5-HT_{1A} AND 5-HT_{2C} RECEPTOR GENE EXPRESSION AND FUNCTIONAL REGULATION DURING RAT HEPATOCYTE PROLIFERATION AND APOPTOSIS

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PYROJA S.

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 **"5-HT_{1A} AND 5-HT_{2C} RECEPTOR GENE EXPRESSION AND FUNCTIONAL REGULATION DURING RAT HEPATOCYTE PROLIFERATION AND APOPTOSIS"** is a bonafide record of the research work carried out by **Ms. PYROJA S.** 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.

Cochin - 682 022
Date : 3 April, 2002

DEPARTMENT OF BIOTECHNOLOGY
COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY
COCHIN-
682 022

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

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

ABBREVIATIONS USED IN THE TEXT

5-HT	5-Hydroxy tryptamine
5-HTP	5-Hydroxy tryptophan
8-OH DPAT	8-Hydroxy-2(di-n-propylamino)-tetralin
ADP	Adenosine diphosphate
AP-1	Activating protein 1
ATP	Adenosine triphosphate
B_{max}	Maximal binding
BS	Brain stem
C/EBP β	CCAAT /Enhancer binding protein β
cAMP	Cyclic adenosine mono phosphate
CC	Cerebral cortex
Cdk	Cyclin dependent kinase
CNS	Central nervous system
CREB	CAMP regulatory element binding protein
DAG	Diacylglycerol
dATP	Deoxy adenosine triphosphate
dCTP	Deoxy cytosine triphosphate
DEPC	Di ethyl pyro carbonate
dGTP	Deoxy guanosine triphosphate
dNTP	Deoxynucleotide triphosphate
DOI	1-(2,5-di-methoxy-4-iodophenyl)-2-aminopropane
dTTP	Deoxynucleotide thymidine triphosphate
EBSS	Earle's balance salt solution
EGF	Epidermal growth factor
ERK	Extracellular signal-regulated kinase
FGF	Fibroblast growth factor
GABA	Gamma aminobutyric acid
GTP	Guanosine triphosphate
HBGF	Heparin binding growth factor
HBSS	Hank's balance salt solution
HGF	Hepatocyte growth factor
HPLC	High performance liquid chromatography

HPTA	Hepatopoietin A
HSS	Hepatic stimulatory substance
HYPO	Hypothalamus
IL	Interleukin
JNK	c-Jun amino-terminal kinase
K_d	Dissociation constant
LN	Lead nitrate
MAPK	Mitogen-activated protein kinase
MuMLV	Murine moloney leukemia virus reverse transcriptase
NADH	Nicotinamide adenine dinucleotide, reduced form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NDEA	*N*-nitrosodiethylamine
NE	Norepinephrine
NF-κB	Nuclear factor kappa B
p	Level of significance
PBS	Phosphate buffered saline
Rb	Retinoblastoma tumour suppressor gene
PCPA	Paracholorophenyl alanine
PH	Partially hepatectomised
PIP_2	Phosphatidylinositol-4,5-biphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PTX	Pertussis toxin
RT-PCR	Reverse-transcription-polymerase chain reaction
S.E.M.	Standard error of mean
SAPK	Stress activated protein kinase
STAT	Signal transducer and activator of transcription
T_3	Tri-iodo thyronine
TGF	Transforming growth factor
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TR	Thyroid hormone receptor

CONTENTS

INTRODUCTION

The maintenance of normal cell function and tissue homeostasis is dependent on the precise regulation of multiple signaling pathways that must accurately control cellular decisions to either proliferate, differentiate, arrest cell growth, or initiate programmed cell death (apoptosis). Cancer arises when clones of mutated cells escape this balance and proliferate inappropriately without compensatory apoptosis. Many studies have revealed that the disruption of multiple pathways is required for the development of cancer. Thus, not only it is critical to understand the normal function of specific cellular pathways, but also equally important is an understanding of how they interconnect to synchronously regulate cell growth versus apoptosis.

