AND TRANSCRIPTIONAL REGULATION DURING HEPATIC PROLIFERATION

Following PH, there is a rapid and highly orchestrated series of biochemical events which occur prior to cellular proliferation. Some of these events are presumably intimately linked with the eventual regeneration of the liver, whereas others are likely to be stress related or required for the continued differentiated function of the liver while regeneration is occurring. There is a progressive increase in *c-jun* mRNA levels after sham operation, one-third PH, and two-thirds PH. A concomitant increase in activating protein 1 (AP-1) binding activity is also observed. The c-Jun protein is a major constituent of the AP-1 complex in quiescent and early regenerating liver. The activity of c-Jun amino-terminal kinase (JNK), which phosphorylates the activation domain of the c-Jun protein, is markedly stimulated after one-third and two-thirds PH. C-Jun amino-terminal kinase-1 is a constituent of this stimulated JNK activity after PH. When primary cultures of adult rat hepatocytes are incubated with EGF or TGFα, AP-1 transcriptional activity is increased

and the activation domain of the c-Jun protein is further potentiated. Phosphopeptide mapping of the endogenous c-Jun protein in proliferating cultured hepatocytes demonstrates phosphorylation of the c-Jun activation domain. Pretreatment of animals prior to PH with a neutralising antibody to TNFα, inhibits hepatocyte DNA synthesis and JNK activation. It is concluded that the stimulation of one-third or two-thirds PH activates JNK through a mechanism that requires TNFα, which phosphorylates the c-Jun activation domain in hepatocytes, resulting in enhanced transcription of AP-1-dependent genes. IL-6 is reported to be an important mediator of liver regeneration (Taub.R *et al.*, 1999). Two transcription factor complexes identified are NF-κB and STAT3, that pre-exist in normal liver in an inactive form. Cytokines such as TNFα, IL-1 and IL-6 induce both NF-κB and STAT pathways and suggests a common mechanism for the activation of both transcription factor complexes, phosphorylation of Jak1 and to a lesser extent Jak2 with STAT phosphorylation. Ras and mitogen activated protein kinase (MAPK) activation has been reported following IL-4 stimulation of hepatocytes (Chuang.L.M *et al.*, 1996). Interferon (IFN) and IL-6, but not IL-1β, TNFα and EGF, activate STAT3 in rat hepatocytes and human hepatoma cells (Kordula.T *et al.*, 1995). IL-1β induces the stress activated protein kinase (SAPK) pathway in HepG2 cells but does not activate Ras or MAPK (Bird.T.A *et al.*, 1994). Oxidative stress can also induce the MAPK, which via phosphorylation of protein phosphatases can dephosphorylate and inactivate hepatic p38, a phosphoprotein constitutively active in the liver (Mendelson.K.G *et al.*, 1996). STATs are transcription factors that require tyrosyl phosphorylation before they can translocate to the nucleus and bind to regulatory elements of genes (Sadowski.H.B *et al.*, 1993). The targets of STAT3 are *c-myc*, *c-fos* and c-*jun* (Taub.R, 1996). EGF and IL-6 have been shown to activate the STAT3 (Ruff-Jamison.S *et al.*, 1993) (Ruff-Jamison.S *et al.*, 1994). The finding of STAT3 activation in liver regeneration supports the importance of EGF in liver regeneration. In addition TNFα can induce specific hepatocyte phosphatase expression, and hence modulate signalling via cytoplasmic or membrane-bound protein kinases (Ahmad.F & Goldstein.B.J, 1997).

Ligand activation of receptors with tyrosine kinase activity appears to play an important role in promoting hepatocyte proliferation (Marshall.C.J, 1995). EGF, TGFα and HGF bind to this class of receptor. Phospholipase C gamma (PLCγ),

phosphatidylinositol-3-kinase (PI-3K), Src-related tyrosine kinase p59 fyn, Grb2 and Ras GAP are among the downstream signal transducing proteins that have been shown to bind directly to specific sequences surrounding the phosphorylated tyrosine residues of receptor tyrosine kinases (RTKs) (Hill.C.S & Treisman.R, 1995) (Heldin.C.H, 1995) (Marshall.C.J, 1995). One of these complexes, Grb2-SOS, interacts with membrane-associated Ras and facilitates the exchange of GDP for GTP. This activates Ras, which then activates cytosolic Raf, which in turn activates another cytosolic kinase, MEK by phosphorylating it. Activated MEK then phosphorylates and activates MAPK and permits them to translocate to the nucleus (Heldin.C.H, 1995) (Marshall.C.J, 1995). Several potential nuclear targets of the MAPKs have been identified, including growth regulatory transcription factors, such as ets-like protein-I (Elk-I), c-Myc and CCAAT/enhancer binding protein β (C/EBPβ) (Hill.C.S & Treisman.R, 1995). Phosphorylation of these transcription factors regulates their transcriptional activity and hence modulates expression of their target genes (Hill.C.S & Treisman.R, 1995) (Hunter.T, 1995).

EGF and HGF increase MAPK phosphorylation in primary hepatocyte cultures (Stolz.D.B & Michalopoulos.G.K, 1994). The Src-homology region of activated c-Met (the HGF receptor) and the EGF receptor bind different downstream signal transduction elements (Songyang.Z *et al.*, 1993). Tyrosine kinase pathways appear to regulate not only entry into the initial phases of the cell cycle but also progression through later pre-replicative stages and into S phase. In cultured cells, EGF and HGF probably activate PLCγ and PI-3K because these mitogens increase PIP_2 hydrolysis to inositol triphosphate (IP_3) and diacylglycerol (DAG) (Graziani.A *et al.*, 1991). DAG, in turn, activates protein kinase C (PKC) and IP_3 facilitated release of Ca^{2+} from intracellular stores (Baffy.G *et al.*, 1992) (Graziani.A *et al.*, 1991). Increased phosphoinositide hydrolysis and calcium transients have also been documented in the regenerating liver after PH (Bucher.N.L.R, 1991). Although this is consistent with mitogenic activation of RTKs during a hepatic growth response *in vivo*, G-protein coupled receptors can also activate these responses (Neer.E.J, 1995).

G_i *PROTEIN AND CELL PROLIFERATION*

A direct role of $G\alpha_1$ in oncogenesis was first postulated when a constitutively active, GTPase-deficient mutant of $G\alpha_1 2$, gip2, was isolated from several human endocrine tumours and subsequently shown to induce neoplastic transformation of Rat-1 fibroblasts (Pace.A.M *et al.*, 1991). There are p21 Ras-dependent and independent mitogenic activations (Gupta.S.K *et al.*, 1992) (Winitz.S *et al.*, 1993). The signals are PTX sensitive, indicating the involvement of a $G_{i/o}$ family heteromeric G-protein. $GABA_B$ receptor which is G_i-protein coupled reported to be over expressed in human breast cancers (Mazurkiewicz.M *et al.*, 1999). Several hepatocyte co-mitogens such as glucagon, epinephrine, norepinephrine and vasopression bind to plasma membrane receptors that activate heterotrimeric G-proteins. Several lines of evidence suggest that activation of receptors that couple to heterotrimeric G-proteins is important in regulating liver regeneration after PH. The expression of the stimulating and inhibitory α subunits of G-proteins that couple various receptors to their effector targets like adenylyl cyclase is differentially regulated during the early pre-replicative period in the liver. Thus, the biphasic increase in hepatic cAMP concentrations that occurs after PH correlates temporally with increased phosphorylation of the cAMP regulatory element binding protein (CREB) and with increased expression of other cAMP regulated transcription factors, influencing induction of the cAMP-inducible genes in the regenerating liver (Diehl.A.M & Rai.R.M, 1996). In Rat-1 fibroblasts, *erk* activation via endogenous insulin like growth factor I (IGF-I) receptor and G_i-coupled lysophosphatidic acid receptor (LPR) is sensitive to PTX treatment suggesting a cross talk between the receptors leading to mitogenesis (Luttrell.L.M *et al.*, 1995). The α-subunits of G_o and G_i can serve as substrates for insulin receptor-mediated tyrosyl phosphorylation *in vitro* (Krupinski.J *et al.*, 1988). Insulin also inhibits PTX catalysed ADP-ribosylation of G_i by about 50% in isolated rat liver plasma membranes (Rothenberg.P.L & Kahn.C.R, 1988) and promotes guanine-nucleotide binding to BC3H-1 myocyte plasma membranes (Luttrell.L *et al.*, 1990).

Several extracellular factors such as TGFβ, activins and inhibins have been identified that abort cell cycle progression in hepatocytes (Park.D.Y & Suh.K.S, 1999) (Kren.B.T *et al.*, 1997). These agents play a crucial role in terminating the regenerative

response to PH, once recovery of liver mass has been accompanied. In some epithelial cells, TGFβ inhibits cellular proliferation largely through its ability to down regulate the activity of cyclin-dependent kinases Cdk2 and Cdk4 (Menjo.M *et al.*, 1998) (Koff.A *et al.*, 1993). TGFβ decreases the transcription of Cdk4 and down regulates Cdk2 activity by inactivating cyclin E-Cdk2 comp'exes. These events lead to accumulation of hypophosphorylated tumour suppressor gene *Rb* (retinoblastoma) and prevent activation of E2F, a transcriptional activator of many S phase genes (Koff.A *et al.*, 1993). Cyclin A, which is essential for the G_1/S transition after PH (Hunter.T, 1993) is known to complex with and activate Cdk2 and E2F (Menjo.M *et al.*, 1998). This may be the signalling pathway that mediates the antiproliferative actions of TGFβ in hepatocytes. In addition, in cultured hepatocytes, recombinant TGFβ increases the binding activity of C/EBPα (Rana.B *et al.*, 1995), a transcription factor that arrests proliferation in hepatocytes (Diehl.A.M, 1998).

PRIMARY RESPONSE GENES

Following rapid intracellular signal transduction in hepatic cells undergoing regeneration, pre-existing transcription factors are modified, resulting in their activation. These transcription factors are responsible for activating the transcription of primary or immediate early response genes within minutes after PH in a protein synthesis-independent manner (Kren.B.T *et al.*, 1997). A rapid change in the rate of RNA synthesis and overall mRNA steady-state levels after post-PH. Genes rapidly induced in transition from the normally quiescent state of the liver to the growth phase are called immediate-early genes and include certain proto-oncogenes (Herschman.H.R, 1991) (McMahon.S.B & Monroe.J.G, 1992). In fact, more than 70 immediate early genes induced in regenerating rat liver after PH have been identified, of which 41 are novel (Mohn.K.L *et al.*, 1991) (Diamond.R.H *et al.*, 1993). Steady-state mRNA levels of *c-fos* and *c-jun* increase almost immediately after PH, peak at 15 to 30 minutes and return to basal levels by 12 hr. It has been shown that c-Fos protein complex with that of c-Jun to form the transcriptional activating factor AP-1. In contrast to the very early expression of *c-fos* and *c-myc*, the levels of the tumour suppressor gene, p53 transcript increase between 8 and 16 hr and return to normal by the onset of the first wave of DNA synthesis. Expression of members

in *ras* gene family of proto-oncogenes begins during the pre-replicative phase peaks at cell division and slowly returns to normal by 72 to 96 hr (Kren.B.T *et al.*, 1997). As immediate-early genes are induced in a protein synthesis independent fashion, their transition must be activated by transcription factors that are pre-existing in hepatic cells.

The ultimate proliferative response is likely to be dictated by the timing with which the various extracellular signals such as growth factors, neurotransmitters and hormones are presented to the hepatocyte. Both $GABA_A$ and $GABA_B$ receptors are present in hepatocytes (Minuk.G.Y *et al.*, 1987) (Castelli.M.P *et al.*, 1999). In the present study, we examined the GABA receptor gene expression and functional changes in models of regulated and deregulated hepatic proliferation *in vivo* to elucidate its role in liver cell proliferation and liver cancers. The role of $GABA_A$ and $GABA_B$ receptors in hepatocyte proliferation was investigated in primary cultures of rat hepatocytes to understand the mechanism by which these receptors regulate hepatic proliferation. Central nervous system GABAergic changes were also studied during controlled hepatic proliferation and liver cancers.

MATERIALS AND METHODS

MATERIALS AND METHODS

BIOCHEMICALS AND THEIR SOURCES

Biochemicals used in the present study were purchased from SIGMA Chemical Co., St. Louis, U.S.A. All other reagents were of analytical grade purchased locally. HPLC solvents were of HPLC grade obtained from SRL and MERCK, India.

Important chemicals used for the present study

Biochemicals: (Sigma Chemical Co., USA.)

γ-aminobutyric acid (GABA), (±)Norepinephrine, (±)Epinephrine, Sodium octyl sulfonate, Ethylenediamine tetra acetic acid (EDTA), Phenylmethyl sulfonyl fluoride (PMSF), HEPES (N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid 2-Methane 2-propyl thiol, Tris buffer, foetal calf serum (heat inactivated), collagenase type IV, Muscimol, Baclofen, Bicuculline methoiodide, Earl's Balanced Salt solution (EBSS), Collagen from rat-tail, William's medium E, Epidermal Growth Factor (EGF), Transforming Growth Factorβ1 (TGFβ1), Insulin.

Radiochemicals

4-Amino-*n*-[2,3-^{3}H]butyric acid (Specific activity - 84.0 Ci/mmol) was purchased from Amersham Life Science, UK.

Baclofen, (-)-[butyl-4-^{3}H(N)] (Specific activity - 42.9 Ci/mmol), Bicuculline methyl chloride, (-)-[methyl-^{3}H] (Specific activity - 82.9 Ci/mmol) were purchased from NEN Life Sciences products, Inc., Boston, USA.

[^{3}H]Thymidine (Specific activity 18 Ci/mmol) and [^{14}C]Glutamate were from Bhabha Atomic Research Centre, Mumbai, India.

Molecular biology chemicals

Random hexamers, human placental RNAse inhibitor and DNA molecular weight markers were purchased from Bangalore Genei, India. Tri-reagent kit was purchased from Sigma Chemical Co., USA. RT-PCR kit and dNTPs were purchased from Roche

Diagnostics, Germany. PCR primers used in this study was synthesised by Genemed, San Francisco, USA.

Animals

Adult male Wistar rats weighing 200-300g were obtained from Central Institute of Fisheries Technology, Cochin and used for all experiments. They were fed lab chow and water *ad libitum* and maintained under a 12 hr light and 12 hr dark cycle and controlled temperature.

Partial Hepatectomy

Two-thirds of the liver constituting the median and left lateral lobes were surgically excised under light ether anaesthesia, following a 16 hr fast (Higgins.G.M & Anderso.R.M, 1931). Sham operations involved median excision of the body wall followed by all manipulations except removal of the lobes. All the surgeries were done between 7 and 9 A.M to avoid diurnal variations in responses.

Lead Nitrate Administration

Rats received a single intravenous injection of lead nitrate (100μmol/kg of body weight) (Kubo.Y *et al.*, 1996) while the control rats received distilled water only.

***N*-Nitrosodiethylamine Treatment**

Liver cancer was induced using NDEA (Narurkar.L.M. & Narurkar.M.V, 1989). Animals received 0.02% NDEA in distilled water (2.5ml/animal by gavage, 5 days a week for 20 weeks). Rats treated only with distilled water served as control. After 20 weeks all the rats were kept without any treatment for one week and sacrificed at 22nd week. Neoplasia was confirmed by histological techniques.

Sacrifice of Rats

The rats were sacrificed by decapitation and the liver was dissected and stored at -70°C after immediate freezing in liquid nitrogen. The brains were rapidly dissected into different regions (Glowinski.J & Iverson.L.L, 1966). The dissection was carried out on a

chilled glass plate into brain stem, hypothalamus, cerebellum and cerebral cortex. These regions were immediately immersed into liquid nitrogen and stored at -70°C for various experiments.

Measurement of DNA synthesis in liver

DNA synthesis was measured by thymidine incorporation. 5μCi of [^{3}H]thymidine (Sp.acivity 18Ci/mmol) was injected intraperitonially into partially hepatectomised/lead nitrate injected rats to study DNA synthesis at 12, 18, 24, 30, 48, 72 and 168 hours. Tritiated thymidine was injected 2 hr before sacrifice. DNA was extracted from rat liver (Schneider.W.C, 1945). A 10% TCA homogenate was made lipid free and DNA was extracted from the lipid free residue by heating with 5% TCA at 90°C for 15 min. DNA was estimated by diphenylamine method (Burton.K, 1955). Radioactivity was measured in liquid scintillation counter (LKB WALLAC, 1409) after adding scintillation cocktail containing Triton X 100 and DNA synthesis expressed as dpm/mg DNA. DNA synthesis in NDEA treated rats were measured by analysing the activity of thymidine kinase (TK). A 10% liver homogenate was prepared in 50 mM Tris HCl buffer pH 7.5. It was centrifuged at 36,000 *g* for 30 min. TK was assayed by determining the conversion of [^{3}H]thymidine to[^{3}H]thymidine monophosphate [TMP] by the binding of the latter nucleotide to DEAE cellulose disc (Tessy.T.M *et al.*, 1997). 60μl reaction mixture contained 5mM [^{3}H]thymidine (0.5μCi), 10mM ATP, 100mM NaF, 10mM $MgCl_2$, 0.1M Tris-HCl buffer, pH 8.0 and the liver supernatent fraction (2.5μg protein). After incubation at 37°C for 15 min the reaction was stopped by placing the mixture in a boiling water bath for 3 min followed by immersing in an ice bath. Aliquots of 50 μl were spotted on Whatman DE 81 paper discs which were washed with 1 mM ammonium formate, water and three times with methanol. Disks were allowed to dry overnight. The dried disks were placed in counting vials and spotted with 0.3 ml of 0.2M KCl in 1M HCl to release bound thymidine monophosphate and then placed in 10ml of scintillation cocktail. Radioactivity was measured using liquid scintillation cocktail. The activity was expressed as pmoles min^{-1} mg^{-1} protein.

GABA HPLC determinations

GABA HPLC determinations were done by electrochemical detection after derivatisation (Gaskins.H.R *et al.*, 1995). A 10% homogenate of the tissue was made in 0.15M sodium acetate buffer. Ten microliters of sample was mixed with 4 µl of derivatisation reagent (27 mg of *o*-phthaldialdehyde in 10 ml of 50% 0.1 M carbonate buffer (pH 9.6), 50% methanol, and 45 µl of *t*-butylthiol) exactly 3 min before injection into a Shimadzu HPLC system with electrochemical detector fitted with C18-CLC-ODS reverse phase column. Mobile phase was 0.15M sodium acetate buffer (pH 5.4) containing 1mmol/l EDTA and 50% (v/v) acetonitrile delivered at a flow rate of 1.0 ml/min. Quantitation was by electrochemical detection, using a glass carbon electrode set at +0.70 V. The peaks were identified by relative retention time compared with standards and concentrations were determined using a Shimadzu integrator interfaced with the detector.

Analysis of circulating catecholamines

Plasma catecholamines were extracted from 1ml of plasma and diluted twice with distilled water. To it 50 µl of 5mM sodium bisulphite was added, followed by 250 µl of 1M Tris buffer, pH 8.6. Acid alumina (20mg) was added, shaken in the cold for 20 min and was washed with 5mM sodium bisulphite. Catecholamines were extracted from the final pellet of alumina with 0.1N perchloric acid, mixed well and 20 µl of filtered sample was analysed (Jackson.J *et al.*, 1997).

GAD assay in liver

GAD activity was measured in crude extract of liver (Tapia.R & Meza-ruiz.G, 1975). Sample extracts were prepared by making 10% homogenate of the tissue in 0.25M sucrose in sodium phosphate buffer. One ml of reaction mixture contained 50mM sodium phosphate buffer (pH 6.8), 0.2mM pyridoxal phosphate and 1mM dithiothreitol (DTT). 400µl of the homogenate was used for the assay. Kinetic parameters were studied by using varying ^{14}C L-glutamic acid concentrations from 0.11µM-0.66µM. Reaction was started by adding substrate to the mixture and was incubated at 37°C for 1 hr. The reaction was stopped by adding 10N H_2SO_4. The reaction vessel incubated for another 30 min to ensure complete absorption of $^{14}CO_2$ in the centrally placed vessel containing hyamine hydroxide.

After the incubation the hyamine hydroxide solution was transferred to a scintillation vial containing 8 ml of Cocktail-O. The enzyme activity was calculated by the formula

$$\mu\text{moles of } ^{14}C \text{ formed/hr/mg protein} = \frac{\text{Net dpm of } ^{14}CO_2 \text{ recovered} \times \mu\text{moles of } ^{14}C \text{ glutamic acid/vessel}}{\text{dpm. of } ^{14}C \text{ glutamic acid/vessel}}$$

GABA RECEPTOR STUDIES USING [^{3}H] RADIOLIGANDS

$GABA_A$ Receptor Binding Assays

[^{3}H]GABA binding to the GABA receptor was assayed in Triton X-100 treated synaptic membranes (Kurioka.S *et al.*, 1981). Crude synaptic membranes were prepared using sodium-free 10mM tris buffer (pH 7.4). Each assay tube contained a protein concentration of 0.3-0.4 mg. In saturation binding experiments, 1-10 nM of [^{3}H]GABA incubated with and without excess of unlabelled GABA (100μM) and in competition binding experiments the incubation mixture contained 2nM of [^{3}H]GABA with and without muscimol at a concentration range of 10^{-9}M to 10^{-4}M. The incubation was continued for 20 min at 0-4°C and terminated by centrifugation at 35,000 *g* for 20 min. [^{3}H]GABA in the pellet was determined by liquid scintillation spectrometry. Specific binding was determined by subtracting non-specific binding from the total binding. The non specific binding determined showed 30-40% in all our experiments. Protein concentrations were estimated (Lowry.O.H *et al.*, 1951) using bovine serum albumin as the standard.

[^{3}H]Bicuculline methochloride binding to the GABA receptor was assayed in Triton X-100 treated synaptic membranes. Crude synaptic membranes were prepared using sodium-free 10mM tris buffer (pH 7.4). Each assay tube contained a protein concentration of 0.3-0.4 mg. In saturation binding experiments, 5-75 nM of [^{3}H]bicuculline methochloride incubated with and without excess of unlabelled bicuculline methoiodide (100μM) and in competition binding experiments the incubation mixture contained 5nM of [^{3}H] bicuculline methochloride with and without bicuculline methoiodide at a concentration range of 10^{-9}M to 10^{-4}M. The incubation was continued for 20 min at 0-4°C and terminated by centrifugation at 35,000 *g* for 20 min. [^{3}H] bicuculline methochloride in the pellet was determined by liquid scintillation spectrometry. Specific binding was determined

by subtracting non-specific binding from the total binding. The non specific binding determined showed 30-40% in all our experiments.

$GABA_B$ Receptor Binding Studies

Tritiated baclofen binding to $GABA_B$ receptor in the synaptic membrane preparations were assayed as previously described (Hill.D.R *et al.*, 1984). Crude synaptic membrane preparation was suspended in 50mM Tris-HCl buffer (pH 7.4) containing 2mM $CaCl_2$ and 0.3-0.4 mg protein. In saturation binding experiments, 10-100nM of [^{3}H]baclofen was incubated with and without excess of unlabelled baclofen (100μM) and in competition binding experiments the incubation mixture contained 2nM of [^{3}H]GABA with and without baclofen at a concentration range of 10^{-9}M to 10^{-4}M. The incubations were carried out at 20°C for 20 min. The binding reactions were terminated by centrifugation at 7500 *g* for 10 min. [^{3}H]Baclofen and [^{3}H]GABA in the pellet was determined by liquid scintillation spectrometry. Specific binding was determined by subtracting non-specific binding from total binding. The non specific binding determined showed 30-40% in all our experiments.

Receptor Binding Parameters Analysis

The receptor binding parameters determined using Scatchard analysis(Scatchard.G, 1949). The maximal binding (B_{max}) and equilibrium dissociation constant (K_d) were derived by linear regression analysis by plotting the specific binding of the radioligand on x-axis and bound/free on y-axis using Sigma plot computer software. This is called a Scatchard plot. B_{max} is a measure of the total number of receptors present in the tissue and the K_d represents affinity of the receptors for the radioligand. The K_d is inversely related to receptor affinity or the "strength" of binding. Competitive binding data were analyzed using non-linear regression curve-fitting procedure (GraphPad PRISM™, San Diego, USA). The concentration of competitor that competes for half the specific binding was defined as EC_{50}. It is same as IC_{50}. The affinity of the receptor for the competing drug is designated as Ki and is defined as the concentration of the competing ligand that will bind

to half the binding sites at equilibrium in the absence of radioligand or other competitors(Cheng.Y & Prusoff.W.H, 1973).

Displacement Curve analysis

The data of the competitive binding assays were represented graphically with the negative log of concentration of the competing drug on X axis and percentage of the radioligand bound on the Y axis. The steepness of the binding curve can be quantified with a slope factor, often called a Hill slope. A one-site competitive binding curve that follows the law of mass action has a slope of -1.0. If the curve is more shallow, the slope factor will be a negative fraction (i.e., -0.85 or -0.60). The slope factor is negative because curve goes downhill. If slope factor differs significantly from 1.0, then the binding does not follow the law of mass action with a single site, suggesting a two-site model of curve fitting.

ISOLATION OF RAT HEPATOCYTES AND PRIMARY CULTURE

Buffers Used for Perfusion

Ca 2+-free perfusion buffer

This buffer contained 142 mM NaCl, 6.7 mM KCl, 10 mM HEPES, and 5.5 mM NaOH, pH 7.4. It was made up in sterile triple distilled water and filtered through 0.22 μm filters (Millipore).

Collagenase Buffer

This buffer contained 67 mM NaCl, 6.7 mM KCl, 100 mM HEPES, 4.76 mM $CaCl_2.2H_2O$, and 66 mM NaOH, pH 7.6. It was made up in sterile triple distilled water. Collagenase type IV (0.05%) was added prior to perfusion and filtered through 0.22 μm filters(Millipore).