The liver plays a primary role in body homeostasis. It regulates levels of circulating nutrients, excretes waste products into the bile, reduces circulating ammonia through production of urea, produces important serum proteins, bile acids required in digestion of lipids and acts as the primary site of a metabolic defense against carcinogens that enter through the gastrointestinal tract. Under normal physiological conditions, liver size is tightly controlled in almost all species. When baboon livers are transplanted into humans, they grow to the mass of a human liver. The liver has a remarkable capacity to regenerate after cellular damage or tissue removal. Liver regeneration is mostly the result of increased mitosis of hepatocytes. Removal of as much as 80-90% of the liver can be restored in the absence of liver disease. Livers from rats have been subjected to partial hepatectomy (removal of $2/3^{rd}$ of the liver) 12 times in a row. Each time, the liver is restored to its normal size within a few weeks. It has been estimated that one rat hepatocyte has the capacity to generate at least 50 livers (Michalopoulos.G.K & DeFrancis.M.C, 1997).

The liver has the unique capacity to regulate its growth and mass both in humans and animals. The mammalian liver is one of the few adult organs capable of completely regenerating itself in response to injury through the release of growth factors that stimulate reentry of terminally differentiated hepatocytes into the cell cycle (Michalopoulos.G.K & DeFrancis.M.C, 1997, Fausto.N *et al.*, 1995 &, Taub.R, 1996). This property is particularly remarkable because hepatocytes are cells, which in their

normal state rarely divide. However, their proliferative capacity and the ability of the liver to adapt to variable metabolic demands are not lost. These properties are quickly displayed when a deficit or excess of hepatic mass occurs (Fausto.N & Webber.E.M, 1994). The surgical removal of 70% of the liver induces a partially synchronised growth response that leads to the rapid restoration of organ mass.

Lead nitrate (LN) is a direct mitogen, which specifically induces liver hyperplasia. A single intravenous injection of LN caused hepatic cell proliferation, which peaked at 48 hours followed by hepatic apoptosis. Apoptosis was found to be markedly increased at 5 days after LN treatment (Shinozuka.H *et al.*, 1996 & Columbano.A *et al.*, 1985). Apoptotic cells die a stereotypical death, regardless of the initiating death signal (Martin.S.J *et al.*, 1994). During apoptosis the cytoplasm shrinks, the plasma membrane blebs and vesiculates, and phosphatidylserine redistributes to the cell surface. Simultaneously, the nucleus shrinks, chromatin condenses and DNA fragments into high molecular weight oligonucleosomal pieces. Insufficient apoptosis because of caspase inactivation may promote oncogenesis by allowing cell accumulation (Martin.S.J & Green.D.R, 1995). Recent evidence supports this hypothesis, and careful study of the caspase knockout animals should provide more definitive answers to this question (Resnicoff.M *et al.*, 1998 & Green.D.R, 1998).

Globally, primary liver cancer was ranked seventh in men in 1975 and by 1990 it reached the fifth position (Perin.N.N, 2001). It is estimated that approximately 1.25 million people die from hepatocellular carcinoma each year. Tumourigenesis is a complex process involving at least three steps, initiation, promotion and progression (Farber.E, 1976). Many oncogenes and protooncogenes are now known and some of these are closely associated with cell growth (Weinberg.RA, 1989, Goustin.A.S *et al.*, 1986 & Bishop.J.M, 1987). The activation of protooncogenes by a variety of mechanisms that affect their expression is believed to play an important role in formation and progression of tumours. *N*-nitrosodiethylamine (NDEA) is a well known carcinogenic agent causing hepatocellular carcinoma (Narurkar.L.M. & Narurkar.M.V, 1989). NDEA induces liver tumours in guinea pigs, rabbits, dogs, and rats and nasal cavity tumours in rats (Nakae.D *et al.*, 1997).

Identification of mutations in tumours that lead to decreased apoptosis is not only of academic interest but rather an important goal in the light of cancer therapy. Clearly, mutations in cell death control do affect sensitivity of tumour cells to anti-cancer therapy which in most cases functions by inducing apoptosis (Zhang.L *et al.*, 2000 & Finkel.E, 1999). It has become clear that, together with deregulated growth, inhibition of programmed cell death (PCD) plays a pivotal role in tumourigenesis.

In vivo and *in vitro*, many substances have been shown to influence the entry of hepatocytes into DNA synthesis. Serum proteins, peptide growth factors, androgens, estrogens, glucocorticoids, thyroid hormones and adrenergic agents all have been implicated in the regulation of growth. Growth factors and growth factor receptors play an important physiological role in the normal process of growth and differentiation. The binding of the growth factor to its receptor leads to receptor dimerisation and cross phosphorylation, activating the receptors. The activated receptors phosphorylate a series of cytoplasmic proteins which in turn sets off a cascade of events leading to the activation of transcription factors in the nucleus resulting in increased mRNA synthesis. The translation of the mRNA results in increased protein synthesis finally leading to either growth or differentiation (Fantl.W.J *et al.*, 1993). Aberrations of growth factor signaling pathways can lead to abnormal growth and development. Cancer is now recognised to be the result of a multistep process. Among the events that can lead to malignant transformation is the unregulated expression of growth factors or components of their signaling pathways. Apoptosis or programmed cell death is an important physiological phenomenon playing crucial role in growth and development of an organism. It also plays an important protective role in DNA damaged cells which fail to have their DNA damage repaired but attempting to enter the cell cycle. By triggering apoptosis, these abnormal cells are destroyed, thereby preventing tumour induction. In the absence or inhibition of apoptosis, these cells survive, cumulate more DNA damage and tend to acquire an altered phenotype.

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 while 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, neurotransmitters and their receptors in mediating these effects are not well characterised.

Neuronal stimulation of rat liver could result in changes in cellular metabolism and liver regeneration through a combination of direct innervation and intercellular communication via gap junctions (Seseke.F.G *et al.*, 1992). Vagotomy was shown to cause a marked depression of cell proliferation following hepatectomy (Maros.T, 1970 & Ohtake.M *et al.*, 1993). Extirpation of the brain cortex was shown to increase the rate of cell proliferation implying that the cortex exerts a normal inhibitory function on liver cell division and growth. It was reported that transection of the spinal cord above the area innervating the liver resulted in decreased DNA synthesis (Vaptzarova.K.I *et al.*, 1973). Norepinephrine (NE) was shown to induce DNA synthesis in a dose-dependent manner in hepatocyte cultures, acting through the α1 adrenergic receptor (Cruise.J.L & Michalopoulos.G, 1985).

Neurotransmitters stimulate or inhibit cell proliferation in non-neuronal cells by activating receptors coupled to various second messenger pathways (Kluess.C *et al.*, 1991). 5-hydroxytryptamine (5-HT) has been recognised to cause proliferation of a variety of cells in culture, including those of vascular smooth muscle cells and hepatocytes. The proliferative effect is synergistic with that of more conventional growth producing polypeptides. Most evidences indicate that cellular cyclic nucleotides play an important role in the intracellular signaling process for growth regulation by 5-HT, and newer studies point to protein phosphorylation pathways as being important in the mitogenic response (Fanburg.B.L & Lee.S.L, 1997). Using a pancreatic cell line, Ishizuka *et al.*, (Ishizuka.J *et al.*, 1992) proposed that activation of cellular proliferation occurs through a pertussis toxin-sensitive 5-$HT_{1A/1B}$ receptor activated through PLC and PKC that resulted in downregulation of cellular cAMP. *Mene et al.*, (Mene.P *et al.*, 1991) suggested activation of a 5-HT_2 receptor in rat renal mesangial cells to account for 5-HT induced cell proliferation. The involvement of 5-HT and its receptor subtype in the induction of hepatocyte DNA synthesis was investigated in primary cultures of adult rat hepatocytes.