Collagen-Coating of Culture Dishes

Sterile rat-tail collagen solution (100μg/ml in 0.1%acetic acid) was added to each 35mm culture dish and spread uniformly. After 2 hr, the unattached collagen is aspirated out and the dishes were washed thrice with sterile phosphate buffered saline. Finally, sterile

Earl's Balanced Salt solution (EBSS, Sigma) was added and the dishes were left in the sterile hood till the seeding of cells.

Hepatocyte Culture

Hepatocytes were isolated from adult male Wistar rats by collagenase perfusion (Seglen.P.O, 1971). The liver was perfused *in situ* with the calcium-free HEPES buffer pH 7.4 and then with Ca^{2+} containing collagenase buffer (pH 7.6). Hepatocytes were dispersed from the perfused liver, filtered through nylon mesh and washed by three centrifugations in EBSS. The final cell pellet was resuspended in William's Medium E. Cell viability was tested by trypan blue exclusion. The hepatocyte preparation having a viability of >90% as assessed by trypan blue exclusion was chosen for culture. Hepatocytes were plated on rat tail collagen coated dishes at a density of 10^6 cells/35mm culture dish in 1ml of William's medium E. Cells were allowed to settle and adhere for 3 hr in medium supplemented with 10% Fetal Calf Serum, 10^{-7} M Insulin and 50μg/ml gentamycin sulphate. After that the plating media was replaced by serum- free media containing 10ng/ml EGF and 2.5 μCi/plate of [^{3}H] thymidine. The cultures were incubated for 48 hrs at 37°C in 5% CO_2.

DNA Synthesis Assays in Cultured Hepatocytes

Hepatocytes were washed twice in the cold PBS after 48 hr of incubation and cold 10% TCA was added . The hepatocytes were solubilized by incubation at 37°C for 30 minutes in 1N NaOH and cold 100% TCA was added to the solution to get a final concentration of 15% to precipitate the macromolecules. Then DNA hydrolysed by heating the precipitate at 90°C for 15 minutes in 10% TCA. DNA synthesis was expressed as dpm of [^{3}H] thymidine incorporated/mg protein (Takai.S *et al.*, 1988).

REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

RT-PCR was carried out using Titan™ one tube RT-PCR system (Roche Diagnostics) using specific primers for $GABA_A$ subunits (Borboni.P *et al.*, 1994). cDNA synthesis was performed with AMV (Avian Melanoma Virus) reverse transcriptase enzyme.

The PCR step was carried out with a high fidelity enzyme blend that consisted of Taq DNA Polymerase and Pwo DNA Polymerase. Enzyme was stored in storage buffer (20mM Tris HCl, 100mM KCl, 0.1mM EDTA, 1mM Dithiothrietol (DTT), 0.5% Tween-20 (v/v), 0.5% Nonidet P40 (v/v), 50% Glycerol (v/v): pH 7.5 (25^0C).

Preparation of RNA

RNA was isolated from the livers of control, partially hepatectomised, lead nitrate treated and NDEA treated experimental rats using the Tri reagent kit. 25-50 mg tissue was homogenised in 0.5 ml Tri-Reagent. The homogenate was centrifuged at 12,000 *g* for 10 min at 4^0C. The clear supernatant was transferred to a fresh tube and it was allowed to stand at room temperature for 5 minutes. 100µl of chloroform was added to it, shaken vigorously for 15 seconds and allowed to stand at room temperature for 15 min. The tube was centrifuged at 12,000 *g* for 15 min at 4^0C. Three distinct phases appear after centrifugation. The bottom red organic phase contained protein, interphase contained DNA and a colourless upper aqueous phase contained RNA. The upper aqueous phase was transferred to a fresh tube and 250µl of isopropanol was added and the tubes allowed to stand at room temperature for 10 min. The tubes were centrifuged at 12,000 *g* for 10 min at 4^0C. RNA precipitate forms a pellet on the sides and bottom of the tube. The supernatant was removed and the RNA pellet was washed with 500µl of 75% ethanol, vortexed and centrifuged at 12,000 *g* for 5 min at 4^0C. The pellet was semi-dried and dissolved in minimum volume of DEPC-treated water. 2 µl of RNA was made up to 1 ml and absorbance was measured at 260nm and 280nm. For pure RNA preparation the ratio of absorbance at 260/280 was ≥ 1.7. The concentration of RNA was calculated as one absorbance $_{260}$ = 42µg.

RT-PCR Primers

The following primers were used for $GABA_A$ receptor subunit mRNA expression studies.

5'-TGA GAT GGC CAC ATC AGA AGC AGT-3'	*β*2
5'-TCA TGG GAG GCT GGA GTT TAG TTC-3'	GABA

5'-GAA ATG AAT GAG GTT GCA GGC AGC-3'	$\beta 3$
5'-CAG GCA GGG TAA TAT TTC ACT CAG-3'	GABA
5'-TGT GAG CAA CCG GAA ACC AAG CAA-3'	$\gamma 2$ GABA
5'-CGT GTG ATT CAG CGA ATA AGA CCC-3'	

RT-PCR of $GABA_A$ receptor subunits (Borboni.P *et al*., 1994)

RT-PCR was carried out according to the procedure mentioned in the kit with modifications. The reaction was carried out within a total volume of 20µl reaction mixture in 0.2ml tubes. RT-PCR was performed on an Eppendorf Personal thermocycler. Three separate master mixes were made. Master mix 1 consisted of 200ng RNA template, 83ng Random hexamers and RNase inhibitor (5units). To this 10 µl of master mix 2 was added, master mix 2 consisted of reaction buffer containing 1.5mM $MgCl_2$, 5mM DTT and 0.2mM dNTPs (10mM stock containing mixture of dATP, dCTP, dGTP and dTTP). The tubes were then incubated at 42°C for one hour. After incubation, master mix 3 containing 30nM of the respective primers were added.

Thermocycling profile for touch down RT-PCR

For obtaining higher stringency conditions a touch down RT-PCR profile was adopted. The strategy of touch down PCR involves starting the cycle with a very high annealing temperature and then lowering the annealing temperature with successive cycles.

Following is the thermocycling profile used for GABA receptor $\beta 2$ & $\beta 3$ subunits

42°C -- 1 hr

RT step

50°C -- 20 min

95°C -- 3 min --- Denaturation

95°C -- 40 sec --- Denaturation
I. 65°C -- 1.0 min --- Annealing temperature
II. 68°C -- 1.0 min --- Extention
} 10 cycles, with reduction in annealing by 1°C every second cycle.

III. 95°C -- 40 sec --- Denaturation
IV. 62°C -- 1.0 min --- Annealing
V. 68°C -- 1.0 min --- Extention
} 24 cycles

VI. 68^0C -- 15 min --- Final extention

Following is the thermocycling profile used for GABA receptor γ-2 subunit

42^0C -- 1 hour

RT step

50^0C -- 20 min

95^0C -- 3 min --- Denaturation

95^0C -- 40 sec --- Denaturation
I. 68^0C -- 1.0 min ---Annealing
temperature
II. 72^0C -- 1.0 min --- Extention
} 10 cycles, with reduction in annealing by 1^0C every second cycle.

III. 95^0C -- 40 sec --- Denaturation
IV. 65^0C -- 1.0 min --- Annealing
V. 72^0C -- 1.0 min --- Extention
} 24 cycles

VI. 72^0C -- 15 min --- Final extention

Analysis of RT-PCR product

After completion of RT-PCR reaction 10µl of BPB gel-loading buffer was added to 20µl reaction mixture and the total volume was applied to a 1.8% agarose gel containing ethidium bromide. The gel was run at 60V constant voltage with 0.5 x TBE buffer. The image of the bands was captured using an Imagemaster gel documentation system (Pharmacia Biotech) and densitometrically analysed using Imagemaster ID software to quantitate the $GABA_A$ receptor subunit mRNA expression in control, partially hepatectomised, lead nitrate treated and NDEA treated rats.

Statistical Analysis: Statistical comparisons were performed by Students *t*-test and ANOVA by using GraphPad InStat software.

RESULTS

RESULTS

DNA synthesis in the regenerating rat liver

Tritiated thymidine incorporation into replicating DNA was used as a biochemical index for quantifying DNA synthesis during liver regeneration after PH. DNA synthesis was increased significantly ($p<0.001$) after 18 hr, reached a maximum at 24 hr, and had reversed to control value by 72 hr after PH. DNA synthesis decreased to basal levels by 7 days post PH (Figure-1).

GABAergic Alterations in Brain Regions After Partial Hepatectomy

GABA content

GABA content in cerebellum, hypothalamus and brain stem decreased significantly ($p<0.05$, $p<0.01$) at 12 hr reaching maximum difference at 24 hr after PH while it remained unaltered in cerebral cortex. The changes came back to basal level by 72 hr after PH (Table-1).

GABA Receptor Binding Parameters

GABA receptor of the brain regions were assayed by [^{3}H]GABA binding to the membrane preparations. Binding maximum (B_{max}) of the receptors decreased significantly ($p<0.01$) in cerebellum, hypothalamus and brain stem at 12 hr reaching the maximum difference from control value at 24 hr after PH. Dissociation constant (K_d) of the receptor increased significantly ($p<0.05$) in hypothalamus 24 hr after PH while it remained unaltered in all other brain regions studied. The changes in B_{max} and K_d reached basal level by 72 hr after PH (Table-2, 3 & 4).

Changes in Circulating GABA and Norepinephrine

Serum GABA levels significantly increased ($p<0.05$) in the serum 12 and 24 hr after PH (Table-5). Though the values were high at 72 hr after PH, it showed a declining trend to basal level. After 168 hr the values reversed to control level. Plasma norepinephrine levels also increased very significantly ($p<0.001$) 12 and 24 hr after PH (Table-6). It reached back to basal levels by 72 hr after PH.

Changes in GABA content and GAD activity in regenerating rat liver

GABA content significantly decreased ($p<0.05$) 12 and 24 hr after PH and came back to control level by 72 hr (Table-7). Maximal velocity (V_{max}) of GAD significantly decreased ($p<0.05$) 24 hr after PH and gradually came back to the levels of resting liver by 168 hr after PH (Table-8 & Figure-2). The Michaelis-Menten constant (K_m) of the enzyme remained unaltered during the course of liver regeneration.

DNA sythesis in Lead Nitrate treated and NDEA treated rats

Tritiated thymidine incorporation was increased significantly ($p<0.001$) after 24 hr, reached a maximum at 48 hr, and had almost returned to control value 72 hr after the LN treatment (Figure-3). Thymidine kinase enzyme specific activity was increased significantly ($p<0.001$) in the rats sacrificed 22nd week after the NDEA treatment (Figure-4). Neoplasia was confirmed by the morphological and histological observations (Plate 1 to 4).

GABA content in the brain regions of LN treated, NDEA treated and PH rats

GABA content significantly decreased in cerebellum, hypothalamus and brain stem ($p<0.05$, $p<0.01$) of PH and NDEA treated rats while it increased ($p<0.01$) in LN treated rats. In cerebral cortex GABA content decreased significantly in NDEA treated rats while it remained unaffected in the other two groups (Table-9).

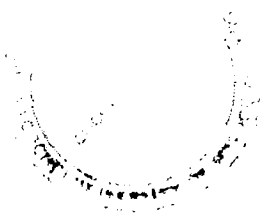
Receptor Alterations in the Brain Regions of Rats

Brain stem

$GABA_A$ binding parameters

$[^3H]$GABA binding parameters

A significant decrease was observed in the B_{max} of $[^3H]$GABA binding to the membrane preparation of NDEA treated ($p<0.001$) and PH rats ($p<0.01$) while it significantly increased ($p<0.05$) in LN treated rats compared with control. The K_d value of NDEA and PH rats increased significantly ($p<0.01$) while it remained unaltered in LN rats compared with control (Table-10 & Figure-5).



Displacement analysis of [^{3}H]GABA by muscimol

The competition curve for muscimol against [^{3}H]GABA fitted for two-sited model in all the groups with Hill slope value away from unity. The log(EC_{50})-1 and $Ki_{(H)}$ increased significantly in NDEA treated rats compared with control denotes a shift in affinity of the receptor towards low-affinity. The binding parameters remained unaltered in other experimental groups compared with control (Table-11 & Figure-6).

[^{3}H]Bicuculline binding parameters

Scatchard analysis of [^{3}H]bicuculline binding to brain stem membrane preparations showed a significant decrease ($p<0.001$) in B_{max} in PH and NDEA treated rats, while it showed an significant increase ($p<0.05$) in LN treated rats compared with the control. K_d showed a significant increase ($p<0.01$) in NDEA treated and PH group while it remained unaltered in LN treated rats (Table-12 & Figure-7).

Displacement analysis of [^{3}H]bicuculline by bicuculline

The competition curve for bicuculline against [^{3}H]bicuculline fitted for two-sited model in all the groups with Hill slope value away from unity. The $Ki_{(H)}$ increased in NDEA and PH treated group without any significant change in log(EC_{50})-1 compared with the control. This indicates a shift in the high-affinity site towards low-affinity. The log (EC_{50})-2 increased in NDEA treated group with an increase in $Ki_{(L)}$ shows a shift in affinity to very low-affinity. In LN treated rats the log (EC_{50})-2 and $Ki_{(L)}$ decreased indicating a shift in affinty of this low-affinity site to high-affinity (Table-13 & Figure-8).

$GABA_B$ binding parameters

[^{3}H]Baclofen binding parameters

The B_{max} of [^{3}H]baclofen binding increased significantly in PH and NDEA ($p<0.01$ and $p<0.001$) with a decrease in K_d ($p<0.05$ and $p<0.01$) compared to control. In LN treated rats these binding parameters remained unaltered (Table-14 & Figure-9).

Displacement analysis of [^{3}H]baclofen by baclofen

The competition curve for unlabelled baclofen inhibited specific [^{3}H]baclofen binding fitted for one site model in all the groups with a Hill slope value near to unity. The log(EC_{50}) and Ki of NDEA treated and PH rats showed a decrease indicating a shift in affinity to high-affinity compared with control. LN treated group did not show any shift in affinity (Table-15 & Figure-10).

Hypothalamus

$GABA_A$ binding parameters

[^{3}H]GABA binding parameters

B_{max} of [^{3}H]GABA binding decreased significantly in NDEA treated and PH treated rats ($p<0.001$ and $p<0.01$) while it significantly increased ($p<0.05$) in LN treated rats compared with control. The K_d value of NDEA treated and PH rats increased significantly ($p<0.01$and $p<0.05$) while it remained unaffected in LN treated rats compared with control (Table-16 & Figure-11).

Displacement analysis of [^{3}H]GABA by muscimol

The competition curve for muscimol against [^{3}H]GABA fitted for two-sited model in all the groups with Hill slope value away from unity. The log(EC_{50})-1 and $Ki_{(H)}$ increased in NDEA treated group compared with control denoting a shift towards low-affinity. Binding parameters remained unaltered in LN treated and PH rats (Table-17, Figure-12).

[^{3}H]Bicuculline binding parameters

Scatchard analysis of [^{3}H]bicuculline binding to hypothalamus membrane preparations of rats showed a significant decrease in B_{max} in PH and NDEA treated rats ($p<0.01$ and $p<0.001$) while it remained unaltered in LN treated rats compared with the control. K_d showed a significant increase in NDEA treated ($p<0.01$) and PH ($p<0.05$) groups while it decreased in ($p<0.05$) LN treated rats compared with control (Table-18 & Figure-13).

Displacement analysis of [^{3}H]bicuculline by bicuculline

The competition curve for bicuculline against [^{3}H]bicuculline fitted for two-sited model in all the groups with Hill slope value near unity. Although the log(EC_{50})-1 value did not show any change the $Ki_{(H)}$ increased significantly in NDEA and PH rats compared with control. This shows a shift from the high-affinity site to low-affinity. In LN rats log(EC_{50})-1 and $Ki_{(H)}$ decreased indicating a shift in affinity to high-affinity (Table-19 & Figure-14).

$GABA_B$ receptor binding parameters

[^{3}H]Baclofen binding parameters

The B_{max} of [^{3}H]baclofen binding increased significantly in PH and NDEA ($p<0.01$ and $p<0.001$) while it remained unaltered in LN treated group. The K_d value decreased significantly ($p<0.01$) in NDEA treated group while in PH and LN treated group it remained unaffected (Table-20 & Figure-15).

Displacement analysis of [^{3}H]baclofen by baclofen

The competition curve for unlabelled baclofen inhibited specific [^{3}H]baclofen binding fitted for one site model in control and experimental group with a Hill slope value near to unity. The log-(EC_{50}) and Ki of NDEA treated rats decreased compared with control indicating a shift in affinity to high-affinity. The binding parameters remained unaltered in LN treated and PH rats (Table-21 & Figure-16).

Cerebellum

$GABA_A$ binding parameters

[^{3}H]GABA binding parameters

A significant decrease was observed in the B_{max} of [^{3}H]GABA binding to membrane preparation of NDEA treated ($p<0.01$) and PH rats ($p<0.05$) while it remained unaltered in LN treated rats compared with control. The K_d value of LN treated rats decreased significantly ($p<0.05$) while it remained unchanged in PH and NDEA treated groups compared with control (Table-22 & Figure-17).

Displacement analysis of [^{3}H]GABA by muscimol

The competition binding curve for [^{3}H]GABA against muscimol fitted for two-sited model in all the groups with Hill slope value away from unity. The log(EC_{50})-1 and $Ki_{(H)}$ increased in LN treated group indicating a shift in affinity to high-affinity compared with control. The binding parameters remained unaltered in NDEA and PH rats (Table-23, Figure-18).

[^{3}H]Bicuculline binding parameters

Scatchard analysis of [^{3}H]bicuculline binding to cerebellum membrane preparations of rats showed a significant decrease ($p<0.05$) in B_{max} of PH and NDEA treated rats, while it showed no change in LN rats. K_d showed an increase in NDEA treated group while it remained unchanged in PH and LN rats (Table-24 & Figure-19).

Displacement Analysis of [^{3}H]bicuculline by bicuculline

The competition curve for bicuculline against [^{3}H]bicuculline fitted for one-sited model in all the groups with Hill slope value near unity. Although the log(EC_{50})-1 remained unaltered in NDEA group its $Ki_{(H)}$ increased compared with control showing a shift in affinity to low-affinity. log(EC_{50})-2 and $Ki_{(L)}$ also increased in NDEA treated group indicating a shift in affinity to very low-affinity. Binding parameters remained unaltered in PH and LN treated groups. (Table-25, Figure-20).

$GABA_B$ binding parameters

[^{3}H]Baclofen binding parameters

The B_{max} of [^{3}H]baclofen binding did not show any change in experimental groups compared with control. The K_d of the receptor decreased significantly in PH and NDEA treated groups ($p<0.05$) compared with control. In LN treated rats it decreased significantly ($p<0.05$) compared with the control (Table-26 & Figure-21).

Displacement analysis of [^{3}H]baclofen by baclofen

The competition curve for unlabelled baclofen against specific [^{3}H]baclofen binding fitted for one site model in all the groups with a Hill slope value near to unity. The log(EC_{50}) and Ki of NDEA and PH rats showed a decrease compared with control indicating a shift in affinity to high-affinity. In LN treated rats log(EC_{50}) and Ki values increased which denoted a shift in affinity towards low-affinity compared with control (Table-27 & Figure-22).

Circulating GABA and NE levels

Serum GABA levels showed a significant increase ($p<0.05$ to $p<0.001$) in all the three experimental groups compared with the control. NDEA treated rats showed maximum increase in serum GABA level ($p<0.001$) compared with the control. Plasma NE levels showed significant increase in PH and NDEA rats ($p<0.001$ and $p<0.01$) compared with the control while it decreased significantly ($p<0.05$) in LN treated rats (Table-28 & 29).

Liver GABAergic Changes During Liver Cell Proliferation

Liver GABA content

GABA content significantly decreased ($p<0.05$; $p<0.01$) in the livers of PH and NDEA treated rats compared with the control. LN treated rats showed a significant increase ($p<0.01$)in hepatic GABA content compared with the control (Table-30).

$GABA_A$ binding parameters

[^{3}H]GABA binding parameters

A significant decrease in the B_{max} of [^{3}H]GABA binding ($p<0.01$) to membrane preparation of NDEA treated and PH rats was observed compared with control while it remained unaltered in LN treated rats. The K_d of the receptor in NDEA treated rats significantly increased ($p<0.01$) while it significantly decreased ($p<0.01$) in LN treated rats compared with control. In PH rats the K_d remained unaltered (Table-31 & Figure-23).

Displacement analysis of [^{3}H]GABA by muscimol

The competition curve for muscimol against [^{3}H]GABA fitted for two-sited model in control, PH and LN treated groups with Hill slope values away from unity. In NDEA treated rats the curve fitted for a one-site model with Hill slop value near unity. In LN treated rats both log (EC_{50})-1, log (EC_{50})-2, $Ki_{(H)}$ and $Ki_{(L)}$ decreased compared with the control indicating a shift in both high-affinity and low-affinity site to respective high-affinity regions. The high-affinity site was shifted to low affinity and low-affinity site was completely lost in NDEA treated rats (Table-32, Figure-24).

[^{3}H]Bicuculline binding parameters

Scatchard analysis of [^{3}H]bicuculline binding to liver membrane preparations of rats showed a significant decrease ($p<0.001$) in B_{max} in PH and NDEA treated rats while it increased ($p<0.05$) in LN treated rats compared with the control. K_d remained unaffected in all the experimental groups compared with control (Table-33 & Figure-25).

Displacement analysis of [^{3}H]bicuculline by bicuculline

The competition curve for bicuculline against [^{3}H]bicuculline fitted for two-sited model in all the groups with Hill slope value away from unity. The binding parameters remained unaltered in all the experimental groups compared with control (Table-34, Figure-26).

$GABA_B$ binding parameters

[^{3}H]Baclofen binding parameters

The B_{max} of [^{3}H]baclofen binding increased significantly in NDEA treated group ($p<0.001$) while it remained unchanged in PH and LN groups compared with control. K_d significantly decreased ($p<0.05$) in PH while it increased in LN treated rats ($p<0.01$). In NDEA treated rats K_d remained unchanged (Table-35 & Figure-27).

Displacement analysis of [^{3}H]baclofen by baclofen

The competition curve for unlabelled baclofen against [^{3}H]baclofen binding fitted for one site model in all the groups with Hill slope value near to or above -1. The

log(EC_{50}) and Ki of PH rats decreased while these were increased in LN treated rats compared with the control. This indicates a shift in affinity towards high-affinity in PH rats andtowards low-affinity in LN treated rats. In NDEA treated group no change in affinity was observed compared with control (Table-36 & Figure-28).

$GABA_A$ Receptor Subunit Gene Expression in the Liver of Rats

$\beta 2$ Subunit Expression

There was a significant decrease in the mRNA expression of $\beta 2$-subunit of $GABA_A$ receptor in NDEA treated rats (50%) and in PH rats (30%) while it remained unaltered in LN treated rats compared with control (Plate-5).

$\beta 3$ Subunit Expression

There was a significant increase (40%) in the mRNA expression of $\beta 3$-subunit of $GABA_A$ receptor in LN treated rats while the expression was almost nil in PH and NDEA treated rats (Plate-6).

$\gamma 2$Subunit Expression

There was a significant decrease in the mRNA expression of $\gamma 2$-subunit of $GABA_A$ receptor (60%) in NDEA treated rats while it increased in LN treated rats (100%) compared with control. The mRNA expression of this subunit remain unaltered in PH rats (Plate-7).

Effect of GABA on cultured hepatocytes

Isolated hepatocytes in serum-free culture medium exhibited very low levels of [^{3}H]thymidine incorporation into DNA. Addition of EGF caused a significant increase ($p<0.001$) in the hepatocyte DNA synthesis. When GABA (100μM) alone was added to cultured hepatocytes there was no significant change in the DNA synthesis from basal level. However, addition of GABA (100μM) in hepatocyte cultures caused a significant inhibition ($p<0.001$) on EGF induced DNA synthesis (Figure-29). Combination of TGF$\beta 1$ and GABA and TGF$\beta 1$ alone did not show any significant change compared with control.

Effect of muscimol on cultured hepatocytes

Addition of muscimol caused a significant decrease ($p<0.001$) in the EGF induced DNA synthesis. When muscimol (100μM) alone was added to cultured hepatocytes there was no significant change in the DNA synthesis compared to control. TGFβ1 significantly inhibited ($p<0.001$) EGF induced DNA synthesis. Combined effect of TGFβ1 and muscimol was found to have greater inhibitory effect ($p<0.01$) on EGF induced DNA synthesis compared with EGF and TGFβ1 treated group (Figure-30).

Dose dependent response of hepatocyte DNA synthesis to muscimol

Different doses of muscimol (10^{-8} M to 10^{-4}M) were added to cultured hepatocytes in the presence of EGF (10 ng/ml). Muscimol at low concentration did not bring about a significant decrease in DNA synthesis. A significant decrease ($p<0.001$) was observed with 10^{-6}M muscimol reaching a maximal effect at 10^{-5}M (Figure-31).

Effect of baclofen on EGF induced DNA synthesis *in vitro*

Addition of baclofen caused a significant increase ($p<0.001$) in the EGF induced DNA synthesis. When baclofen (100μM) alone was added to cultured hepatocytes there was no significant change in the DNA synthesis compared with control. Baclofen also significantly decreased ($p<0.01$) the inhibitory effect of TGFβ1 on EGF induced DNA synthesis (Figure-32). Addition of pertussis toxin abolished DNA synthesis triggered by baclofen (Figure-33).

Dose dependent response of hepatocyte DNA synthesis to baclofen

Varying concentrations of baclofen (10^{-8}M to 10^{-4} M) were added to primary cultures of rat hepatocytes in the presence of fixed concentrations of EGF (10 ng/ml). Lower concentrations of baclofen did not cause any significant change in the DNA synthesis compared with EGF-treated cultures alone. However, 10^{-6}M of baclofen caused a significant increase ($p<0.001$) in DNA synthesis compared with the EGF induced DNA synthesis. The maximal effect of baclofen was observed at 10^{-4}M of baclofen (Figure-34).