5-HT caused a dose-dependent increase in DNA synthesis in primary cultures of rat hepatocytes in the presence of epidermal growth factor (EGF) and insulin. 5-HT can act as a potent hepatocyte co-mitogen and induce DNA synthesis in primary cultures of rat hepatocytes, which is suggested to be mediated through the 5-HT_2 receptors of hepatocytes (Sudha.B & Paulose.C.S, 1997). 5-HT_{1A} receptor is known to mediate cell differentiation and cessation of proliferation in neuronal cells (Azmitia.E.C, 2001).

The work that is presented here is an attempt to understand the role of 5-HT, 5-HT_{1A} and 5-HT_{2C} receptors in the regulation of liver cell proliferation using *in vivo* and *in vitro* models. The work also focuses on the brain serotonergic changes associated with hepatocyte proliferation and apoptosis to delineate its regulatory function. The investigation of mechanisms involving different models of hepatocyte proliferation may contribute to our knowledge about serotonergic regulation of cell growth, apoptosis and carcinogenesis of liver.

OBJECTIVES OF THE PRESENT STUDY ARE:

1. To induce controlled liver cell proliferation by partial hepatectomy and lead nitrate treatment, hepatic apoptosis by lead nitrate treatment and hepatocellular carcinoma by *N*-nitrosodiethylamine treatment in male Wistar rats.
2. To study DNA synthesis by [^{3}H]thymidine incorporation/thymidine kinase assay in regenerating, lead nitrate induced hepatic hyperplasia and apoptosis and NDEA induced hepatic neoplasia in rats.
3. To study the 5-hydroxytryptamine (5-HT) content in brain stem, cerebral cortex, hypothalamus and liver during controlled cell proliferation, controlled cell death and uncontrolled cell proliferation in rat liver using High Performance Liquid Chromatography (HPLC) integrated with an electrochemical detector.
4. To study the plasma norepinephrine levels in the experimental animals using HPLC.
5. To study 5-HT_{1A} and 5-HT_{2C} receptor status in brain stem, cerebral cortex, hypothalamus and liver during active hepatocyte proliferation and apoptosis in male Wistar rats.
6. To study the alteration of 5-HT_{1A} and 5-HT_{2C} receptor mRNA in brain stem, cerebral cortex, hypothalamus and liver of experimental rats using reverse transcription polymerase chain reaction (RT-PCR).
7. To study the effect of 5-HT_{1A} and 5-HT_{2C} receptor ligands in DNA synthesis in primary hepatocyte culture in combination with epidermal growth factor and/or transforming growth factor β1.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

The mammalian liver is one of the few adult organs capable of completely regenerating itself in response to injury through the release of growth factors that stimulate re-entry of terminally differentiated hepatocytes into the cell cycle (Fausto.N *et al.*, 1995, Taub.R, 1996 & Michalopoulos.G.K & DeFrancis.M.C, 1997). The liver responds to many forms of injury, including traumatic, chemical, metabolic, or infectious injuries, with a proliferative response in the remnant tissue (Michalopoulos.G.K & DeFrancis.M.C, 1997 & Fausto.N, 2000). Unlike other regenerating tissues (e.g. skin, gastrointestinal epithelium, and bone marrow) the liver does not require a stem cell population for regeneration. Instead, liver regeneration can proceed by stimulation of existing, normally quiescent, mature cellular populations. After proliferation and restructuring, the regenerative response stops and the cells of the liver return to a state of quiescence. Hepatocytes are characterised by a longer G1 phase when compared to the other cells. Adult mammalian hepatocytes are in a quiescent state with respect to cell division and have long life spans (Hunter.T, 1993). Experimentally, liver cell proliferation can be induced by any acute treatment, surgical or chemical, that will remove or kill a large percentage of hepatic parenchyma. Loss of parenchyma rapidly induces a wave of cell proliferation so that the total mass of the liver is restored to normal. Several chemical treatments will induce necrosis of the central zone of the hepatic lobule. CCl_4 is frequently used for this purpose. Although all cells of the liver participate in regeneration, most studies have focussed on the main functional cells of the liver, the parenchymal hepatocytes. These cells constitute the largest portion (80-90%) of liver cell mass. Several different experimental model systems will be necessary to fully elucidate the mechanisms, which regulate hepatocyte proliferation. Controlled cell proliferation during liver regeneration and 48 hours after LN treatment, hepatic apoptosis 5 days after LN treatment, NDEA induced hepatic cellular carcinoma and primary hepatocyte culture are the well established model systems to study hepatocyte replication (Futakuchi.M, 1999).