Table-1

GABA content in brain regions after partial hepatectomy in rats

(μmoles/g wet weight of the tissue)

Animal Status	Hours after partial hepatectomy	Cerebellum	Hypothalamus	Brain stem	Cerebral Cortex
Sham Operated		3.39 ± 0.16	3.50 ± 0.17	1.57 ± 0.08	1.71 ± 0.11
Partially Hepatectomised	12	2.75 ± 0.10*	2.78 ± 0.10*	1.07 ± 0.09*	1.58 ± 0.17
	24	2.38 ± 0.34*	2.34 ± 0.24**	0.99 ± 0.11*	1.46 ± 0.24
	72	3.12 ± 0.11	3.18 ± 0.23	1.46 ± 0.13	1.59 ± 0.31
	168	3.40 ± 0.15	3.37 ± 0.17	1.38 ± 0.17	1.64 ± 0.15

** $P<0.01$ Compared to the sham-operated rat

* $P<0.05$ Compared to the sham-operated rat

Values are Mean ± S.E.M of 4-6 separate experiments

Table-2

[^{3}H]GABA binding parameters in the cerebellum of rats

Animal Status	Hours after partial hepatectomy	B_{max} (fmoles/mg protein)	K_d (nM)
Sham Operated		1124.96 ± 40.57	11.53 ± 0.41
Partially Hepatectomised	12	1071.20 ± 35.56*	11.78 ± 0.59
	24	1001.20 ± 31.49*	12.49 ± 0.70
	72	1110.00 ± 30.55	12.75 ± 0.16
	168	1120.35 ± 30.55	13.59 ± 0.37

*p<0.05;**p<0.01;***p<0.001 compared to the sham-operated rat

Values are Mean ± S.E.M of 4-6 separate experiments

B_{max}-Binding maximum, K_d-Dissociation constant

Table-3

[^{3}H]GABA binding parameters in the hypothalamus of rats

Animal Status	Hours after hepatectomy	B_{max} (f moles/mg protein)	K_d (nM)
Sham Operated		856.24 ± 37.48	13.78 ± 0.38
Partially Hepatectomised	12	748.33 ± 36.33*	13.17 ± 1.89
	24	628.33 ± 36.33**	18.41 ± 1.46*
	72	322.50 ± 12.50	14.96 ± 0.89
	168	370.00 ± 10.50	14.93 ± 0.44

*$p<0.05$; **$p<0.01$ compared to the sham-operated rat

Values are Mean ± S.E.M of 4-6 separate experiments

B_{max}-Binding maximum, K_d-Dissociation constant

Table-4

[^{3}H]GABA binding parameters in the brain stem of rats

Animal Status	Hours after hepatectomy	B_{max} (fmoles/mg protein)	K_d (nM)
Sham Operated		927.14 ± 20.84	12.44 ± 0.59
Partially Hepatectomised	12	710.40 ± 32.54**	14.47 ± 0.90
	24	697.30 ± 31.54**	13.49 ± 0.70
	72	898.45 ± 25.98	12.56 ± 1.01
	168	957.36 ± 34.25	12.45 ± 0.76

**$p<0.01$ compared to the sham-operated rat

Values are Mean ± S.E.M of 4-6 separate experiments

B_{max}-Binding maximum, K_d-Dissociation constant

Table-5

Serum GABA levels after partial hepatectomy in rats

(nmoles/ml serum)

Animal Status	Hours after hepatectomy	Serum GABA
Sham Operated		4.80 ± 0.10
Partially Hepatectomised	12	6.75 ± 0.15**
	24	6.59 ± 0.19**
	72	5.51 ± 0.21*
	168	4.76 ± 0.14

"P<0.01;** P<0.05 Compared with the sham-operated rat

Values are Mean ± S.E.M of 4-6 separate experiments

Table-6

Levels of plasma norepinephrine during liver regeneration in rats

(nmoles/ml plasma)

Animal status	Hours after PH	Norepinephrine
Sham-operated		0.30 ± 0.10
Partially Hepatectomised	12	1.78 ± 0.25***
	24	1.86 ± 0.03***
	72	0.57 ± 0.03
	168	0.35 ± 0.04

***p<0.001 compared to control

Values are Mean ± S.E.M of 4-6 separate experiments

Table-7

Liver GABA content after partial hepatectomy in rats

(nmoles/g wet wt. of the tissue)

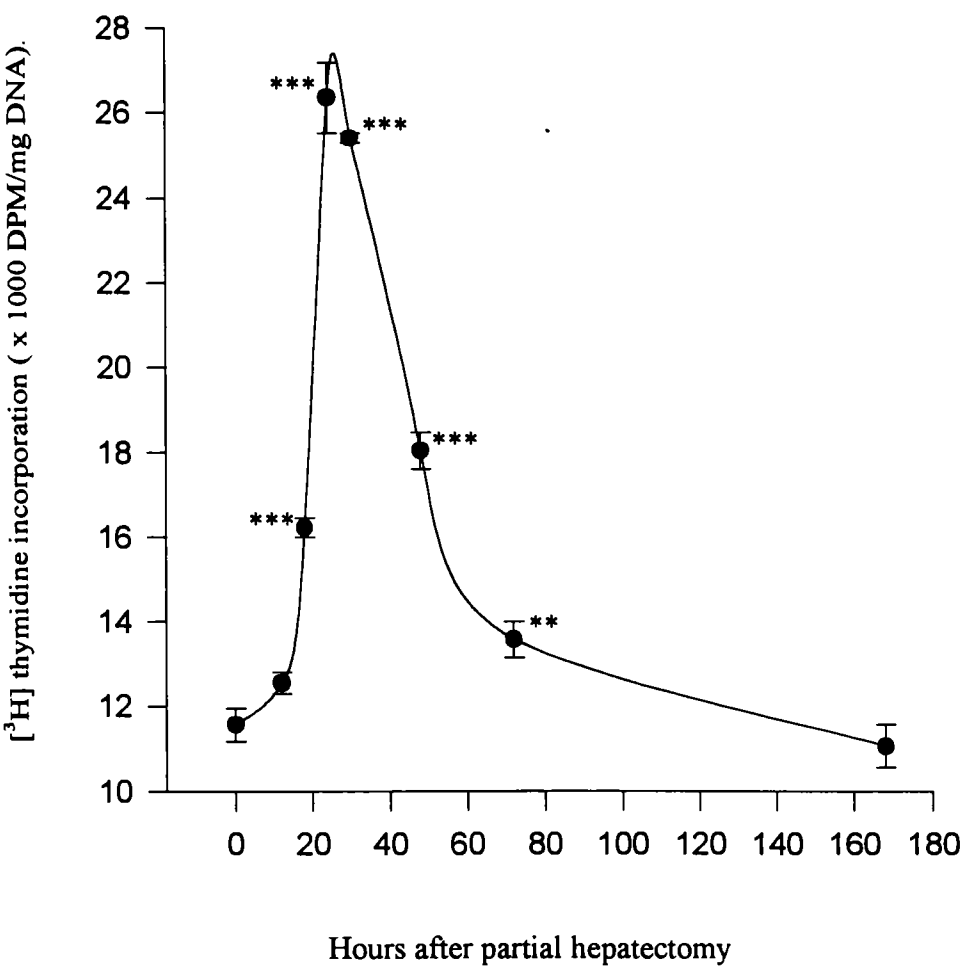
Animal Status	Hours after hepatectomy	Liver GABA
Sham Operated		73.94 ± 6.55
Partially Hepatectomised	12	59.87 ± 4.68*
	24	45.79 ± 5.78*
	72	68.45 ± 7.89
	168	75.45 ± 6.75

*P<0.05 Compared to the sham-operated rat

Values are Mean ± S.E.M of 4-6 separate experiments

Figure-1

DNA synthesis in the liver of rats after partial hepatectomy



*** $p<0.001$ compared with the control
** $p<0.01$ compared with the control

Figure-2

Glutamic acid decarboxylase activity in regenerating rat liver at different time intervals after partial hepatectomy

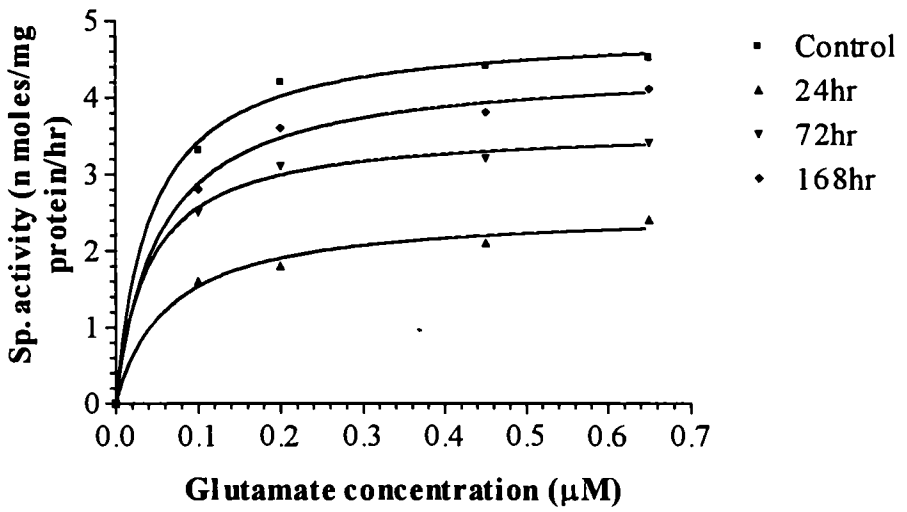


Table-8

Liver GAD activity after partial hepatectomy in rats

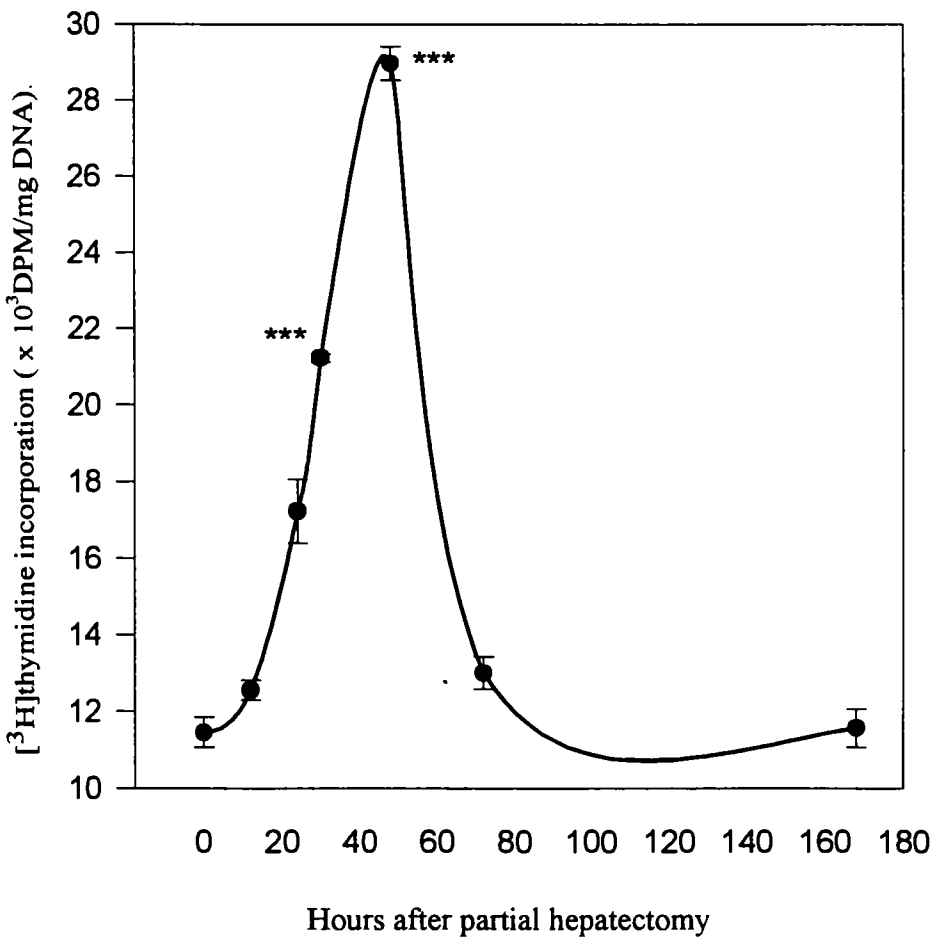
Animal Status	Hours after hepatectomy	V_{max} (nmoles/mg protein/hr)	K_d (μM)
Sham Operated		4.50 ± 0.50	0.05 ± 0.01
Partially Hepatectomised	24	2.40 ± 0.45*	0.08 ± 0.02
	72	3.40 ± 0.35	0.03 ± 0.01
	168	4.20 ± 0.55	0.04 ± 0.01

*P<0.05 Compared to the sham-operated rat

Values are Mean ± S.E.M of 4-6 separate experiments

Figure-3

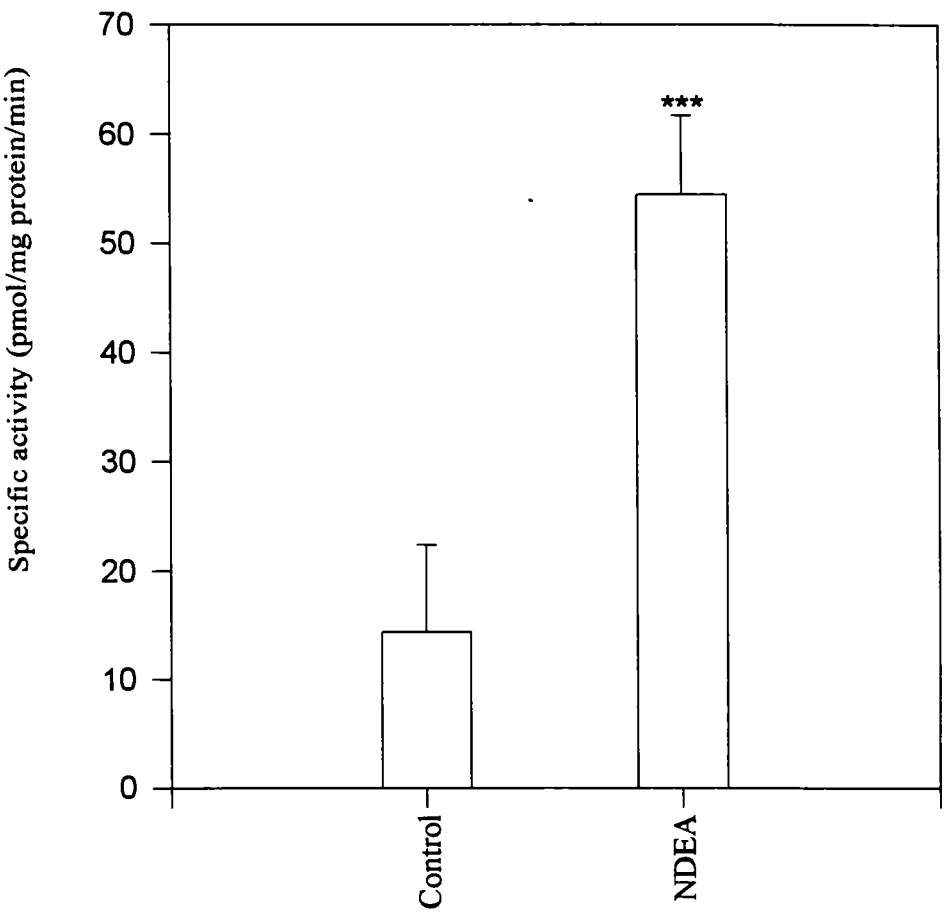
Effect of lead nitrate on liver DNA synthesis



*** $p<0.001$ compared with the control

Figure-4

Thymidine kinase activity in the liver of control and NDEA treated rats



NDEA- *N*-nitrosodiethylamine treated

*** $p<0.001$ compared with the control

Plate-1

Normal rat liver

Plate-2

Rat liver after *N*-nitrosodiethylamine treatment

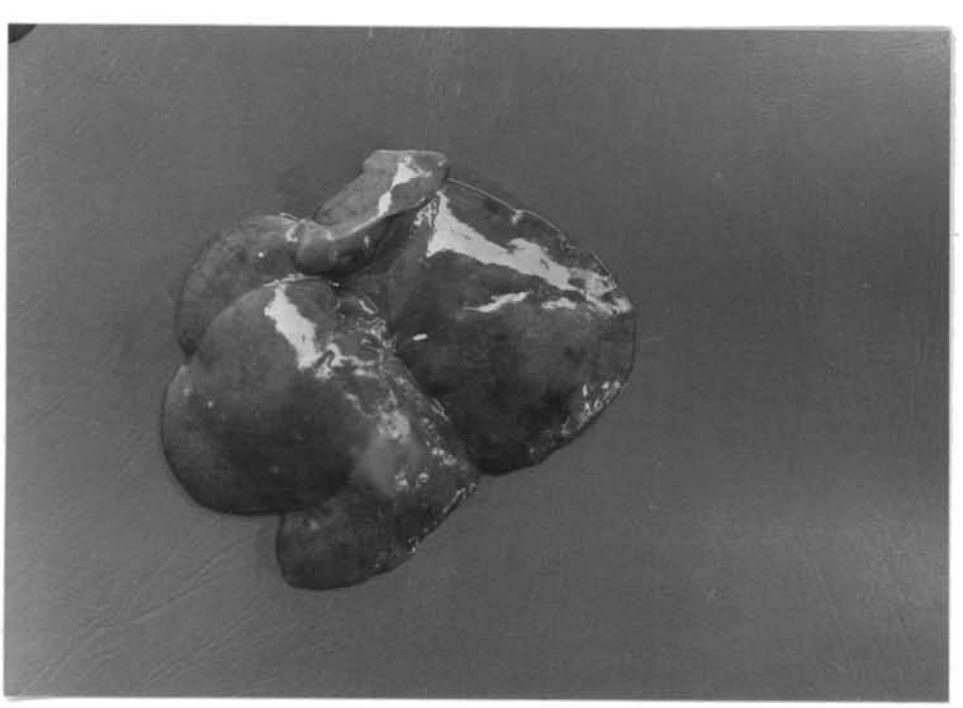


Plate-3

Histological section of normal rat liver

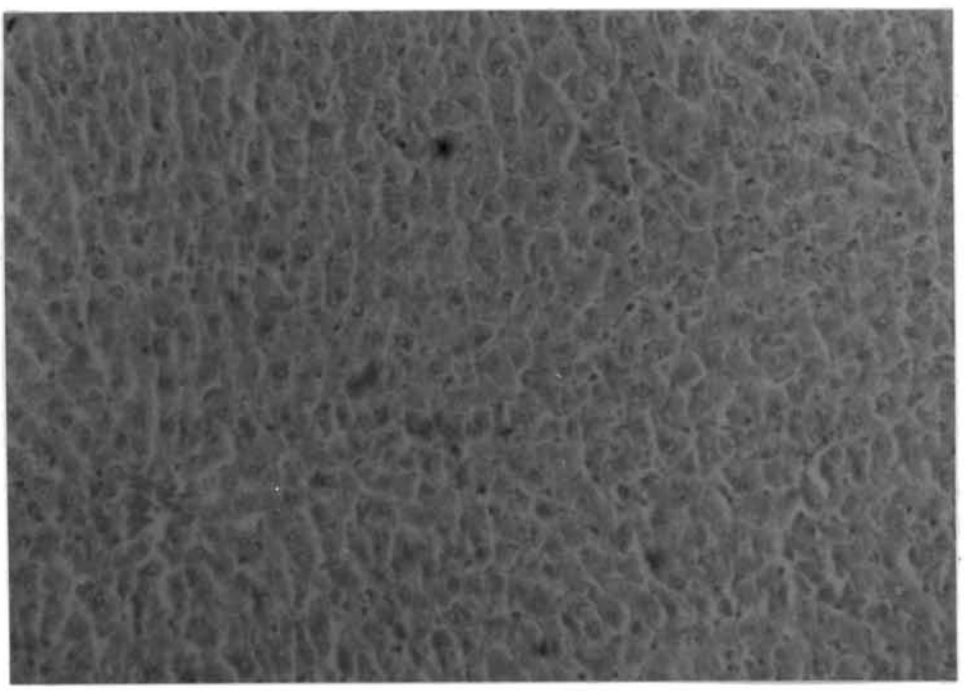


Plate-4

Histological section of rat liver after *N*-nitrosodiethylamine treatment

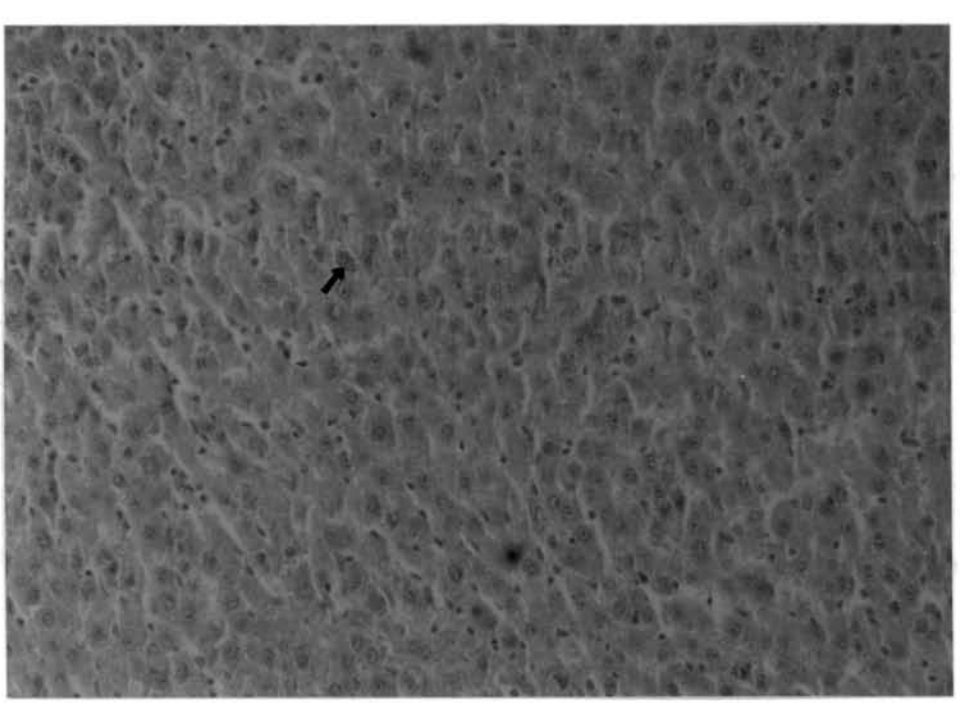


Table-9

GABA content in the brain regions of rats

(μmoles/g wet wt. of tissue)

Brain regions	Control	Partial Hepatectomy	NDEA treated	Lead Nitrate treated
Cerebellum	3.33 ± 0.17¶	2.38 ± 0.34*	1.86 ± 0.10**	5.11 ± 0.35**
Cerebral cortex	1.61 ± 0.15¶	1.46 ± 0.24	1.05 ± 0.11*	1.82 ± 0.34
Brain stem	1.47 ± 0.05¶	0.99 ± 0.11*	0.88 ± 0.11*	2.42 ± 0.11**
Hypothalamus	3.16 ± 0.11¶	2.34 ± 0.24*	2.30 ± 0.12*	4.19 ± 0.08**

**p<0.01, *p<0.05 with respect to control

Values are mean ± S.E.M. of 4-6 separate experiments

¶Control value given is a pooled data from different control experiments since there was no significant difference in values among groups.

Figure-5

Scatchard analysis of [^{3}H]GABA binding against GABA in the brain stem of rats

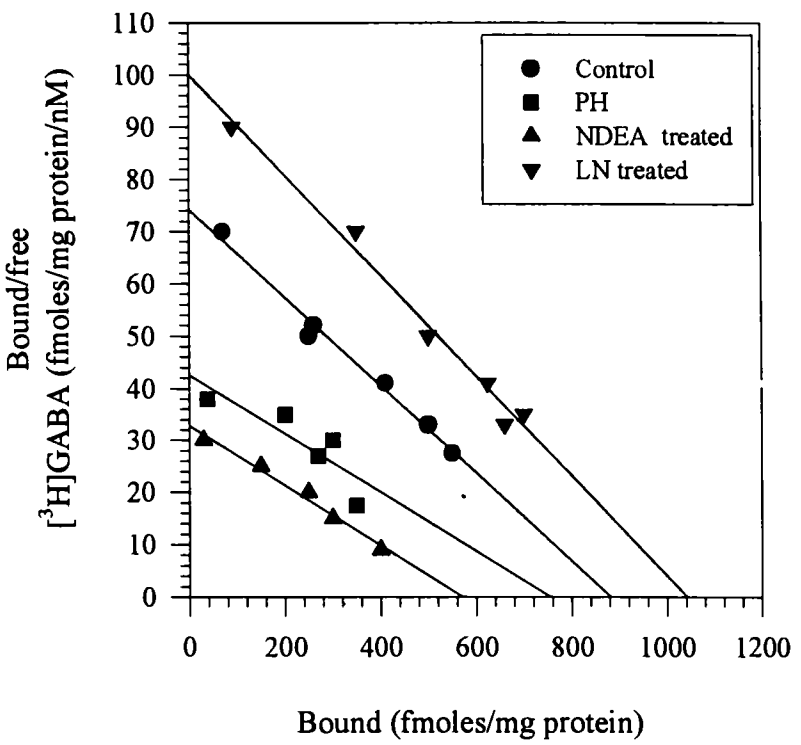


Table-10

[^{3}H]GABA binding parameters in the brain stem of rats

	B_{max} (fmoles/mg protein)	K_d (nM)
Control	938.13 ± 40.84¶	12.64 ± 0.48
Partial Hepatectomy	697.30 ± 31.54**	17.79 ± 1.30**
NDEA Treated	563.33 ± 37.12***	18.42 ± 0.65**
Lead Nitrate Treated	1117.20 ± 43.21*	11.53 ± 1.68

***p<0.001, **p<0.01, *p<0.05 with respect to control

Values are mean ± S.E.M. of 4-6 separate experiments

¶Control value given is a pooled data from different control experiments since there was no significant difference in values among groups

Binding parameters of [^{3}H]GABA against muscimol in brain stem of rats

Animal status	Best-fit model	log (EC_{50})-1	log (EC_{50})-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slopes
Control	Two-site	-8.67	-5.67	1.76×10^{-9}	1.80×10^{-6}	-0.43
Partial Hepatectomy	Two-site	-8.66	-5.82	1.78×10^{-9}	1.27×10^{-6}	-0.52
NDEA Treated	Two-site	-7.81	-5.49	1.29×10^{-8}	2.66×10^{-6}	-0.49
Lead Nitrate Treated	Two-site	-8.65	-5.59	1.86×10^{-9}	2.14×10^{-6}	-0.40

Values are mean of 4-6 separate experiments

Data were fitted with an iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki -The affinity of the receptor for the competing drug. The affinity for the first and second site of the competing drug are designated as $Ki_{(H)}$ (for high affinity) and $Ki_{(L)}$ (for low affinity). EC_{50} is the concentration of the competitor that competes for half the specific binding.