LIVER REGENERATION AFTER PARTIAL HEPATECTOMY

The adult hepatocytes have distinctive replication potential and therefore liver has the ability to regenerate, allowing for rapid recovery after partial hepatectomy, liver transplant, or toxic injury (Michalopoulos.G.K, 1990, Sandgren.E.P *et al.*, 1991 & Taub.R, 1996). The most preferred approach, however, for inducing liver regeneration is by performing two-thirds partial hepatectomy (2/3 PH) in rats as described by Higgins and Anderson in 1931 (Higgins.G.M & Anderson.R.M, 1931). In this simple surgical procedure, easily performed under light ether anaesthesia, two-thirds of the liver becomes externalised through a small mid-abdominal incision. The externalised portion of the liver is resected and the rats return to normal activity in minutes. This procedure requires no major abdominal surgery and is well tolerated by experimental animals. Regeneration of liver has been demonstrated in most vertebrate organisms and proceeds extremely fast (within 6-8 days after two-thirds resection) in all species examined, including humans.

After 70% partial hepatectomy, in which the two largest lobes of the liver are removed intact without injury to remnant liver cells, more than 95% of the mature, normally quiescent cells in the remnant liver rapidly proliferate, restoring liver mass in a few days. The hepatocytes undergo one or more rounds of semisynchronous replication after partial hepatectomy (PH) before returning to quiescence (Yamada.Y *et al.*, 1997).

In young rats and mice, 95% of hepatocytes replicate after PH, and the hepatic mass is restored in 7–10 days. The growth process is tightly regulated and terminates when it reaches a set point, defined as the optimal ratio between hepatic functional mass and body mass. The same principles that govern liver regeneration after PH in rats and mice apply to the growth response of human livers transplanted to a new host. In this situation, a small transplant grows, but a large transplanted liver decreases in size, so in each case, the optimal liver/body mass set point for the individual host is attained (Yamada.Y *et al.*, 1997). Liver regeneration after partial hepatectomy in animal models have indicated that this process is precisely regulated in its initiation, duration, and termination, with the regenerative response proceeding only until the liver to body weight of the animals has been restored (Higgins.G.M & Anderson.R.M, 1931). Moreover, regeneration occurs while the liver continues to perform its critical functions including glucose homeostasis, protein synthesis, bile secretion and toxin degradation.

The kinetics of the regenerative response in hepatocytes has been well described (Grisham.J.W, 1962 & Rabes.H.M *et al.*, 1976). DNA synthesis in these cells starts within 12-16 hours after two-thirds PH and first reaches a peak within 22-24 hours. A second smaller peak occurs at 48 hours, reflecting DNA synthesis occurring in the inner third (centrilobular portion) of the hepatic lobule. The DNA synthesis in the non-parenchymal cells, endothelial cells, macrophages and bile ductule cells starts 24 hours after parenchymal DNA synthesis. Cellular proliferation begins in the periportal region (i.e., around the portal triads) and proceeds towards the centres of lobules (Leevy.C.B, 1998). Proliferating hepatocytes initially form clumps and are soon transformed into classical plates. Proliferating endothelial cells develop into the type of fenestrated cells typical of those seen in sinusoids. The consensus of evidence suggests that after regeneration there is no substantial increase in the number of hepatic lobules, but that each lobule enlarges in size to encompass a large number of cells. A central concept in understanding cell kinetics of liver regeneration is that hepatocytes are generated by mitotic events occurring in other mature hepatocytes. The genesis of hepatocytes does not proceed through a stem-cell stage, comparable to that of occurring in bone marrow, intestinal crypts etc.