NDEA- *N*-Nitrosodiethylamine

LN- Lead Nitrate

PH- Partial Hepatectomy

Figure-6

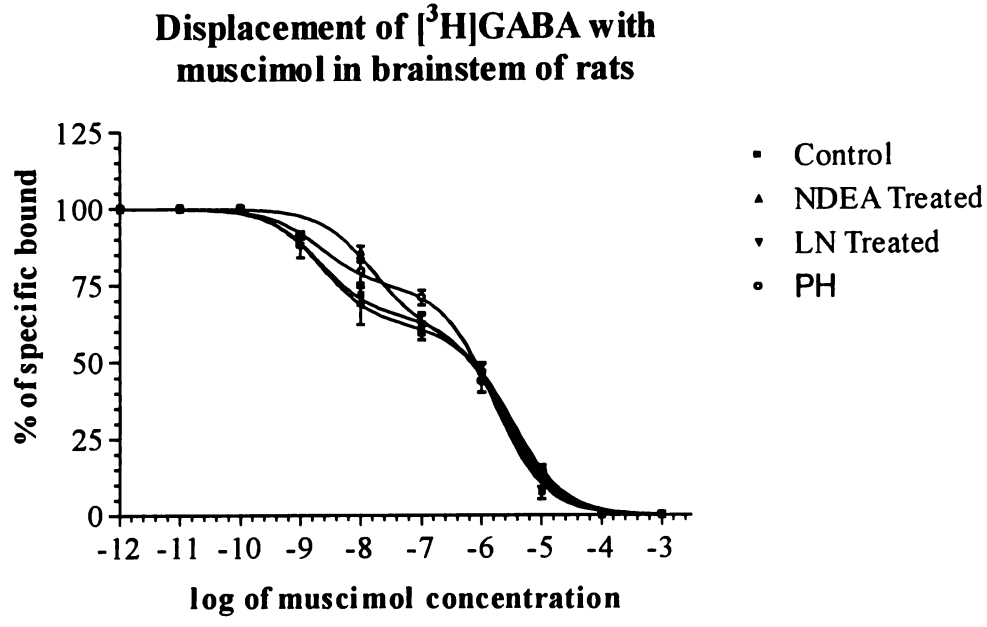


Figure-7

Scatchard analysis of [^{3}H]bicuculline binding against bicuculline in the brain stem of rats

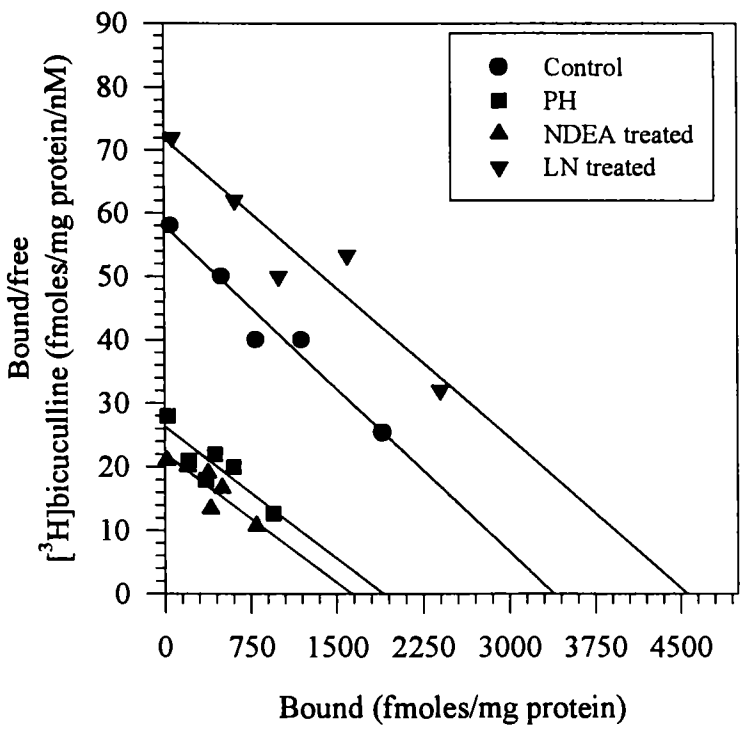


Table-12

[^{3}H]bicuculline binding parameters in the brain stem of rats

Animal status	B_{max} (pmol/mg protein)	K_d (nM)
Control	3.89 ± 0.14¶	58.62 ± 2.20
Partial Hepatectomy	2.20 ± 0.17***	73.45 ± 3.15**
NDEA Treated	1.60 ± 0.16***	76.54 ± 2.45**
Lead Nitrate Treated	4.58 ± 0.265*	63.40 ± 3.13

***p<0.001, **p<0.01 with respect to control

Values are mean ± S.E.M. of 4-6 separate experiments

¶Control value given is a pooled data from different control experiments since there was no significant difference in values among groups

Binding parameters of [^{3}H]bicuculline against bicuculline in brain stem of rats

Animal status	Best-fit model	log (EC_{50})-1	log (EC_{50})-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slopes
Control	Two-site	-7.98	-5.76	9.91×10^{-9}	1.62×10^{-6}	-0.52
Partial Hepatectomy	Two-site	-7.47	-5.70	3.17×10^{-8}	1.87×10^{-6}	-0.65
NDEA Treated	Two-site	-7.46	-4.91	3.28×10^{-8}	1.16×10^{-5}	-0.52
Lead Nitrate Treated	Two-site	-8.12	-6.02	7.19×10^{-9}	9.00×10^{-7}	-0.52

Values are mean of 4-6 separate experiments

Data were fitted with an iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki - The affinity of the receptor for the competing drug. The affinity for the first and second site of the competing drug are designated as $Ki_{(H)}$ (for high affinity) and $Ki_{(L)}$ (for low affinity). EC_{50} is the concentration of the competitor that competes for half the specific binding.

NDEA- *N*-Nitrosodiethylamine

LN- Lead Nitrate

PH- Partial Hepatectomy

Figure-8

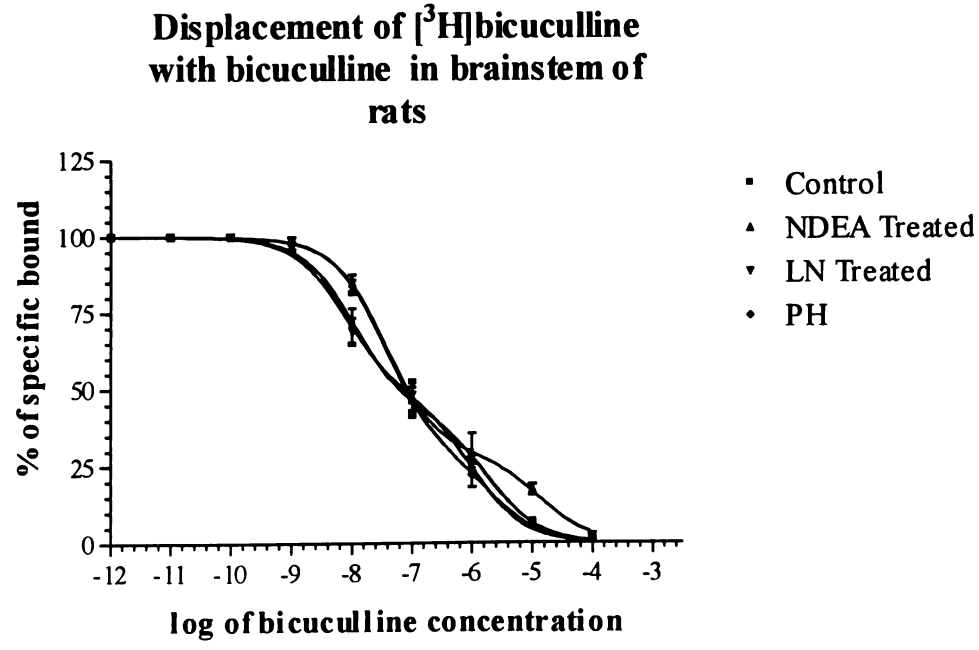


Figure-9

Scatchard analysis of [^{3}H]baclofen binding against baclofen in the brain stem of rats

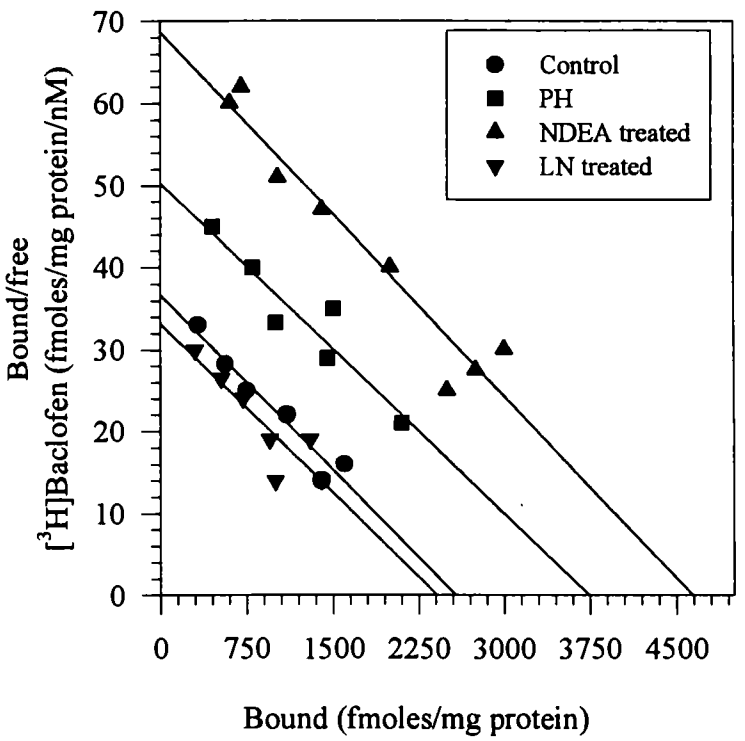


Table-14

[^{3}H]baclofen binding parameters in the brain stem of rats

Animal status	B_{max} (pmol/mg protein)	K_d (nM)
Control	2.18 ± 0.23¶	67.84 ± 3.68
Partial Hepatectomy	3.57 ± 0.13**	52.19 ± 3.20*
NDEA Treated	4.38 ± 0.14***	46.63 ± 4.40**
Lead Nitrate Treated	2.27 ± 0.09	76.53 ± 3.23

***p<0.001, **p<0.01, *p<0.05 with respect to control

Values are mean ± S.E.M. of 4-6 separate experiments

¶Control value given is a pooled data from different control experiments since there was no significant difference in values among groups

Table-15

Binding parameters of [^{3}H]baclofen against baclofen in brain stem of rats

Animal status	Best-fit model	log (EC_{50})	Ki	Hill slopes
Control	One-site	-5.82	1.18×10^{-6}	-1.03
Partial Hepatectomy	One-site	-6.90	9.66×10^{-8}	-0.91
NDEA Treated	One-site	-7.91	9.62×10^{-9}	-0.99
Lead Nitrate Treated	One-site	-5.89	9.88×10^{-7}	-0.95

Values are mean of 4-6 separate experiments

Data were fitted with an iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki - The affinity of the receptor for the competing drug. EC_{50} is the concentration of the competitor that competes for half the specific binding.

NDEA- *N*-Nitrosodiethylamine

LN- Lead Nitrate

PH- Partial Hepatectomy

Figure-10

Displacement of [^{3}H]baclofen with baclofen in brainstem of rats

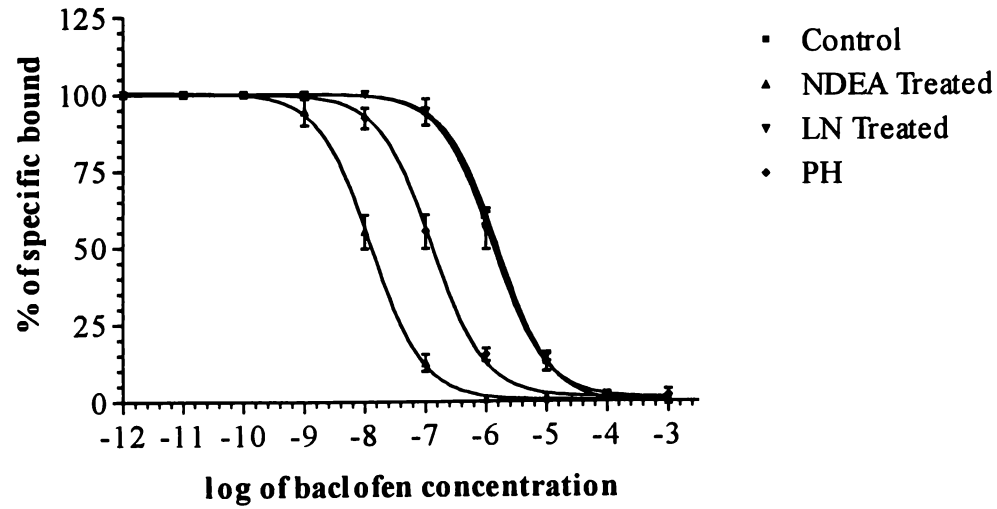


Figure-11

Scatchard analysis of [^{3}H]GABA binding against GABA in the hypothlamus of rats

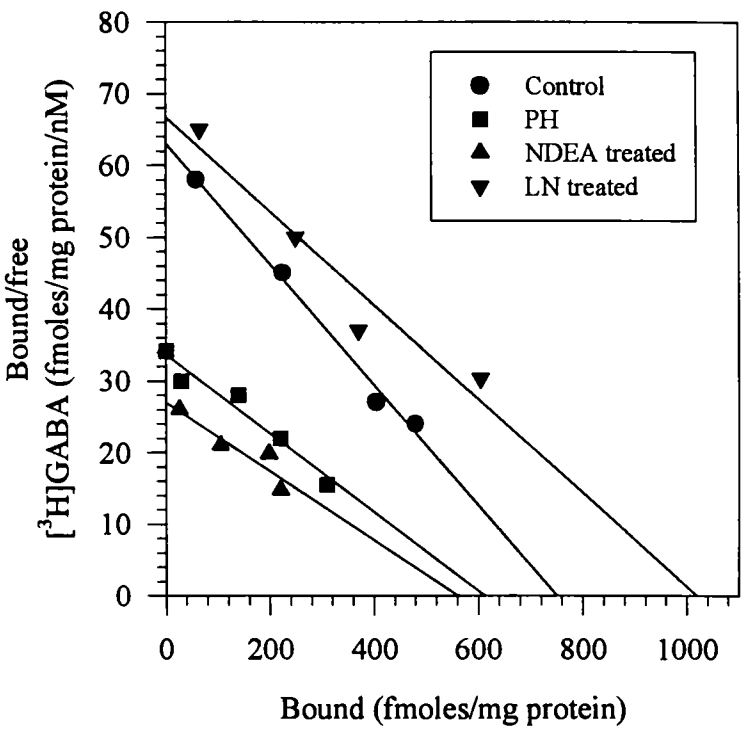


Table-16

[^{3}H]GABA binding parameters in the hypothalamus of rats

Animal status	B_{max} (fmol/mg protein)	K_d (nM)
Control	856.24 ± 37.48¶	13.78 ± 0.38
Partial Hepatectomy	628.33 ± 36.33**	18.41 ± 1.46*
NDEA Treated	527.64 ± 40.15***	19.39 ± 0.98**
Lead Nitrate Treated	980.45 ± 48.57*	14.47 ± 1.45

***p<0.001, **p<0.01, *p<0.05 with respect to control

Values are mean ± S.E.M. of 4-6 separate experiments

¶Control value given is a pooled data from different control experiments since there was no significant difference in values among groups

Binding parameters of [^{3}H]GABA against muscimol in hypothalamus of rats

Animal status	Best-fit model	log (EC_{50})-1	log (EC_{50})-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slopes
Control	Two-site	-8.65	-5.71	1.85×10^{-9}	1.61×10^{-6}	-0.41
Partial Hepatectomy	Two-site	-8.83	-5.93	1.22×10^{-9}	9.73×10^{-7}	-0.60
NDEA Treated	Two-site	-7.39	-5.66	3.41×10^{-8}	1.83×10^{-6}	-0.64
Lead Nitrate Treated	Two-site	-8.65	-5.59	1.85×10^{-9}	2.14×10^{-6}	-0.40

Values are mean of 4-6 separate experiments

Data were fitted with an iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki -The affinity of the receptor for the competing drug. The affinity for the first and second site of the competing drug are designated as $Ki_{(H)}$ (for high affinity) and $Ki_{(L)}$ (for low affinity). EC_{50} is the concentration of the competitor that competes for half the specific binding.

NDEA- *N*-Nitrosodiethylamine

LN- Lead Nitrate

PH- Partial Hepatectomy

Figure-12

Displacement of [^{3}H]GABA with muscimol in hypothalamus of rats

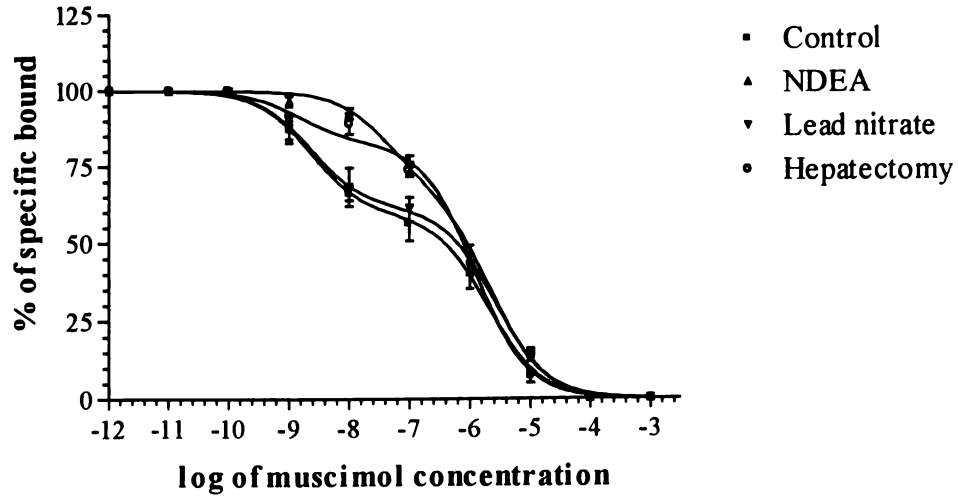


Figure-13

Scatchard analysis of [^{3}H]bicuculline binding against bicuculline in the hypothalamus of rats

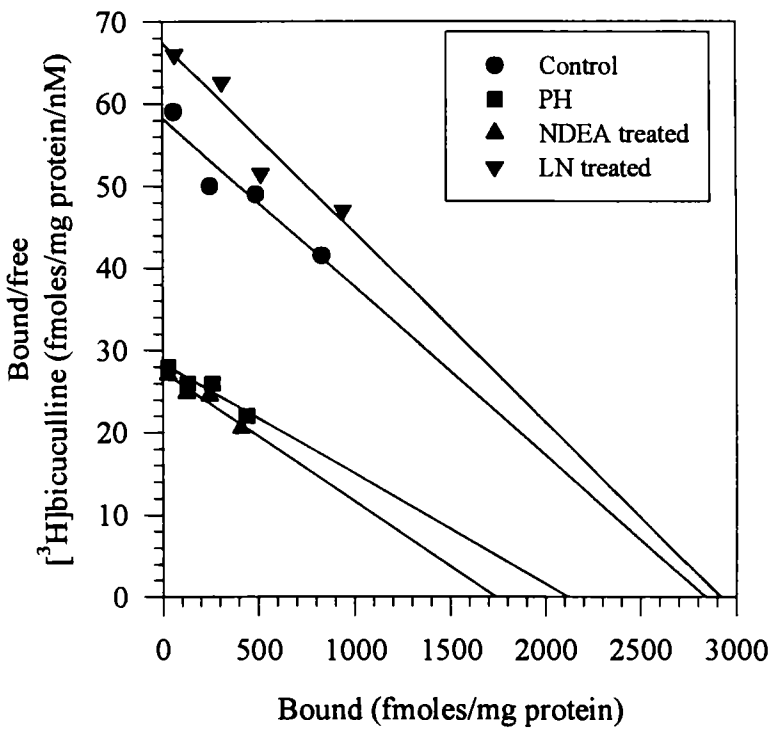


Table-18

[^{3}H]bicuculline binding parameters in the hypothalamus of rats

Animal status	B_{max} (pmol/mg protein)	K_d (nM)
Control	2.84 ± 0.17¶	49.62 ± 2.45
Partial Hepatectomy	1.90 ± 0.15**	62.33 ± 4.02*
NDEA Treated	1.45 ± 0.12***	66.67 ± 2.44**
Lead Nitrate Treated	2.74 ± 0.13	40.44 ± 2.62*

***p<0.001, **p<0.01, *p<0.05 with respect to control

Values are mean ± S.E.M. of 4-6 separate experiments

¶Control value given is a pooled data from different control experiments since there was no significant difference in values among groups

Table-19

Binding parameters of [^{3}H]bicuculline against bicuculline in hypothalamus of rats

Animal status	Best-fit model	log (EC_{50})-1	log (EC_{50})-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slopes
Control	Two-site	-7.98	-5.76	9.91×10^{-9}	1.62×10^{-6}	-0.52
Partial Hepatectomy	Two-site	-7.47	-5.70	3.17×10^{-8}	1.87×10^{-6}	-0.65
NDEA Treated	Two-site	-7.28	-4.79	4.86×10^{-8}	1.51×10^{-5}	-0.58
Lead Nitrate Treated	Two-site	-8.36	-5.95	4.07×10^{-9}	1.06×10^{-6}	-0.47

Values are mean of 4-6 separate experiments

Data were fitted with an iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki -The affinity of the receptor for the competing drug. The affinity for the first and second site of the competing drug are designated as $Ki_{(H)}$ (for high affinity) and $Ki_{(L)}$ (for low affinity). EC_{50} is the concentration of the competitor that competes for half the specific binding.

NDEA- *N*-Nitrosodiethylamine

LN- Lead Nitrate

PH- Partial Hepatectomy

Figure-14

Displacement of [^{3}H]bicuculline with bicuculline in hypothalamus of rats

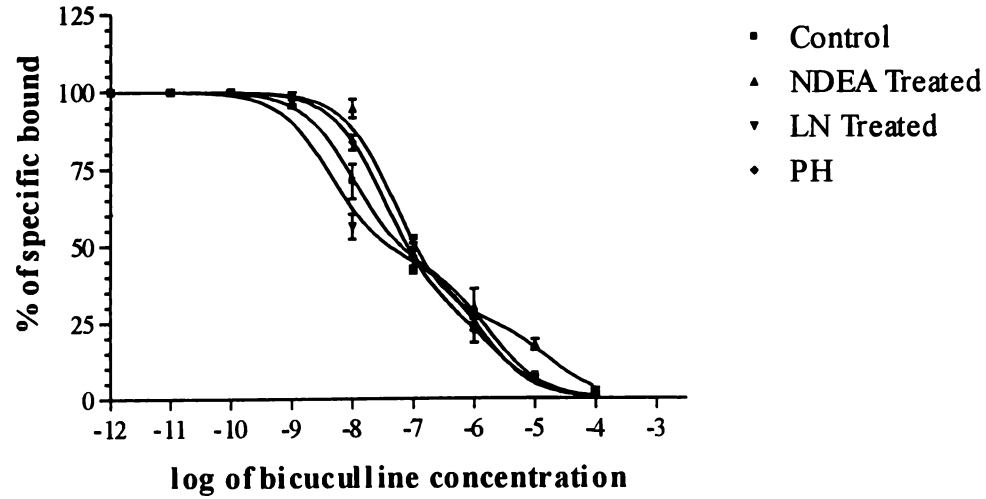


Figure-15

Scatchard analysis of [^{3}H]baclofen against baclofen in the hypothalamus of rats

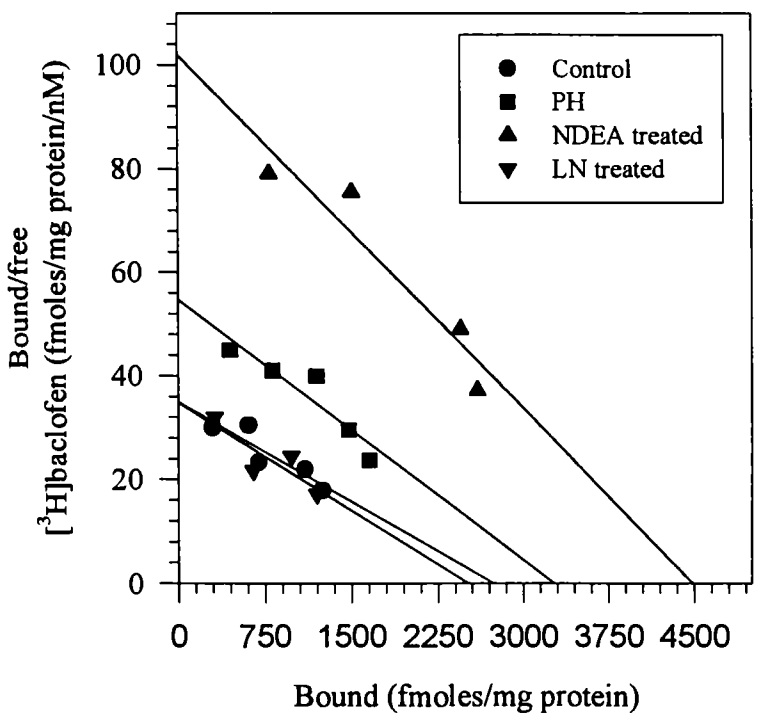


Table-20

[^{3}H]baclofen binding parameters in the hypothalamus of rats

Animal status	B_{max} (pmol/mg protein)	K_d (nM)
Control	2.46 ± 0.20[¶]	67.48 ± 3.33
Partial Hepatectomy	3.46 ± 0.24**	65.78 ± 4.10
NDEA Treated	4.60 ± 0.20***	46.49 ± 4.68**
Lead Nitrate Treated	2.45 ± 0.10	73.27 ± 3.14

***p<0.001, **p<0.01 with respect to control

Values are mean ± S.E.M. of 4-6 separate experiments

[¶]Control value given is a pooled data from different control experiments since there was no significant difference in values among groups

Binding parameters of [^{3}H]baclofen against baclofen in hypothalamus of rats

Animal status	Best-fit model	log (EC_{50})	Ki	Hill slopes
Control	One-site	-5.82	1.18×10^{-6}	-1.03
Partial Hepatectomy	One-site	-5.81	1.20×10^{-6}	-0.94
NDEA Treated	One-site	-7.05	6.90×10^{-8}	-1.00
Lead Nitrate Treated	One-site	-5.76	1.34×10^{-6}	-1.00

Values are mean of 4-6 separate experiments

Data were fitted with an iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki -The affinity of the receptor for the competing drug. EC_{50} is the concentration of the competitor that competes for half the specific binding.

NDEA- *N*-Nitrosodiethylamine

LN- Lead Nitrate

PH- Partial Hepatectomy

Figure-16

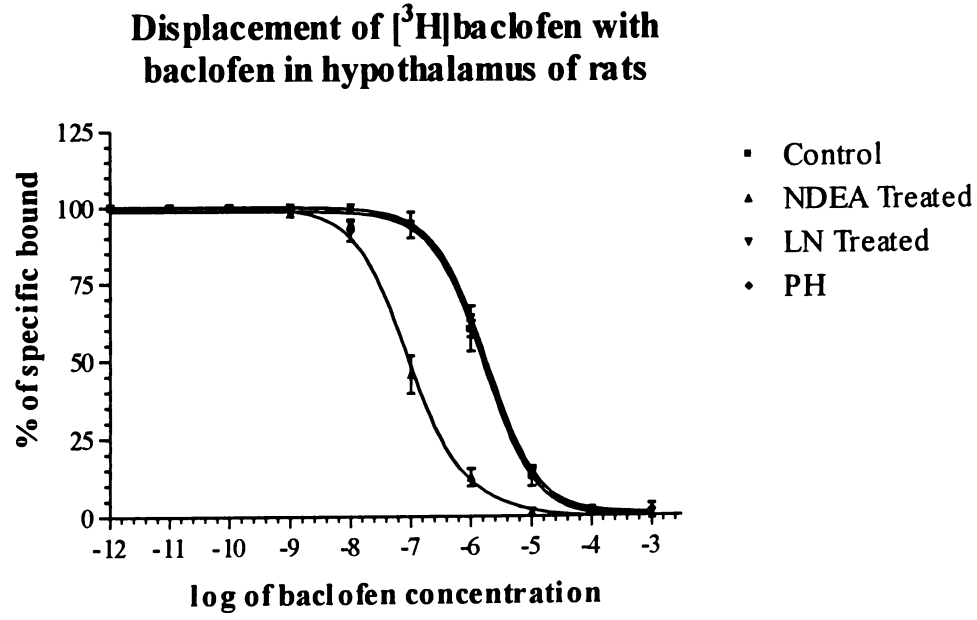


Figure-17

Scatchard analysis of [^{3}H]GABA binding against GABA in the cerebellum of rats

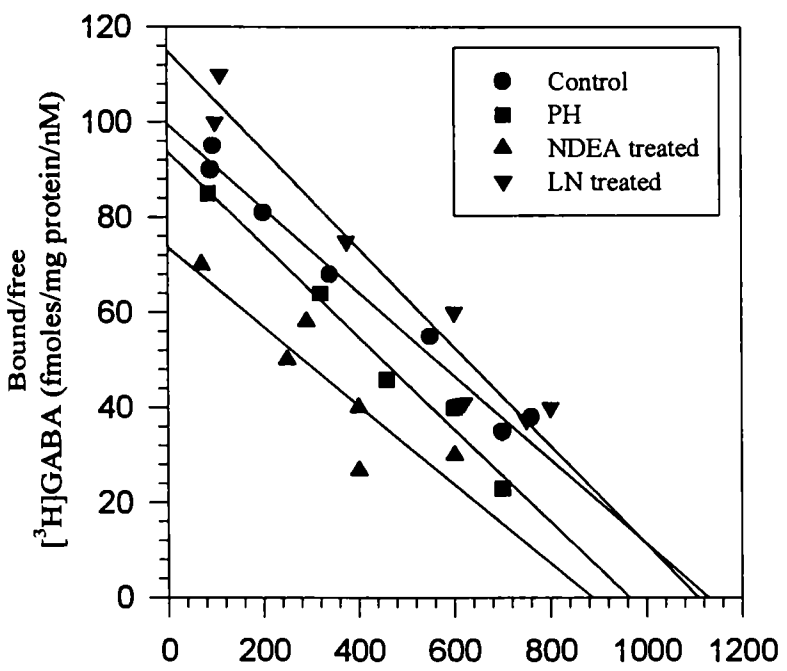


Table-22

[^{3}H]GABA binding parameters in the cerebellum of rats

	B_{max} (fmol/mg protein)	K_d (nM)
Control	1167.96 ± 40.57[¶]	11.53 ± 0.41
Partial Hepatectomy	1001.20 ± 31.49[*]	11.78 ± 0.59
NDEA Treated	895.23 ± 38.45[**]	11.38 ± 0.89
Lead Nitrate Treated	1057.29 ± 51.34	9.89 ± 0.38[*]

[**]p<0.01, [*]p<0.05 with respect to control

Values are mean ± S.E.M. of 4-6 separate experiments.

[¶]Control value given is a pooled data from different control experiments since there was no significant difference in values among groups

Binding parameters of [^{3}H]GABA against muscimol in cerebellum of rats

Animal status	Best-fit model	log (EC_{50})-1	log (EC_{50})-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slopes
Control	Two-site	-7.68	-5.46	1.74×10^{-8}	2.91×10^{-6}	-0.50
Partial Hepatectomy	Two-site	-7.84	-5.76	1.19×10^{-8}	1.44×10^{-6}	-0.57
NDEA Treated	Two-site	-7.46	-5.39	2.91×10^{-8}	3.32×10^{-6}	-0.52
Lead Nitrate Treated	Two-site	-8.88	-5.62	1.09×10^{-9}	1.98×10^{-6}	-0.39

Values are mean of 4-6 separate experiments

Data were fitted with an iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki -The affinity of the receptor for the competing drug. The affinity for the first and second site of the competing drug are designated as $Ki_{(H)}$ (for high affinity) and $Ki_{(L)}$ (for low affinity). EC_{50} is the concentration of the competitor that competes for half the specific binding.

NDEA- *N*-Nitrosodiethylamine

LN- Lead Nitrate

PH- Partial Hepatectomy

Figure-18

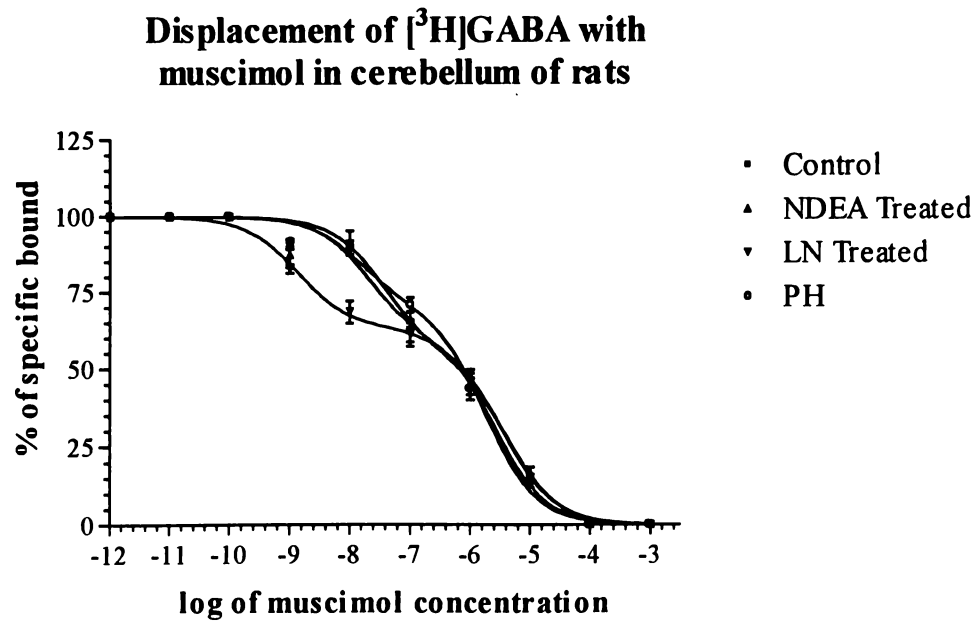


Figure-19

Scatchard analysis of [^{3}H]bicuculline binding against bicuculline in the cerebellum of rats

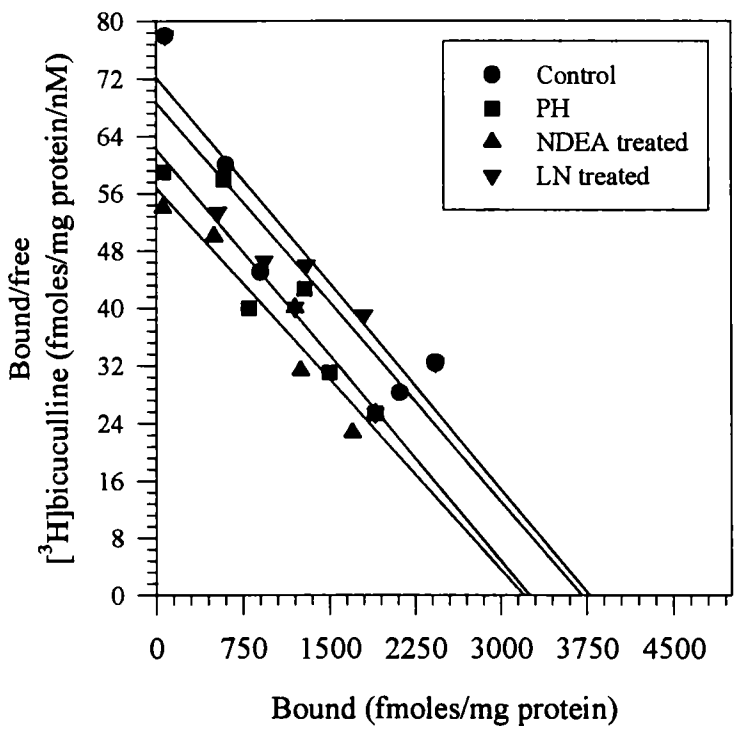


Table-24

[^{3}H]bicuculline binding parameters in the cerebellum of rats

	B_{max} (p mol/mg protein)	K_d (nM)
Control	3.72 ± 0.11¶	54.40 ± 1.8
Partial Hepatectomy	3.31 ± 0.09*	58.22 ± 4.28
Liver Cancer	3.20 ± 0.08*	57.22 ± 3.40
Lead Nitrate Treated	3.82 ± 0.12	52.77 ± 2.87

*$p<0.05$ with respect to control

Values are mean ± S.E.M. of 4-6 separate experiments.

¶Control value given is a pooled data from different control experiments since there was no significant difference in values among groups.

Table-25

Binding parameters of [^{3}H]bicuculline against bicuculline in cerebellum of rats

Animal status	Best-fit model	log (EC_{50})-1	log (EC_{50})-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slopes
Control	Two-site	-7.98	-5.76	9.61×10^{-9}	1.57×10^{-6}	-0.52
Partial Hepatectomy	Two-site	-8.01	-6.02	8.80×10^{-9}	8.77×10^{-7}	-0.65
NDEA Treated	Two-site	-7.88	-5.12	1.19×10^{-8}	6.82×10^{-6}	-0.52
Lead Nitrate Treated	Two-site	-8.23	-6.11	5.34×10^{-9}	7.03×10^{-7}	-0.52

Values are mean of 4-6 separate experiments

Data were fitted with an iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki- The affinity of the receptor for the competing drug. The affinity for the first and second site of the competing drug are designated as $Ki_{(H)}$ (for high affinity) and $Ki_{(L)}$ (for low affinity). EC_{50} is the concentration of the competitor that competes for half the specific binding.

NDEA- *N*-Nitrosodiethylamine

LN- Lead Nitrate

PH- Partial Hepatectomy

Figure-20

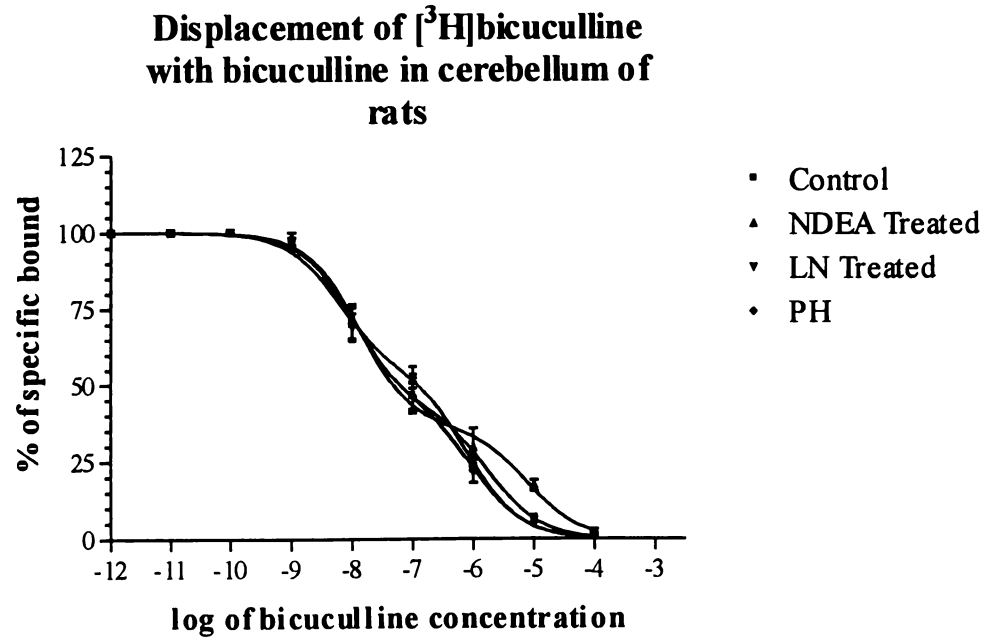


Figure-21

Scatchard analysis of [^{3}H]baclofen binding against baclofen in the cerebellum of rats

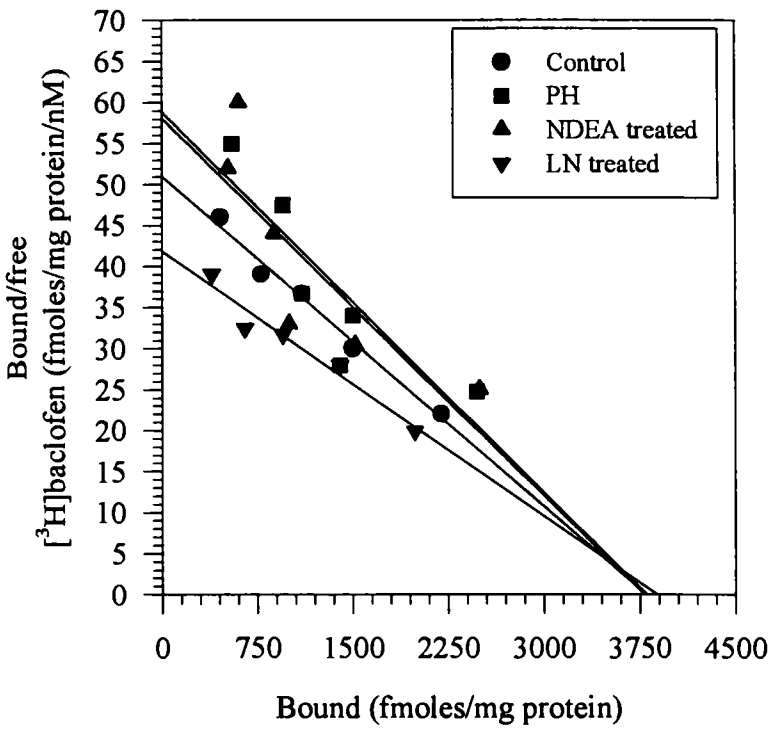


Table-26

[^{3}H]baclofen binding parameters in the cerebellum of rats

	B_{max} (pmol/mg protein)	K_d (nM)
Control	3.60 ± 0.16[¶]	72.17 ± 2.54
Partial Hepatectomy	3.58 ± 0.25	60.78 ± 2.18*
NDEA treated	3.48 ± 0.17	62.45 ± 1.95*
Lead Nitrate Treated	3.56 ± 0.20	83.54 ± 3.01*

*$p<0.05$ with respect to control

Values are mean ± S.E.M. of 4-6 separate experiments

[¶]Control value given is a pooled data from different control experiments since there was no significant difference in values among groups

Binding parameters of [^{3}H]baclofen against baclofen in cerebellum of rats

Animal status	Best-fit model	log (EC_{50})	Ki	Hill slopes
Control	One-site	-5.82	1.18×10^{-6}	-1.03
Partial Hepatectomy	One-site	-6.90	9.66×10^{-8}	-0.91
NDEA Treated	One-site	-6.98	8.07×10^{-8}	-0.92
Lead Nitrate Treated	One-site	-5.11	6.07×10^{-6}	-1.43

Values are mean of 4-6 separate experiments

Data were fitted with an iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki -The affinity of the receptor for the competing drug. EC_{50} is the concentration of the competitor that competes for half the specific binding.

NDEA- *N*-Nitrosodiethylamine

LN- Lead Nitrate

PH- Partial Hepatectomy

Figure-22

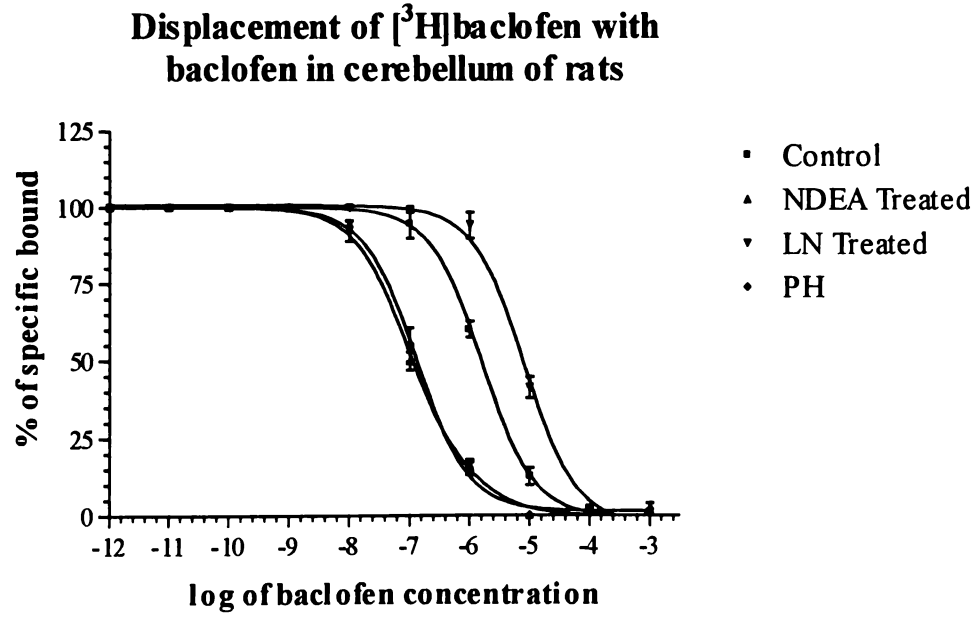


Table-28

Serum GABA levels in rats

(nmoles/ml serum)

Control	Partial Hepatectomy	NDEA Treated	LN Treated
4.25±0.18¶	6.59 ± 0.19**	8.22 ± 0.35***	6.24 ± 0.31*

***p<0.001, **p<0.01, *p<0.05 with respect to control

Values are mean ± S.E.M. of 4-6 separate experiments

¶Control value given is a pooled data from different control experiments since there was no significant difference in values among groups

Table-29

Levels of plasma norepinephrine in rats

(nmoles/ml plasma)

Animal status	Norepinephrine
Control	0.30 ± 0.10¶
Partial Hepatectomy	1.86 ± 0.03***
NDEA Treated	1.24 ± 0.09**
Lead Nitrate Treated	0.15 ± 0.08*

***p<0.001; **p<0.01, *p<0.05 with respect to control

Values are mean ± S.E.M. of 4-6 separate experiments

¶Control value given is a pooled data from different control experiments since there was no significant difference in values among groups

Table-30

Liver GABA content in rats

(nmoles/gr. wet wt. of tissue)

Control	Partial Hepatectomy	NDEA Treated	LN Treated
80.47±3.55¶	45.79 ± 5.78*	37.13 ± 4.58**	134.95 ± 8.45**

**p<0.01, *p<0.05 with respect to control

Values are mean ± S.E.M. of 4-6 separate experiments

¶Control value given is a pooled data from different control experiments since there was no significant difference in values among groups

Figure-23

Scatchard analysis of [^{3}H]GABA binding against GABA in the liver of rats

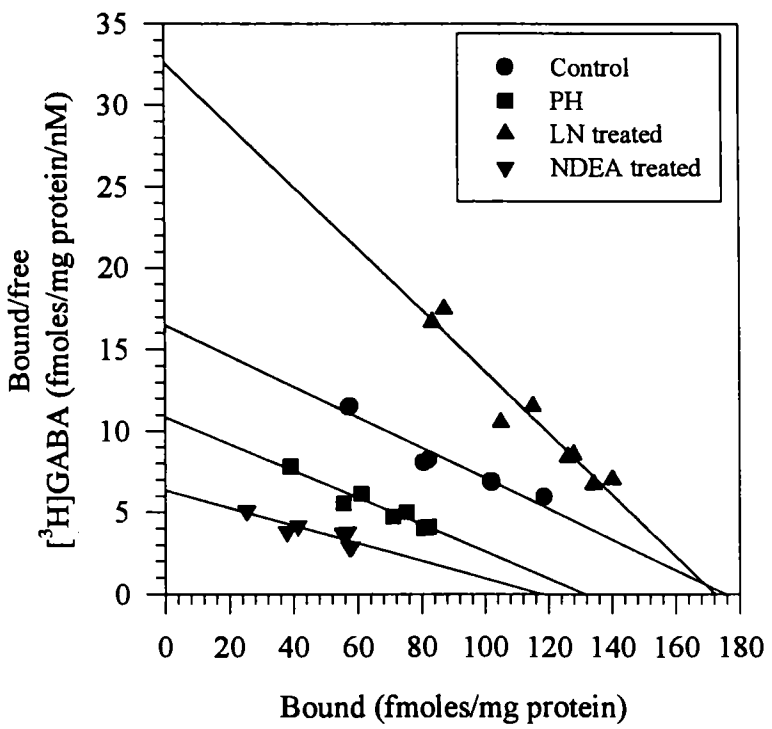


Table-31

[^{3}H]GABA binding parameters in the liver of rats

	B_{max} (f mol/mg protein)	K_d (nM)
Control	175.39 ± 5.175	11.84 ± 0.87
Partial Hepatectomy	135.13 ± 8.45**	11.13 ± 1.13
Lead Nitrate Treated	168.24 ± 10.56	5.11 ± 0.19**
NDEA Treated	127.50 ± 9.54**	19.62 ± 1.02**

**$p<0.01$ with respect to control

Values are mean ± S.E.M. of 4-6 separate experiments

Control value given is a pooled data from different control experiments since there was no significant difference in values among groups

Binding parameters of [^{3}H]GABA against muscimol in liver of rats

Animal status	Best-fit model	log (EC_{50})-1	Log (EC_{50})-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slopes
Control	Two-site	-7.84	-5.32	1.21×10^{-8}	4.01×10^{-6}	-0.48
Partial Hepatectomy	Two-site	-7.91	-5.46	1.02×10^{-8}	2.91×10^{-6}	-0.46
NDEA Treated	One-site	-6.09		6.79×10^{-7}		-1.01
Lead Nitrate Treated	Two-site	-8.58	-7.23	2.21×10^{-9}	4.90×10^{-8}	-0.71

Values are mean ± S.E.M. of 4-6 separate experiments,

Data were fitted with an iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki -The affinity of the receptor for the competing drug. The affinity for the first and second site of the competing drug are designated as $Ki_{(H)}$ (for high affinity) and $Ki_{(L)}$ (for low affinity). EC_{50} is the concentration of the competitor that competes for half the specific binding.