A large number of studies applying inhibitors of polyamine biosynthesis have indicated that these compounds are required for animal cell proliferation. Studies using a transgenic rat model with activated polyamine catabolism has proven that a certain critical concentration of the higher polyamines spermidine and spermine is required for liver regeneration (Alhonen.L *et al.*, 2002). An upregulation of the telomerase activity was found at 24 hours after PH. There was no remarkable change in the telomere length after PH. Preoperative treatment with EGF and hepatocyte growth factor (HGF) increased the *in vivo* telomerase activity. In a hepatocyte primary culture, upregulation of the telomerase activity required the presence of EGF, and this upregulation was accelerated by the addition of HGF.

The construction of the hepatocyte tight junction is one of the most important events during liver regeneration leading to the reorganisation of the bile canaliculi and the repolarisation of hepatocytes after cell division. The levels of tight junction components such as claudin-3, ZO-1 and atypical protein kinase C (PKC)-specific interacting protein

(ASIP) increased two to three fold over control levels in coordination with a peak 2-3 days after partial hepatectomy (Takaki.Y *et al.*, 2001).

Prostaglandin signaling is required during liver regeneration which is mediated through CREB activation (Rudnick.D.A *et al.*, 2001). Exogenous HGF may promote DNA synthesis and protein synthesis during liver regeneration after partial hepatectomy with cirrhosis (Ogura.Y *et al.*, 2001).

Answers to central questions about liver regeneration remain elusive, including the identity of the initiating signals in regeneration and the mechanism by which liver cells continue to function while they are regenerating.

LEAD NITRATE AND LIVER CELL PROLIFERATION

Lead is a ubiquitous environmental pollutant and still remains a serious health concern (Hammond.P.B & Dietrich.K.N, 1990 & Folinsbee.L.J, 1993). It is reported that liver is one of the main targets of lead accumulation. Lead also induces a variety of pathologic conditions in liver (Krasovskii.G.N *et al.*, 1979). Pani and his associates (Columbano.A *et al.*, 1983 & Dessi.S *et al.*, 1984) showed that LN when injected to rats induced a hyperplastic response in the liver of these animals, as shown by increased DNA synthesis and enhanced mitotic index. A single intravenous injection of LN to rats induces a synchronised wave of hepatocyte proliferation without accompanying liver cell necrosis (Shinozuka.H *et al.*, 1994). However, the mechanism of this proliferation and its effect on hepatocytes remain unknown. Hepatocyte proliferation occurred by 24 hours and reached a peak 48 hours after a single intravenous injection of LN (100 μmol/kg). TNFα mRNA expression in the liver was increased 1, 6, and 12 hours after the injection, whereas no alteration was observed in liver or blood level of HGF. Pretreatment with TNFα inhibitors, dexamethasone (4.0 mg/kg), E3330 (100 mg/kg), adenosine (0.3 mmol/kg), and pentoxifylline (100 mg/kg), inhibited both TNFα mRNA expression and hepatocyte proliferation 48 hours after the injection. This clearly indicates that TNFα positively regulates the hepatocyte proliferation induced in rats by the mitogen, LN (Kubo.Y *et al.*, 1996).

Lead nitrate was shown to injure hepatocytes through an increased lipid peroxidation. Response to the injury included increase in the proliferative activity of

parenchymal and sinusoidal liver cells. In addition, activation of Ito cells has been noted, which manifested an increased expression of desmin and increased proliferation (Gumerova.A.A *et al.*, 1999).

A strong and prolonged activation of NF-κB was observed in LN-induced hyperplasia. LN also induced an increase in hepatic levels of TNFα and inducible nitric oxide synthase (iNOS) mRNA (Menegazzi.M *et al.*, 1997).