NDEA- *N*-Nitrosodiethylamine

LN- Lead Nitrate

PH- Partial Hepatectomy

Figure-24

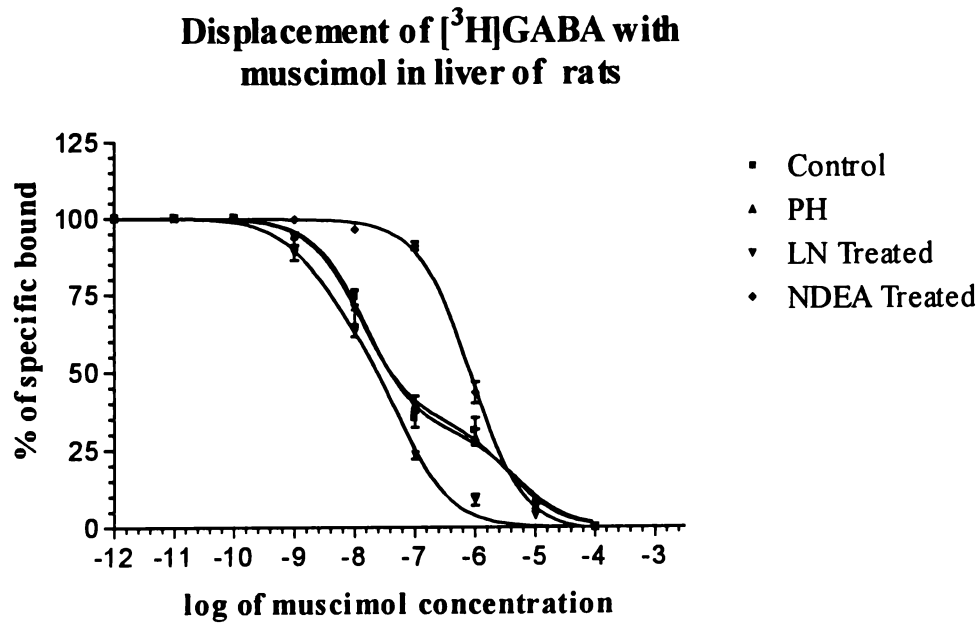


Figure-25

Scatchard analysis of [^{3}H]bicuculline binding against bicuculline in the liver of rats

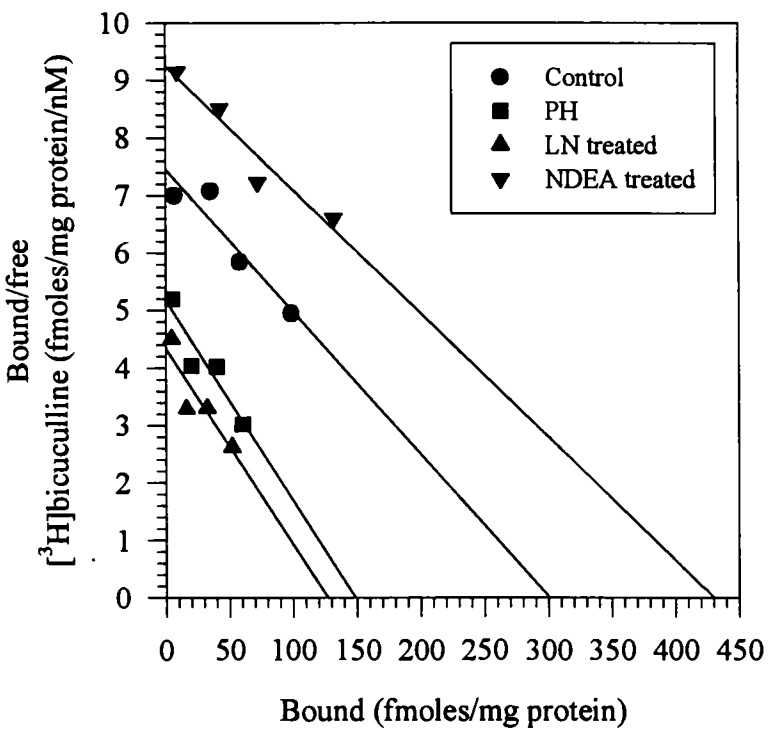


Table-33

[^{3}H]bicuculline binding parameters in the liver of rats

	B_{max} (fmol/mg protein)	K_d (nM)
Control	324.76 ± 10.78	40.84 ± 1.87
Partial Hepatectomy	197.54 ± 13.24***	45.13 ± 2.13
Lead Nitrate Treated	398.45 ± 9.45*	43.24 ± 0.19
NDEA Treated	148.65 ± 11.23***	39.62 ± 1.02

***p<0.001; *p<0.05 with respect to control

Values are mean ± S.E.M. of 4-6 separate experiments.

'Control value given is a pooled data from different control experiments since there was no significant difference in values among groups.

Binding parameters of [^{3}H]bicuculline against bicuculline in liver of rats

Animal status	Best-fit model	log (EC_{50})-1	log (EC_{50})-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slopes
Control	Two-site	-8.02	-5.75	8.95×10^{-9}	1.67×10^{-6}	-0.38
Partial Hepatectomy	Two-site	-7.11	-5.00	7.26×10^{-8}	9.35×10^{-6}	-0.34
NDEA treated	Two-site	-7.03	-5.20	8.72×10^{-8}	1.86×10^{-6}	-0.38
Lead Nitrate Treated	Two-site	-8.12	-6.02	7.19×10^{-9}	9.00×10^{-7}	-0.37

Values are mean of 4-6 separate experiments

Data were fitted with an iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki -The affinity of the receptor for the competing drug. The affinity for the first and second site of the competing drug are designated as $Ki_{(H)}$ (for high affinity) and $Ki_{(L)}$ (for low affinity). EC_{50} is the concentration of the competitor that competes for half the specific binding.

NDEA- *N*-Nitrosodiethylamine

LN- Lead Nitrate

PH- Partial Hepatectomy

Figure-26

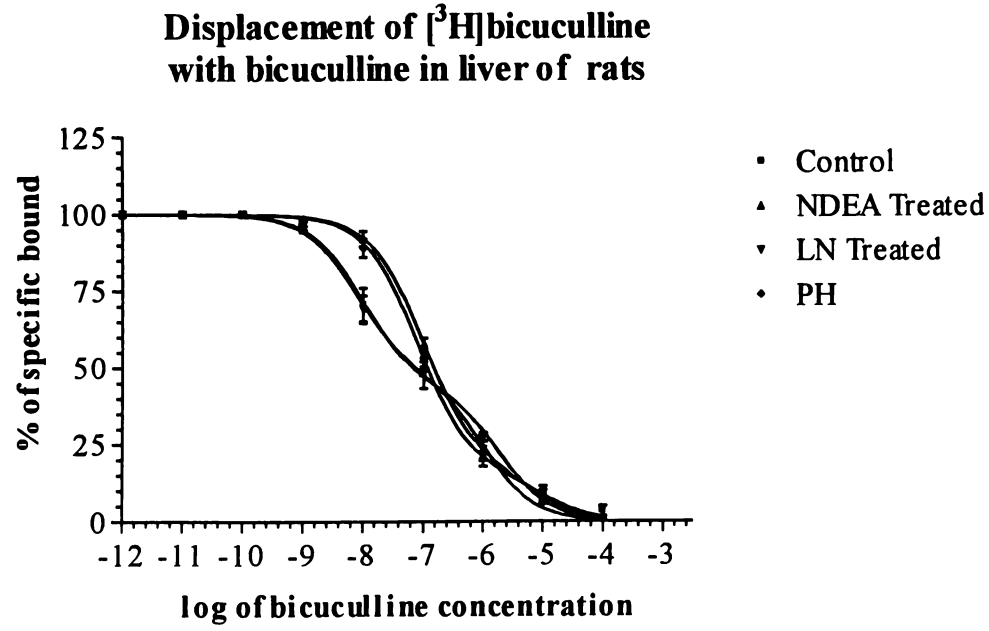


Figure-27

Scatchard analysis [^{3}H]baclofen binding against baclofen in the liver of rats

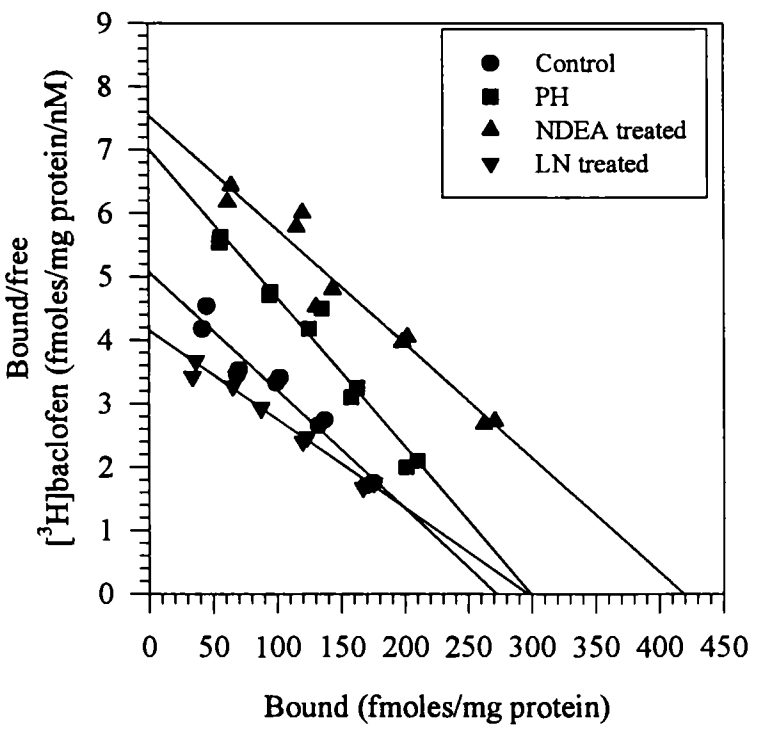


Table-35

[^{3}H]baclofen binding parameters in the liver of rats

Animal status	B_{max} (pmol/mg protein)	K_d (nM)
Control	286.21 ± 5.91	57.22 ± 1.54
Partial Hepatectomy	294.54 ± 10.56	42.16 ± 3.57*
Lead Nitrate Treated	294.98 ± 6.99	70.24 ± 4.20**
NDEA Treated	422.6 ± 17.61***	56.35 ± 3.25

*p<0.005, **p<0.01, ***p<0.001 with respect to control,

Values are mean ± S.E.M. of 4-6 separate experiments.

[1]Control value given is a pooled data from different control experiments since there was no significant difference in values among groups.

Binding parameters of [^{3}H]baclofen against baclofen in liver of rats

Animal status	Best-fit model	log (EC_{50})	Ki	Hill slopes
Control	One-site	-7.18	5.56×10^{-8}	-1.22
Partial Hepatectomy	One-site	-8.32	3.98×10^{-9}	-1.49
NDEA Treated	One-site	-7.25	4.74×10^{-8}	-1.00
Lead Nitrate Treated	One-site	-6.20	5.26×10^{-7}	-0.88

Values are mean of 4-6 separate experiments

Data were fitted with an iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki -The affinity of the receptor for the competing drug. EC_{50} is the concentration of the competitor that competes for half the specific binding.

NDEA- *N*-Nitrosodiethylamine

LN- Lead Nitrate

PH- Partial Hepatectomy

Figure-28

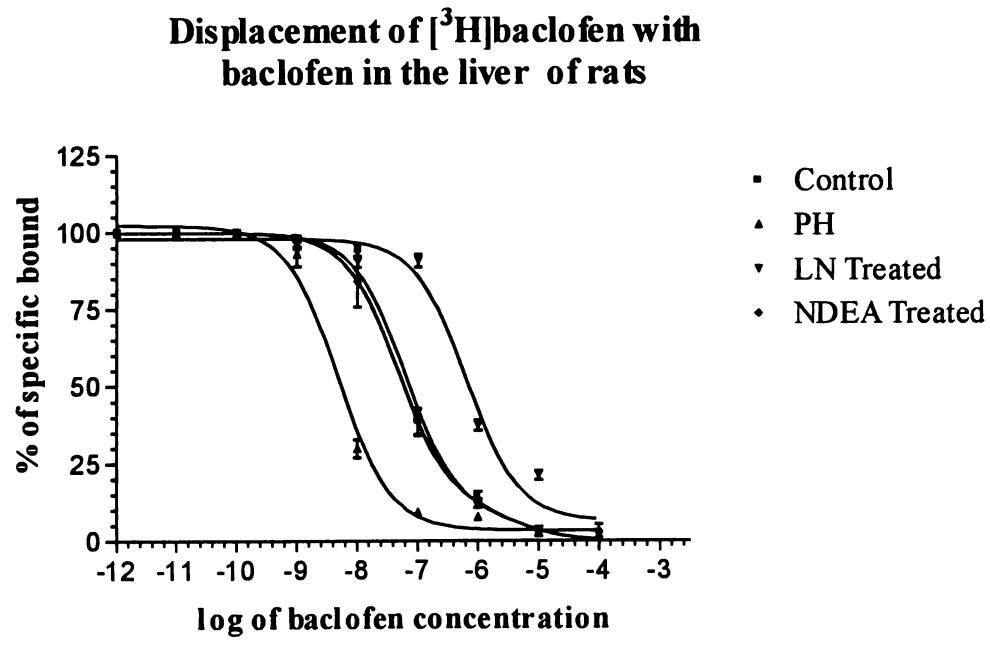
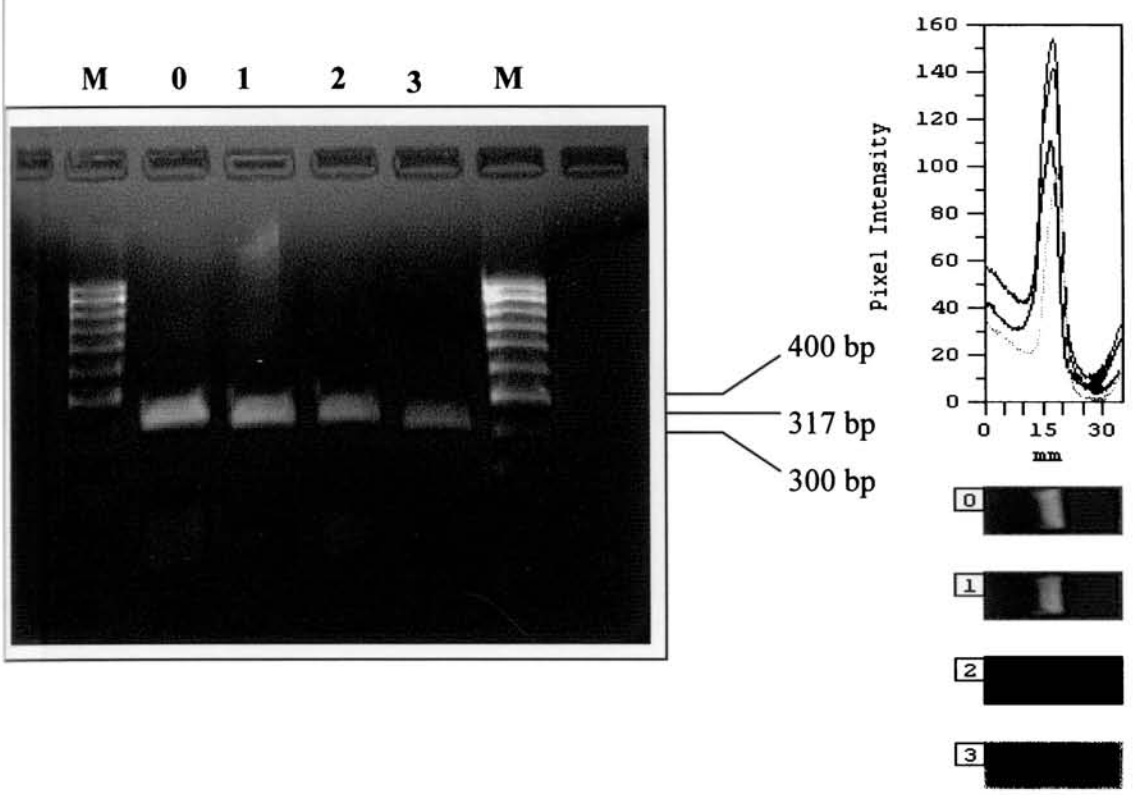


Plate-5

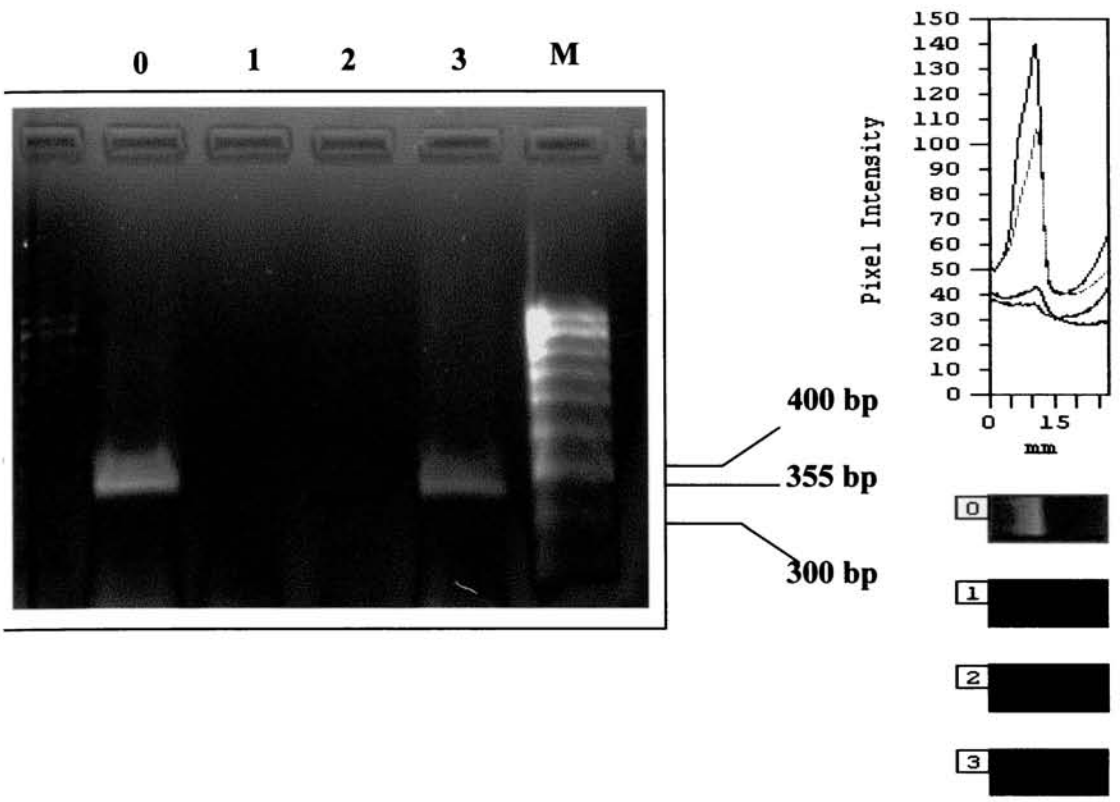
RT-PCR amplification product of Beta-2 subunit of $GABA_A$ receptor mRNA from the liver of rats



M - DNA molecular weight ladder (100 bp-1000 bp)
Lane 0 - Control
Lane 1 - Lead nitrate treated
Lane 2 - Partial hepatectomy
Lane 3 - NDEA treated

Plate-6

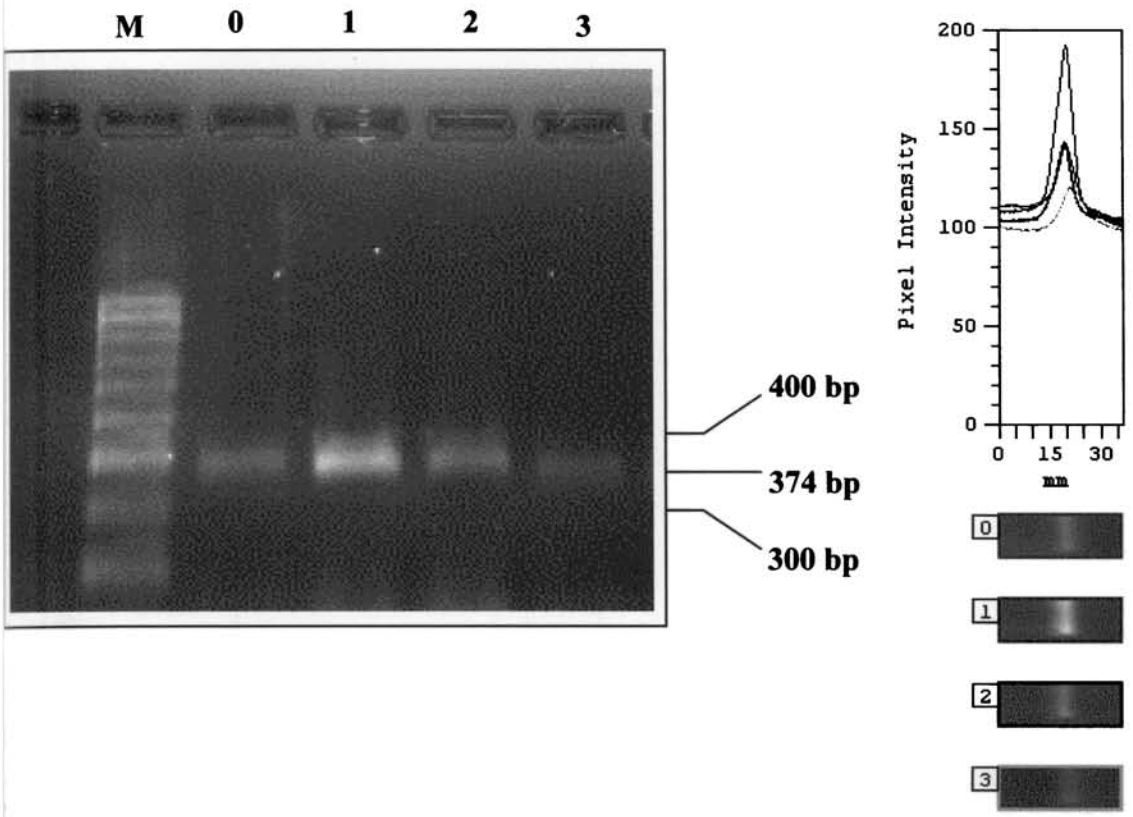
RT-PCR amplification product of Beta-3 subunit of $GABA_A$ receptor mRNA from the liver of rats



M - DNA molecular weight ladder (100 bp-1000 bp)
Lane 0 - Lead nitrate treated
Lane 1 - Partial hepatectomy
Lane 2 - NDEA treated
Lane 3 - Control

Plate-7

RT-PCR amplification product of Gamma -2 subunit of $GABA_A$ receptor mRNA from liver of rats

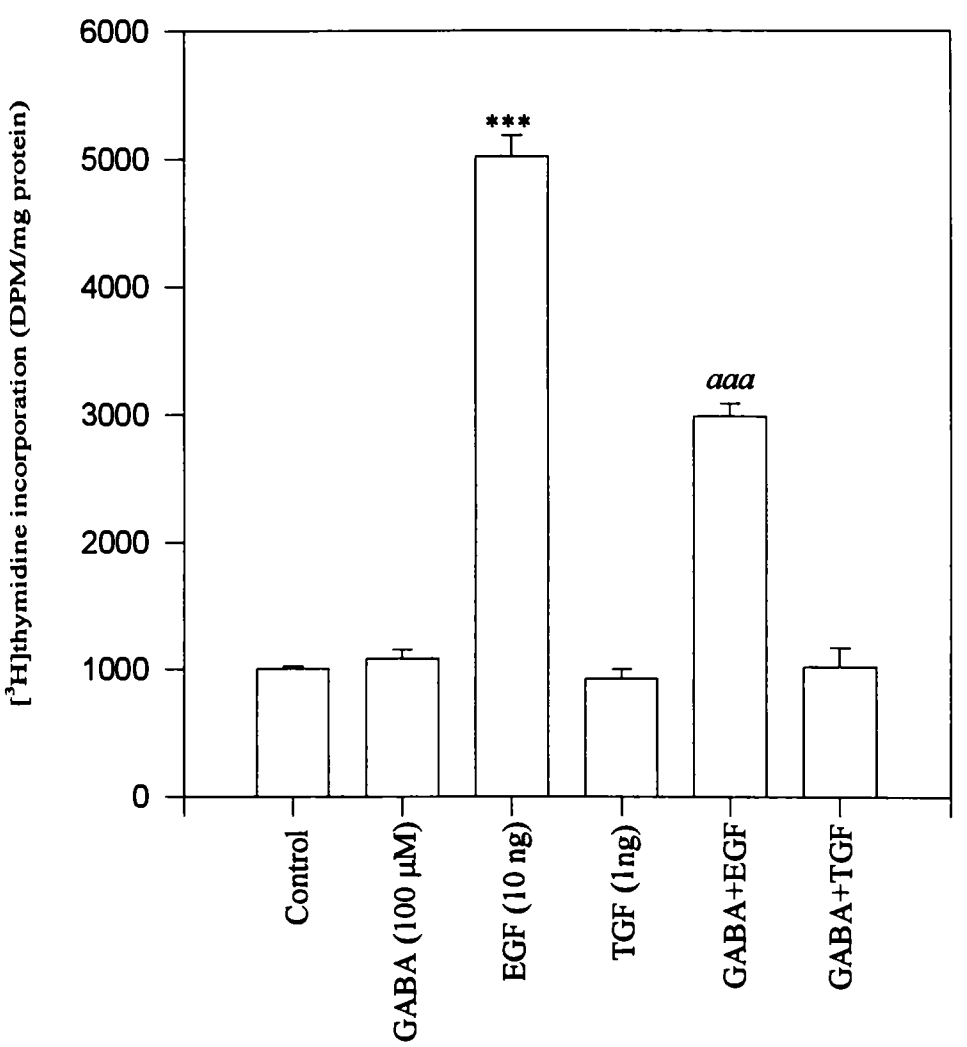


M - DNA molecular weight ladder (100 bp-1000 bp)
Lane 0 - Control
Lane 1 - Lead nitrate treated
Lane 2 - Partial hepatectomy
Lane 3 - NDEA treated

G8301

Figure-29

Effect of GABA on hepatocyte DNA synthesis

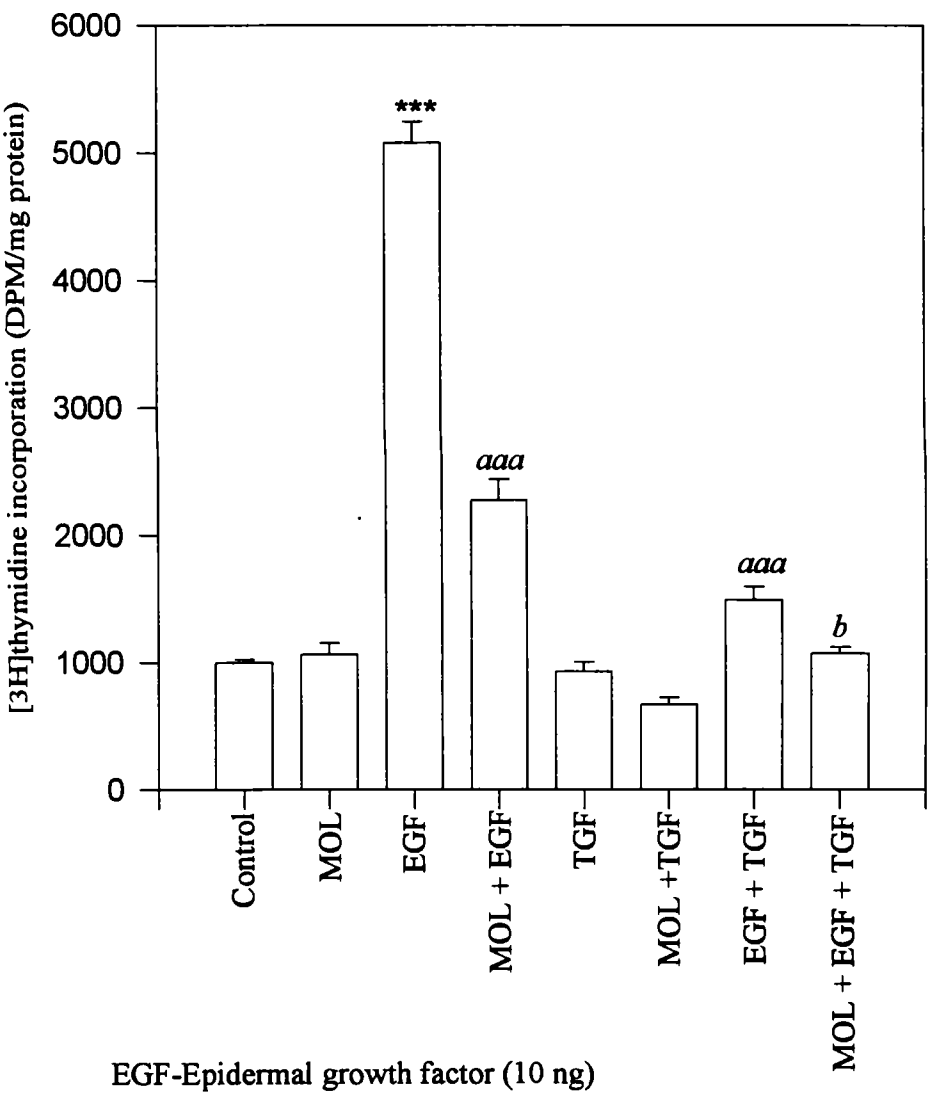


EGF-Epidermal growth factor
TGF-Transforming growth factorβ1

*** $p<0.001$ compared with the control
aaa $p<0.001$ compared with EGF

Figure-30

Effect of muscimol on hepatocyte DNA synthesis

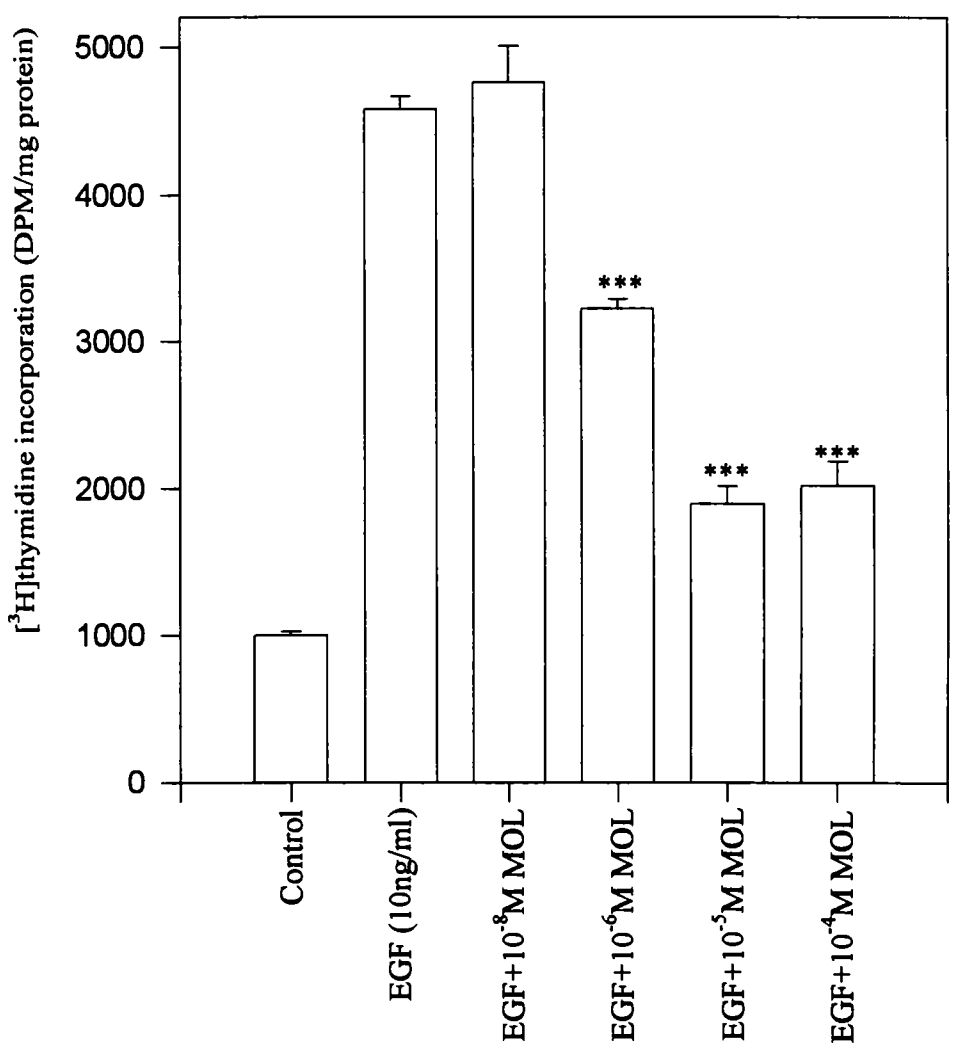


EGF-Epidermal growth factor (10 ng)
TGF- Transforming growth factor β1 (1ng)
MOL- Muscimol (100μM)

*** $p<0.001$ compared with the control
aaa $p<0.001$ compared with the EGF group
b $p<0.01$ compared with the EGF+TGF group

Figure-31

Dose-dependent response of hepatocyte DNA synthesis to muscimol

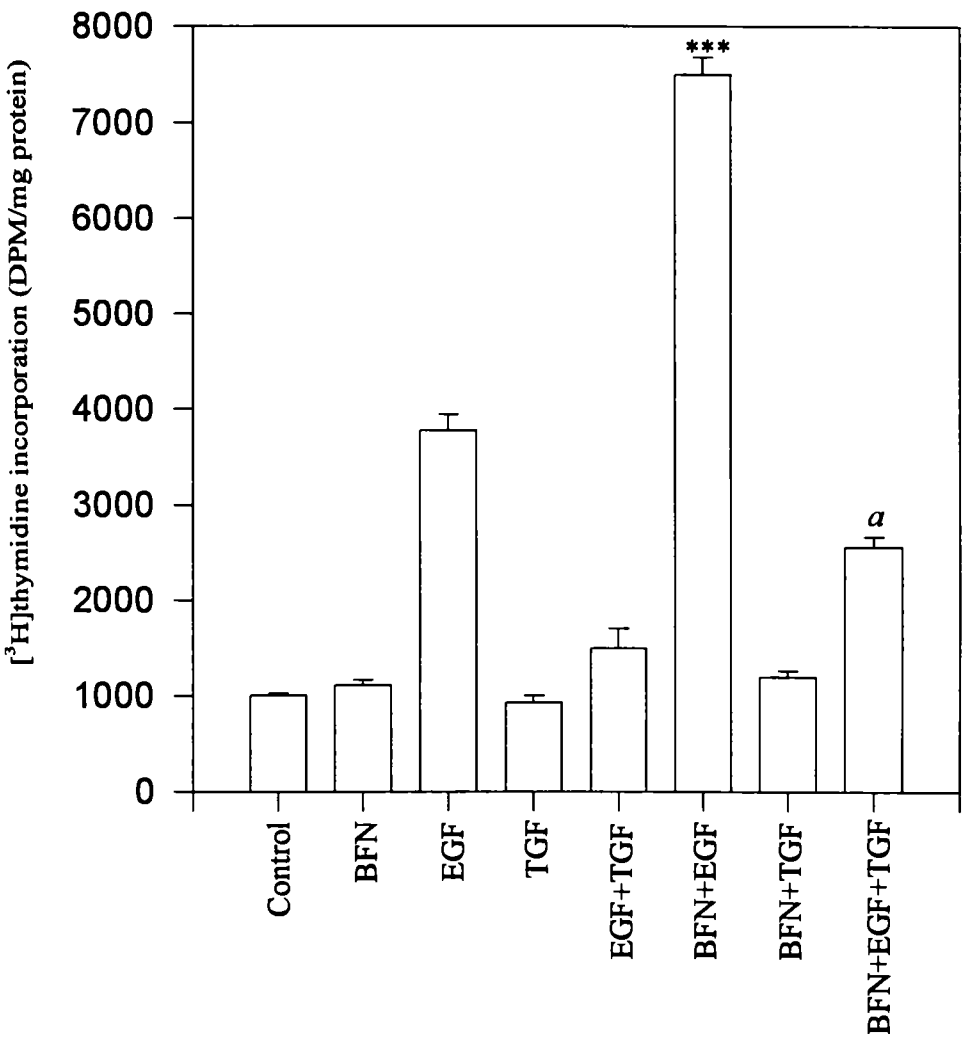


MOL-Muscimol
EGF-Epidermal growth factor

***p<0.001 compared with the EGF

Figure-32

Effect of baclofen on hepatocyte DNA synthesis

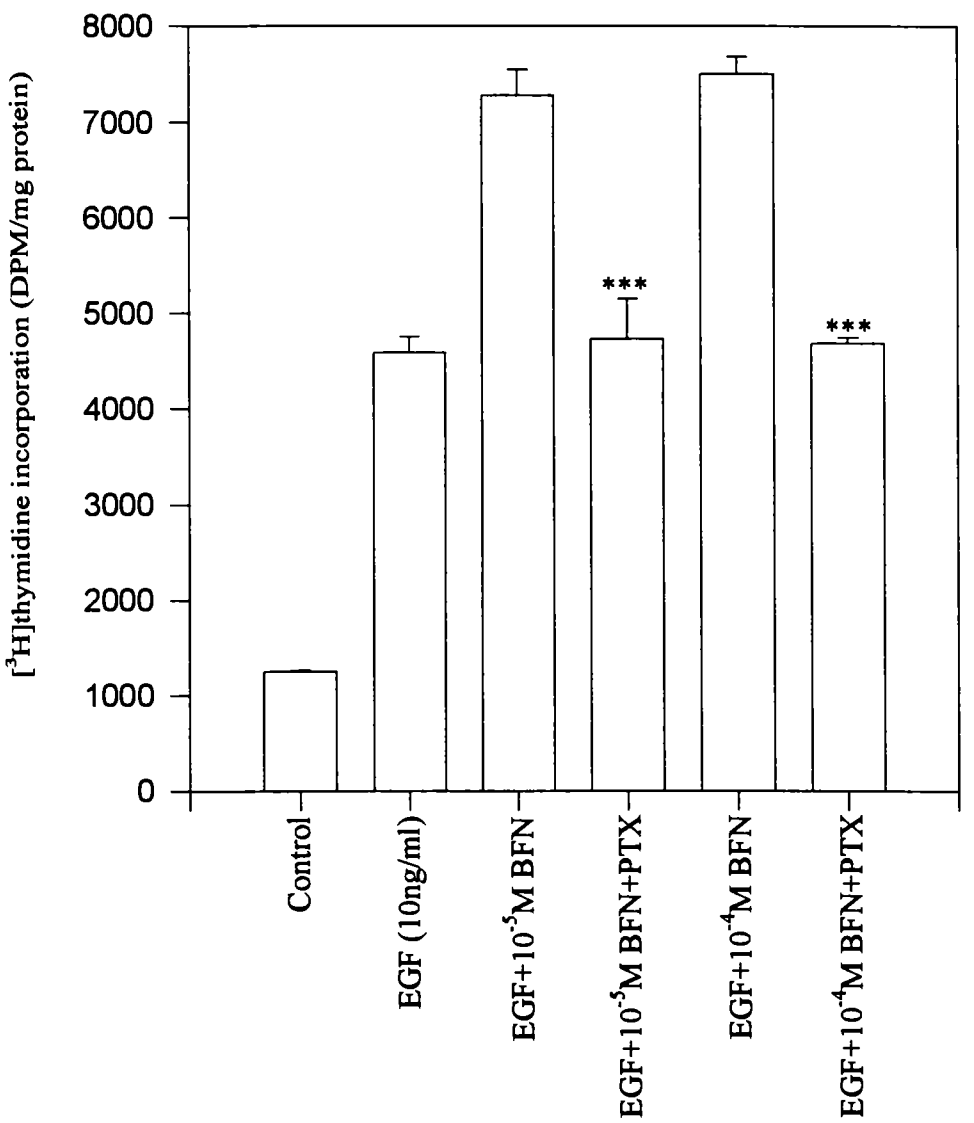


BFN-Baclofen (100 μM)
EGF-Epidermal growth factor (10 ng)
TGF-Transforming growth factor β1(1 ng)

*** $p<0.001$ compared to the EGF group
a $p<0.001$ compared to the EGF+TGF group

Figure-33

Effect of pertussis toxin on baclofen-induced hepatocyte DNA synthesis

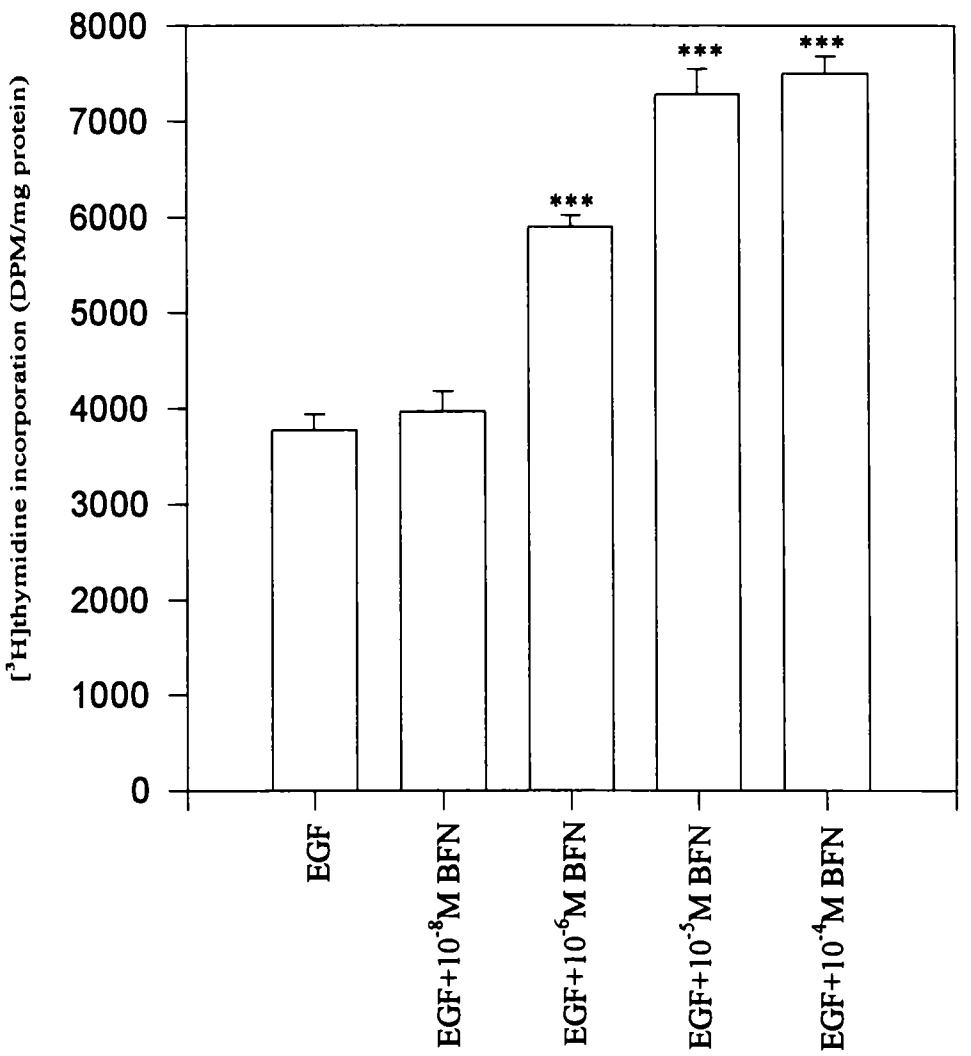


BFN-Baclofen
EGF-Epidermal growth factor
PTX-Pertussis toxin (50ng/ml)

*** $p<0.001$ compared to the BFN+EGF group

Figure-34

Dose-dependent response of hepatocyte DNA synthesis to baclofen



EGF-Epidermal growth factor (10 ng)
BFN-Baclofen

*** $p<0.001$ compared to the EGF group

DISCUSSION

DISCUSSION

DNA synthesis during liver regeneration

DNA synthesis, which is negligible in the intact liver of rats, showed an abrupt rise at 18 hr after PH. The maximal rate of [^{3}H]thymidine incorporation was observed at 24 hr of liver regeneration. DNA synthesis remained significantly higher in the liver at 48 hr of regeneration and declined to near basal levels by 72 hr after PH. This pattern of DNA synthesis observed by us is concordant with the previous reports (Michalopoulose.G.K & DeFrancis.M.C, 1997) (Grisham.J.W, 1962). The hepatocytes, which constitute the major part of the liver mass, are the first to enter the DNA synthetic phase and the 24 hr peak observed corresponds to hepatocyte DNA synthesis. DNA synthesis in the non-parenchymal cells starts after a lag of 24 hr. The [^{3}H]thymidine incorporation observed at 48 hr of hepatectomy indicates DNA synthesis of the non-parenchymal cells (Michalopoulose.G.K & DeFrancis.M.C, 1997). The activity of thymidine kinase is used as an index for liver regeneration (Bresnick.E *et al.*, 1971). Our laboratory have also reported an increase in the activity of thymidine kinase, a rate-limiting enzyme for DNA synthesis, during liver regeneration (Tessy.T.M *et al.*, 1997) (Waliuala.M.P. *et al.*, 1996).

Brain GABA content during liver regeneration

PH induced significant decrease in the content of GABA in different brain regions immediately before the period (12 hr) of active DNA synthesis and during peak DNA synthesis (24 hr) in the liver. These changes were reversed to basal level when liver DNA synthesis declined to control level. This indicates that the decrease in brain GABA content is important in the DNA synthesis in liver. Brain GABAergic changes are reported to regulate autonomic nerve function in rats (Martin.D.S & Haywood.J.R, 1998). Also, elevated intra-cerebral concentrations of GABA significantly decreased ornithine decarboxylase activity in the liver (Lapinjoki.S.P *et al.*, 1983), which is an index for decreased hepatic proliferation. The liver is richly innervated (Rogers.R.C & Hermann.G.E, 1983) and autonomic nervous system has an important role in the process of hepatic cell proliferation (Tanaka.K *et al.*, 1987). Lateral lesions of hypothalamus caused an increase in DNA synthesis during liver regeneration and sympathectomy and vagotomy

blocked this effect (Kiba.T *et al.*, 1994). Brain GABA is reported to have an inhibitory effect on sympathetic outflow (Martin.D.S & Haywood.J.R, 1998). Plasma NE levels increased during the time at which brain GABA content decreased where we observed maximum DNA synthesis. So the results show that a reduction in the GABA content in the brain regions may enhance DNA synthesis in liver by facilitating the sympathetic tone.

[^{3}H]GABA binding parameters in the brain regions during liver regeneration

The decrease in B_{max} of the [^{3}H]GABA binding in brain stem, hypothalamus and cerebellum indicates a decreased number of receptors in these regions immediately before and during active DNA synthesis in liver regeneration. In hypothalamus, increase in K_d of the receptor indicates a decreased affinity of the receptor to ligand. GABA antagonists have a higher affinity for the slowly dissociating high-affinity $GABA_A$ sites (Sieghart.W, 1995) (Olsen.R.W & Snowman.A.M, 1983). So we have investigated the high-affinity binding site using [^{3}H]GABA as a ligand. Brain GABA receptor number is reported to increase in animal models of liver diseases with impaired liver regeneration (Ferenci.P *et al.*, 1984). Intracerebroventricular injection of muscimol, a specific $GABA_A$ agonist demonstrated to have a sympatho-inhibitory effect (Coleman.M.J & Dampney.R.A, 1998) (Dimicco.J.A *et al.*, 1979), which is important for liver regeneration after PH. So the decrease in $GABA_A$ receptor may be a homeostatic regulatory mechanism to facilitate cell proliferation in liver by increasing the sympathetic activity.

Peripheral GABA and liver regeneration

Liver is rich in GABA transaminase, the GABA metabolising enzyme, for the removal and degradation of GABA (Minuk.G.Y, 1993). Liver possess a high-affinity sodium dependent GABA transport system by which regulates the uptake of GABA from blood (Minuk.G.Y *et al.*, 1984). The increase in serum GABA levels during liver regeneration suggests a decreased uptake of GABA into liver. GABA inhibits ornithine decarboxylase enzyme, which convert the ornithine to a hepatic growth promoter substance, putrescine (Theocharis.S.E *et al.*, 1998). The GABA content in the liver decrease at the time of peak DNA synthesis. This suggests that the reduced uptake of GABA into the liver may be responsible for the elevated serum GABA levels. The reduced

GABA content in the liver may in turn enhance putrescine production and thus induce epatic cell proliferation (Minuk.G.Y, 1993).

DNA synthesis in LN and NDEA treated rats

Animal models of liver cell proliferation can be divided into at least two major groups, those of compensatory regeneration and those of mitogenic hyperplasia. LN is known to have mitogenic effect on rat liver (Columbano.A & Shinozuka.H, 1996). The results of the present study clearly showed that a single intravenous injection of LN induced a synchronised wave of hepatocyte proliferation, which is evident from DNA synthesis profile. DNA synthesis peaks at 48 hr after LN administration and returns to basal level by 72 hr, which is concordant with the previous report (Kubo.Y *et al.*, 1996).

NDEA treatment caused morphological neoplastic changes in the rat liver compared to saline treated control rats (Plate 1 & 2). Histological sections showed enlarged nuclei with reduced cytoplasm and abnormal cell shape indicating the liver neoplasia (Plate 3 & 4). Increased thymidine kinase activity indicates an increased DNA synthesis in neoplastic liver (Tessy.T.M *et al.*, 1997).

GABA content in the brain regions of PH, LN and NDEA treated rats

The decreased GABA content in the brain stem, hypothalamus and cerebellum of PH rats was observed during active hepatic proliferation. PH induces the remnant liver to enter into cell cycle from quiescent state to compensate the lost mass of liver. Sympathetic innervation is important for liver regeneration (Kiba.T *et al.*, 1995). So the decrease in GABA content may be a homeostatic adjustment by the brain to trigger the sympathetic innervation, thereby elevating DNA synthesis in the liver. In LN treated rats the increased hepatic proliferation has to be suppressed in order to restore the normal liver mass (Columbano.A & Shinozuka.H, 1996). So the increase in brain GABA content in LN treated rats may be an adaptive mechanism to bring back the normal liver mass by decreasing the liver DNA synthesis through sympathetic activity regulation. Increased hepatic proliferation was also observed in NDEA treated rats but the changes in brain GABA content was reciprocal to that of LN treated rats. This suggests that the brain

GABAergic adaptive adjustment that was observed in the LN treated rats to suppress the excess hepatic proliferation is disturbed in NDEA treated rats leading to hepatic neoplasia.

Brain stem GABA receptor alterations

Brain stem has direct connection with liver through the vagus nerve. The brain stem rostral nucleus ambigus as well as dorsal motor nucleus of the vagus, the centres of autonomic nerves that innervate liver are under the regulation of GABA (Coleman.M.J & Dampney.R.A, 1998). Autonomic regulation of GABA is mediated through $GABA_A$ and $GABA_B$ receptors (Sved.A.F & Sved.J.C, 1990). Therefore we studied the brain stem GABAergic receptor alterations to elucidate its role on hepatic proliferation regulation. Previous reports have referred to [^{3}H]GABA as a $GABA_A$ receptor agonist in the high-affinity concentration (Paulose.C.S & Dakshinamurti.K, 1984). The decreased B_{max} and increased K_d in PH and NDEA treated rats denote the decreased receptor density and affinity of the [^{3}H]GABA binding. $GABA_A$ receptor density increased in LN treated rats. Muscimol, a selective $GABA_A$ receptor agonist specifically identifies high affinity receptors (Sieghart.W, 1995). The two affinity sites for muscimol binding is already reported (Bowery.N.G *et al.*, 1979). The displacement analysis of the [^{3}H]GABA against muscimol indicates a shift in the high-affinity site towards the low-affinity in NDEA treated rats. This suggests an altered receptor function during the neoplastic transformation.

Bicuculline has a higher affinity for rapidly dissociating low-affinity $GABA_A$ sites (Olsen.R.W & Snowman.A.M, 1983). So we have used [^{3}H]bicuculline to study the status of low-affinity $GABA_A$ receptors. The decreased B_{max} and increased K_d of [^{3}H]bicuculline binding indicates a reduction in receptor density as well as affinity of the low-affinity $GABA_A$ receptors in PH and NDEA treated rats. The two affinity sites for bicuculline binding is already described (Mohler.H, 1979). The high-affinity sites of this receptor shifted to low-affinity in NDEA and PH rats denote a decreased functioning of the receptor. The low-affinity site of this receptor also shifted to very low-affinity in NDEA treated rats denoting the total decrease in the low-affinity $GABA_A$ receptor functioning. Since GABA has a sympatho-inhibitory effect (Coleman.M.J & Dampney.R.A, 1998), these changes may be facilitating the increased sympathetic activity observed.

[^{3}H]Baclofen was used to study the $GABA_B$ receptor binding parameters Kerr.D.I.B *et al.*, 1990). In PH and NDEA treated rats the number and affinity of $GABA_B$ receptor increased. The shift in $GABA_B$ binding towards high-affinity in PH and NDEA treated rats indicates increased functioning of this receptor. $GABA_B$ receptor activation in central nervous system is reported to stimulate the sympathetic nervous system Nonogaki.K *et al.*, 1994) (Takenaka.K *et al.*, 1996).

Brain $GABA_A$ and $GABA_B$ receptor systems differentially regulate the sympathetic neural activity (Takenaka.K *et al.*, 1995). From our results it is clear that the brain stem $GABA_A$ and $GABA_B$ receptors functions were in a reciprocal manner. $GABA_A$ receptor demonstrated to have an inhibitory effect on sympathetic stimulation while $GABA_B$ receptor activates sympathetic stimulation (Coleman.M.J & Dampney.R.A, 1998) Nonogaki.K *et al.*, 1994). Brain GABAergic changes are in favour of enhancing sympathetic stimulation to compensate the lost liver mass by activating the remnant liver. The increase in plasma NE level substantiates the hypothesis of sympathetic stimulation during liver regeneration and involvement of brain GABA receptors. In LN treated rats since the hepatic proliferation has to be suppressed (Columbano.A & Shinozuka.H, 1996), the $GABA_A$ and $GABA_B$ receptor changes are in favour of decreasing the sympathetic tone. This is evident from the results of plasma NE levels obtained from different experimental groups, which is a well established growth triggering substance (Michalopoulose.G.K & DeFrancis.M.C, 1997) for hepatocytes. This regulatory mechanism was found to be totally altered in NDEA treated rats. In NDEA treated rats the changes in GABA receptor status and plasma NE levels were similar to that of PH rats. This may be responsible for uncontrolled hepatic cell proliferation.

Hypothalamus GABA receptor alterations

Hypothalamus is the centre of autonomic nervous system reinforcement. Lateral lesions of hypothalamus caused an increase in DNA synthesis during liver regeneration. Sympathectomy and vagotomy blocked this effect (Kiba.T *et al.*, 1995) (Kiba.T *et al.*, 1994). Hypothalamic $GABA_B$ergic innervation is reported have a stimulatory effect on sympathetic nervous system. The receptor number and affinity decreased in PH and NDEA treated rats while in LN treated rats, the receptor number increased without any change in

affinity. This indicates a decreased high-affinity $GABA_A$ receptor activity in PH and NDEA group while it increased in LN treated rats. The affinity change was confirmed by displacement analysis with muscimol against [^{3}H]GABA in NDEA treated rats where we found a shift in the high-affinity site towards low-affinity.

The low-affinity $GABA_A$ receptor binding parameters as determined by [^{3}H]bicuculline against bicuculline indicate a decrease in number and affinity of the receptor in PH and NDEA treated rats. The decrease in activity of the receptor was more pronounced in NDEA treated group. Displacement analysis showed a shift in the high-affinity site to low-affinity site in PH and NDEA groups. In LN treated group the shift in this site was more towards the high-affinity indicating an increased $GABA_A$ receptor function.

$GABA_B$ receptor density was increased in the PH and NDEA treated groups. In NDEA treated group the affinity of the receptor to baclofen also increased significantly. The affinity change in NDEA treated group was confirmed by displacement analysis where we have observed a shift in affinity towards high-affinity indicating the increased $GABA_B$ receptor function.

The results showed that hypothalamic high-affinity and low-affinity $GABA_A$ receptor activity decreased during liver regeneration and neoplasia. It is already reported that intra-hypothalamic administration of $GABA_A$ receptor antagonist bicuculline methoiodide decreased the sympathetic innervation and blood pressure in a dose dependent manner (Tellioglu.T *et al.*, 1996). So the decreased $GABA_A$ receptor activity observed may be facilitating the sympathetic innervation. $GABA_B$ receptor activity was increased in PH and NDEA treated rats. This kind of differential functioning of $GABA_A$ and $GABA_B$ receptor system and its importance in sympathetic innervation is already reported (Takenaka.K *et al.*, 1995). Plasma NE levels of different experimental groups in the present study are also in accordance with the differential functioning of these $GABA_A$ and $GABA_B$ receptors.

Cerebellum GABA receptor alterations

GABA has been established as a post-synaptic inhibitory neurotransmitter in the central nervous system particularly in the cerebellum (DeFeudis.F.V, 1977). The

high-affinity $GABA_A$ receptor number decreased in PH and NDEA treated rats while it was unaffected in LN treated rats. The affinity of the receptor in LN treated rats increased significantly. The shift of the high-affinity site of the receptor towards further high-affinity region in LN treated group was evident from the displacement analysis with [^{3}H]GABA against muscimol. Low-affinity $GABA_A$ receptor number decreased in PH and NDEA treated rats as evident from [^{3}H]bicuculline Scatchard. The affinity of the receptor decreased in NDEA treated group. Displacement analysis of the receptor with [^{3}H]bicuculline against bicuculline confirmed a shift in the low-affinity to very low-affinity in NDEA treated group.

$GABA_B$ receptor affinity was increased in PH and NDEA treated rats while it decreased in LN treated rats. The displacement data are in accordance with the affinity change obtained from Scatchard plot. The changes in $GABA_B$ receptor activity and its importance in sympathetic stimulation was demonstrated by pyridoxine-deficient animal model (Paulose.C.S & Dakshinamurti.K, 1984).

Although there is no direct link between the cerebellum and liver, our findings suggest significant alterations in GABAergic function in this region during hepatic proliferation. We cannot consider different brain regions as separate entities. So the changes in one brain region may affect the total brain functional balance. Compared to hypothalamus and brain stem the changes in cerebellar region was less pronounced.

Hepatic GABAergic alterations

Serum GABA increased in all experimental groups but GABA receptor changes in liver was independent of serum GABA status. The results suggest that the receptor functional status is more important in hepatic cell proliferation than the circulating GABA levels. GABA content in liver was in accordance with the pattern of hepatic proliferation. The decreased GABA content in PH and NDEA treated rats enhances ornithine decarboxylase activity and thereby putrescine production (Minuk.G.Y, 1993). In LN treated rats the increased GABA content functions as a negative feedback for decreasing the ornithine decarboxylase activity and putrescine production. Putrescine is the precursor of polyamines and is a hepatic promoting substance (Theocharis.S.E *et al.*, 1998). The

hanges in hepatic GABA content may be due to changes in production and/or uptake nd/or clearance.

The high-affinity $GABA_A$ receptor binding parameters in the liver showed the resence of very less number of receptors on liver membranes than the membranes isolated om the brain. There was a significant decrease in the number of receptors in PH and \DEA treated rats. In NDEA treated rats the low-affinity site is completely lost and the igh-affinity site shifted to low-affinity. The low-affinity $GABA_A$ receptor binding also howed a decrease in the number of receptors in PH and NDEA treated rats while the umber of receptors increased in LN treated rats as evident from the [^{3}H]bicuculline catchard. Here we haven't seen any shift in affinity of this receptor when displaced with icuculline in all the groups. [^{3}H]GABA binding to membrane is reported to be decreased t the time of peak DNA synthesis in liver during liver regeneration (Minuk.G.Y *et al.*, 992). Studies using $GABA_A$ receptor antagonist, ciprofloxacine, demonstrated the nhibitory effect of GABA on hepatic proliferation (Minuk.G.Y *et al.*, 1995) (Kaita.K.D.E *t al.*, 1998) (Kaita.K.D.E *et al.*, 1998). The decrease in $GABA_A$ receptor number in PH us thus facilitate the hepatic cell proliferation. In LN treated rats the receptor functioning s high may be to suppress the excess cell proliferation for maintaining the normal liver ass. $GABA_A$ receptor-induced neuronal apoptosis is well established (Ikonomidou.C *et l*, 2000). In LN induced direct hyperplasia the removal of excess liver mass by apoptosis s already described (Columbano.A *et al.*, 1985). So increased $GABA_A$ergic activity may e responsible for the involution of liver hyperplasia and maintain normal liver mass. In \DEA treated rats the loss of one of the affinity sites as well as decreased $GABA_A$ receptor umber and function may facilitate uncontrolled cell proliferation. The $GABA_A$ergic echanism to suppress cell proliferation that was observed in LN treated rats was totally ltered in NDEA treated rats. Thus, $GABA_A$ receptor functional alteration may be one of e contributing factors for tumorigenesis in NDEA administered rats.

Although biochemical studies using radioreceptor assay described the presence of $GABA_A$ receptor in liver, no study to date was carried at the mRNA expression level. So e have gone for the sensitive RT-PCR technique to study the mRNA expression of the ree of the six highly expressed receptor subunits in brain (Schousboe.A & Redburn.D.A, 995) i.e. $\beta 2$, $\beta 3$ and $\gamma 2$ subunits. The primers have been used successfully to amplify

$GABA_A$ receptor subunit mRNA in primary cultures of rat cerebellar neurones (Bovolin.P *et al.*, 1992) and rat pancreas (Borboni.P *et al.*, 1994). Beta subunits are used to find out the subcellular localisation of $GABA_A$ receptor (Connolly.C.N *et al.*, 1996) and $\gamma 2$ subunit important in benzodiazepam modulation of $GABA_A$ receptor (Stephenson.F.A, 1995).

The $\beta 3$ subunit expression was found to be almost nil in NDEA treated and PH rats, suggesting its importance in active cell proliferation. This also suggests the importance of this subunit gene expression in normal cell proliferation rather than in carcinogenesis. In LN treated rats the expression of this receptor increased significantly in accordance with our radioreceptor assay results. This increased expression of mRNA may be responsible for the increased receptor number. Beta2 subunit mRNA expression also decreased significantly in PH and NDEA treated rats. Gamma2 subunit expression increased in LN treated rats, while it was decreased in NDEA treated rats. In PH rats, there was no change in expression of this subunit compared with control. This suggests a link between $\gamma 2$ subunit expression and malignant growth and cell proliferation suppression in liver. But it is not involved in normal hepatic proliferation as evident from the PH experimental data.

Disappearance of functional $GABA_A$ receptor is reported in maliganant tumours and immortal cell lines (Labrakakis.C *et al.*, 1998). Our radioreceptor assay and RT-PCR analysis clearly indicate that the gene expression as well as functional status of these receptors significantly decreased in normal and cancerous hepatic cell proliferation. The alterations of the receptor were more drastic in neoplastic liver than in regenerating liver. Also, our result is supported by the recently reported study on hepatocellular carcinoma cell line HepG2 overexpressing $GABA_A$ receptor $\beta 2$ and $\gamma 2$ subunits (Zhang.M *et al.*, 2000). $GABA_A$ receptor activity is markedly down regulated in malignant hepatocytes. Transfection studies in HepG2 cells co-transfected with $GABA_A$ receptor $\beta 2$ and $\gamma 2$ subunit were followed by exposure to muscimol inhibited α-foetoprotein (a hepatic malignant marker) mRNA expression and also the cell proliferation (Zhang.M *et al.*, 2000).

$GABA_B$ receptor number significantly increased in NDEA treated rats while in the PH rats affinity of the receptor increased without any change in density of the receptor. In LN treated rats the affinity of the receptor was significantly reduced. The affinity changes were supported by displacement data of [^{3}H]baclofen against baclofen. The shift in affinity was more towards the high-affinity region in PH rats while it shifted to low-affinity in LN

reated rats. The increase in number of $GABA_B$ receptors is reported in breast cancer cell ines (Mazurkiewicz.M *et al.*, 1999) supportive to our findings. $GABA_B$ receptor is a G_i protein coupled receptor. Stimulation of G_i proteins can act as mitogenic signals in various ell types (Biesen.T.V *et al.*, 1996). So the increase in the number of $GABA_B$ receptor bserved in the NDEA treated rats may enhance the uncontrolled cell proliferation in liver. The decreased affinity of the receptor in LN treated rats may thus help to reduce hepatic ell proliferation.

GABA regulation of hepatocyte proliferation in *in vitro*

The important progress in defining the key factors in hepatic cell proliferation was chieved by using hepatocyte cultures in serum-free medium (Michalopoulose.G.K, 1990). A large number of viable hepatocytes can be produced by perfusing the rat liver with collagenase (Seglen.P.O, 1971). These hepatocytes can be placed in primary culture. Primary cultures of adult rat hepatocytes retain many liver functions and have been used for biochemical studies on liver function. They do not enter into DNA synthesis when kept in chemically defined media or media supplemented with foetal bovine serum. Insulin is a supplement required for all these media. In the absence of insulin, hepatocytes degenerate within 24-48 hr (Michalopoulose.G.K, 1990). However, Insulin despite its strong trophic effects on hepatocytes, does not by itself stimulate DNA synthesis in chemically defined media. So in our present study we have used insulin at a concentration of 100nM to support the primary hepatocyte culture. Primary cultures of rat hepatocytes can be used for liver regeneration studies and the optimal conditions had been described (Michalopoulose.G.K *et al.*, 1982). The study of hepatocyte proliferation in cultures has several well recognised advantages compared to studies of *in vivo* regeneration in defining the factors regulating the cell proliferation. The hepatocytes grow in a controlled environment virtually without interference from the other cell types in primary culture. Growth modulators added to culture medium act directly on the hepatocytes and interference of other factors such as hormones can be excluded (Michalopoulose.G.K, 1990). Most of the replicating hepatocytes enter into multiple consecutive rounds of DNA synthesis in culture and this replicating system of hepatocytes can be used to investigate the trophic factors that control growth of normal and neoplastic hepatocytes.

Effect of GABA on primary hepatocyte cultures

Hepatocytes do not replicate when kept in chemically defined media even supplemented with foetal bovine serum. However, addition of EGF caused a marked increase in DNA synthesis from basal level (Vintermyr.O.K & Doskeland.S.O, 1987). When EGF is added to cultures of freshly isolated hepatocytes, DNA synthesis did not start for 24 hr. The active DNA synthesis occurred from 48 to 72 hr (Sudha.B & Paulose.C.S, 1997) and hence we chose 48 hr of culture to study the effect of growth factors and GABA. The time lag in culture is in contrast to DNA synthesis in liver regeneration *in vivo*, which starts 16-18 hr and peaks at 24 hr (Michalopoulose.G.K & DeFrancis.M.C, 1997). The difference in time course might reflect repair processes after collagenase perfusion and adaptation of hepatocyte to the *in vitro* environment (Michalopoulose.G.K, 1990). Addition of GABA (100μM) alone did not elicit any significant change in hepatocyte DNA synthesis but it significantly inhibited the EGF mediated mitogenicity. This clearly demonstrates the inhibitory effect of GABA on hepatocyte proliferation. This result supports the already demonstrated effect of GABA on terminating rapid growth in developing tissue *in utero* (Gilon.P *et al.*, 1987) (Gilon.P *et al.*, 1987) (Seiler.N *et al.*, 1980). Since $GABA_A$ and $GABA_B$ receptors are present in liver (Castelli.M.P *et al.*, 1999) (Minuk.G.Y *et al.*, 1987) we studied the effect of these receptors on hepatocyte DNA synthesis independently by using specific agonists for these receptors.

Effect of muscimol on primary hepatocyte cultures

Muscimol, a specific agonist for $GABA_A$ receptor (Sieghart.W, 1995), was used to study the $GABA_A$ receptor mediated hepatocyte proliferation regulation. Muscimol (100μM) significantly inhibited the EGF induced hepatocyte DNA synthesis. It also enhanced TGFβ1 inhibition on EGF mediated DNA synthesis. Although there was a decrease in DNA synthesis in muscimol and TGFβ1 treated groups compared with TGFβ1 alone treated group, the changes were not statistically significant. Growth inhibitory property of GABA is reported in HCC cell line - HepG2, HeLa cell lines and squamous murine carcinoma (Zhang.M *et al.*, 2000) (Minuk.G.Y, 1993). Decreased $GABA_A$ receptor function is reported in malignant tumours and immortal cell lines (Labrakakis.C *et al.*, 1998). These reports and our results confirm the inhibitory effect of GABA on rapid

cell proliferation by reducing the activity of mitogenic growth factors and enhancing the inhibitory power of growth inhibitors. The inhibitory effect was confirmed by dose-dependent study of muscimol on EGF induced mitogenicity. Muscimol functions as an inhibitor of human HCC cell line over expressing $GABA_A$ receptor which supports our present findings (Zhang.M *et al.*, 2000).

Effect of baclofen on primary hepatocyte cultures

$GABA_B$ receptor mRNA is reported to be present in rat liver (Castelli.M.P *et al.*, 1999). Unlike $GABA_A$ receptor, $GABA_B$ receptor agonist, baclofen, induced EGF mediated hepatocyte proliferation. It also reduced TGFβ1 inhibition on EGF induced mitogenesis. Baclofen alone couldn't elicit any mitogenic response suggesting the co-mitogenic role of $GABA_B$ receptor in hepatocyte proliferation. The growth stimulatory action of $GABA_B$ receptors in human breast cancer cell line (Mazurkiewicz.M *et al.*, 1999) supports its co-mitogenic role. There was a dose-dependent increase in $GABA_B$ receptor mediated EGF mitogenicity and this was abolished by G_i protein inhibitor PTX. $GABA_B$ receptor is coupled to G_i protein (Kerr.D.I.B *et al.*, 1990). The stimulation of these G_i protein coupled receptors induce cell proliferation in various tissues (Biesen.T.V *et al.*, 1996). Several lines of evidence suggest that activation of receptors that couple to heterotrimeric G-proteins is important in regulating liver regeneration after PH (Michalopoulose.G.K, 1990). The expression of the stimulating and inhibitory α-subunits of G-proteins that couple various receptors to their effector targets like adenylyl cyclase is differentially regulated during the early pre-replicative period in the liver (Mahler.S.M & Wilce.T.A, 1988). G-protein coupled receptors influencing EGF function is already reported (Daub.H *et al.*, 1997). In Rat-1 fibroblasts, Erk activation via endogenous IGF-I receptor and G_i-coupled LPA receptor is sensitive to PTX treatment suggesting a cross talk between the receptors leading to mitogenesis (Luttrell.L.M *et al.*, 1995). Such a cross talk between $GABA_B$ receptor α-subunit and EGF receptor may be responsible for the triggering of hepatocyte DNA synthesis. Neurotransmitter receptors like α1 adrenergic and S2 class serotonin receptors acts as co-mitogenic signals in EGF mediated DNA synthesis in hepatocyte cultures (Sudha.B & Paulose.C.S, 1997) (Michalopoulose.G.K & DeFrancis.M.C, 1997) supports our findings.

CONCLUSION

We conclude from our studies that the changes in $GABA_A$ and $GABA_B$ receptor function of the brain stem, hypothalamus and cerebellum play an important role in the sympathetic regulation of cell proliferation and neoplastic growth in liver. Though many studies implicated the sympathetic nervous system to be an essential part of the regenerative response, the involvement of specific neurotransmitters, their receptors and their regulatory function were not given emphasis. We have observed decrease in GABA content in brain stem, hypothalamus and cerebellum during regeneration and neoplasia in liver. The time course of brain GABAergic changes was closely correlated with that of hepatic DNA synthesis. The functional significance of these changes was further explored by studying the changes in the $GABA_A$ and $GABA_B$ receptors in the brain. The $GABA_A$ and $GABA_B$ receptors of the brain regions showed reciprocal changes. The hypothalamic high-affinity and low-affinity $GABA_A$ receptors exhibited a down regulation during liver regeneration and cancerous growth, while the $GABA_B$ receptors showed reciprocal changes. The receptor functional changes in brain stem and cerebellum were similar to that of hypothalamus. In LN treated rats, the $GABA_A$ and $GABA_B$ receptor changes were opposite to that of PH and NDEA treated rats. These alterations of the $GABA_A$ and $GABA_B$ receptors of the brain is suggested to govern the regenerative response, growth regulation and neoplastic transformation of the liver through sympatheic innervation. The hepatic high-affinity and low-affinity $GABA_A$ receptors were also down regulated during liver regeneration and NDEA induced HCC. The receptor subunit gene expression was significantly reduced during liver regeneration and neoplasia. The $GABA_A$ receptor changes and enhanced gene expression in LN treated rats were in favour of removing the excess liver mass. $GABA_B$ receptor function was increased in liver regeneration and liver cancers suggesting a stimulatory role of this receptor in hepatic proliferation and neoplastic transformation. Thus, the functional balance of these $GABA_A$ and $GABA_B$ receptors in brain and liver is critical in the regulation of hepatic proliferation and neoplastic transformation. $GABA_A$ receptors significantly inhibited the EGF mediated DNA synthesis and enhanced the mito-inhibitory effect of TGFβ1 in primary hepatocyte cultures. $GABA_B$ receptors enhanced the EGF induced mitogenesis and offset the mito-inhibitory effect of

TGFβ1 in primary hepatocyte cultures. Thus, the $GABA_A$ and $GABA_B$ receptor mediated mechanisms and their functional balance in the brain and liver is suggested to exert a major regulatory role in the transition of hepatocytes from quiescent to proliferative state. GABAergic functional modification could lead to therapeutic intervention in liver diseases and cancers which will have immense clinical implications.

SUMMARY

1. Liver cell proliferation after partial hepatectomy and lead nitrate administration was used as models of controlled cell proliferation in rats. The hepatocellular carcinoma induced by *N*-nitrosodiethylamine treatment was used as a model system to study the deregulated cell proliferation in rat liver.

2. Primary cultures of rat hepatocytes were used as the *in vitro* system to study liver cell proliferation.

3. [^{3}H] Thymidine incorporation into the hepatic DNA was used as the index to study DNA synthesis after partial hepatectomy, lead nitrate administration and in cultured hepatocytes. Hepatic thymidine kinase activity was used as index of DNA synthesis in NDEA treated rats. DNA synthesis peak was at 24 hr after partial hepatectomy whereas in lead nitrate treated rats DNA synthesis peaked at 48 hr. Histological studies and thymidine kinase activity showed tumorigenesis and an increased DNA synthesis in NDEA treated group.

4. GABA content was significantly decreased in brain stem, hypothalamus and cerebellum during peak DNA synthesis in liver regeneration and hepatic neoplasia. $GABA_A$ receptor function was decreased and $GABA_B$ function was increased in the brain regionsof these rats. GABA content and these receptors showed reciprocal changes in lead nitrate induced direct hyperplasia.

5. Down regulation of hepatic $GABA_A$ receptors and up regulation of $GABA_B$ receptors were observed in hepatocellular carcinoma and liver regeneration. In lead nitrate treated rats the activity of $GABA_A$ receptor was increased and $GABA_B$ receptor was decreased.

6. $GABA_A$ receptor involvement in inhibiting the mitogenicity of EGF was confirmed in hepatocyte cultures. Co-mitogenic effect of $GABA_B$ receptor was established in primary hepatocyte cultures. Muscimol inhibited the EGF mediated DNA synthesis and baclofen showed a stimulatory effect in a dose-dependent manner.

7. Three of the six highly expressed $GABA_A$ subunit mRNAs in brain were also detected for the first time in rat liver.

8. Beta3, $\beta 2$ *and* $\gamma 2$ subunit mRNAs expression of $GABA_A$ receptor was found to be significantly impaired in hepatocellular carcinoma. In regenerating rat liver $\beta 2$ and $\beta 3$

subunits mRNAs were significantly decreased while $\gamma 2$ subunit remained unaltered. In lead nitrate rats $\beta 3$ and $\gamma 2$ subunit mRNAs were significantly increased while $\beta 2$ subunit remained unaltered.

Thus it is evident from our results that $GABA_A$ and $GABA_B$ receptors gene expression and functional alterations in brain and liver are important for the hepatic cell proliferation. The functional balance between these two receptors is one of the key regulatory factors that decide the progression of quiescent hepatocyte into replicative phase. This suggests GABA function as a possible target for curing deleterious hepatic proliferation.

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1. **M.P.Biju** and Paulose. C.S. "Brain glutamate dehydrogenase changes in streptozotocin diabetic rats as a function of age" ***Biochemistry and Molecular Biology International***, 44(1); 1-7, **(1998).**
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1. **M.P.Biju**, S.Pyroja, N.V.Rajeshkumar, Ani V. Das, T.R. Renuka, P.N. Eswar Shankar, and C.S.Paulose "Altered $GABA_A$ receptor expression and function in direct hyperplasia and liver neoplasia of rat: inhibitory effect on EGF mediated hepatocyte DNA synthesis", ***'19th Annual Convention & National Symposium on Biology of Cancer (Modern concepts and Recent Developments)'***, Jan. 21-23, 2000.
2. **M.P.Biju** and C.S.Paulose "Brain GABAergic function decrease and DNA synthesis in liver after partial hepatectomy", ***67th Annual Meeting of Society of Biological Chemists (India)*** December 19-21, 1998.
3. **M.P. Biju** and Paulose C.S. "Kinetic parameters of glutamate dehydrogenase in the cerebellum of young and one year old streptozotocin induced diabetic rats", ***Symposium on Molecular Markers of Ageing,*** April 7-9, 1997.