PKC is found to induce many cellular responses including proliferation, differentiation, gene expression and tumour promotion (Blobe.G.C *et al.*, 1996). Twelve isozymes (α, βI, βII, γ, δ, ε, ζ, η, θ, λ, μ and ι) of PKC have been identified (Blobe.G.C *et al.*, 1996). These isoenzymes exhibit differences in structure and function in cell signalling. Reports showed that PKC α, PKC δ and PKC ζ are present in rat liver (Wetsel.W.C *et al.*, 1992), and either PKC α or PKC δ are involved in liver cell proliferation (Alessenko.A *et al.*, 1992). It is suggested that PKC isozymes may have different roles in liver cell proliferation. However, it is known whether the effects of LN on liver enlargement and cell proliferation may be the result of the stimulation of the second messenger involving PKC. Lead alters calcium mediated cellular processes in several biological systems. Calcium enhances the activity of protein kinase C (PKC) which takes part in eliciting cell mitosis. PKC activity of the purified particulate fraction was increased and reached a maximum at 24 hours and lasted for 48 hours. Moreover, the frequency of mitotic cells exhibited a significant increase in PKC like activity, reached its maximum at 24 hours with accompanying signs of liver enlargement. Studies suggest that the PKC activation may be involved in promoting liver cell proliferation in LN treated rats (Liu.J.Y *et al.*, 1997). In most tissues, cell division occurs continuously without an increase in organ size or tumour development, suggesting that the rate of cell division is balanced by the rate of cell death.

LEAD NITRATE & HEPATIC APOPTOSIS

Intravenous administration of LN has been successfully used as a model of *in vivo* induction of hepatic parenchymal cell death (Columbano.A *et al.*, 1983 & Columbano.A *et al.*, 1985). Removal of excess liver which follows the initial hyperplasia caused by lead is due to a controlled mode of cell death, namely, apoptosis (Columban

1985). Apoptosis plays a major role in the regression of mitogen (LN) induced hepatic hyperplasia (Nakajima.T *et al.*, 1995). The regression after liver hyperplasia by apoptosis was found at 5 days after LN injection. Five days after the LN injection corresponds to the peak of hepatocyte apoptosis (Dini.L *et al.*, 1999). LN induces apoptosis in alveolar macrophages, which could be attenuated by phosphodiesterase inhibitors, such as caffeine, suggesting a possible mechanism involving cAMP (Shabani.A & Rabbani.A, 2000).

During the involution of LN-induced hyperplasia in rat liver a significant increase of transglutaminase activity, enzyme concentration, transglutaminase messenger RNA and protein-bound epsilon-(gamma-glutamyl) lysine (product of transglutaminase action) coincided with programmed death (apoptosis) of hepatocytes (Fesus.L *et al.*, 1987).

NDEA INDUCED HEPATOCELLULAR CARCINOGENESIS

N-nitrosodiethylamine is one of the potent carcinogenic dialkyl nitrosamine present in the tobacco smoke, cheddar cheese, cured and fried meats and in a number of alcoholic beverages. It is used as a solvent in the fibre industry as a softner for copolymers and as an additive in lubricants (Brown.J.L, 1999). NDEA induces DNA single strand breaks in male Wistar rats (Preat.V *et al.*, 1987). There is sufficient evidence for the carcinogenicity of NDEA in experimental animals. 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).

NDEA is metabolised by a microsomal enzyme system that requires NADPH and oxygen. This metabolism leads to an unstable product, which decomposes to yield a reactive alkylating species. This species is too reactive chemically to influence significantly organs other than those in which it was generated. Alkylation of cellular components, particularly DNA, is a critical event in the initiation of tumours by these carcinogens. The greatest capacity to metabolise this nitrosamine to alkylating agents is found in the liver, but other organs, including the oesophagus, lung and kidney, are also

capable of activation. These organs may be more susceptible to alkylation than the liver because they have a lesser ability to catalyse the removal of O^6-alkylguanine from their DNA. However, orally administered doses of NDEA formed by nitrosation reactions within the gastrointestinal tract are rapidly absorbed from the upper part of the small intestine and carried to the liver in the portal blood supply. When small doses are given in this way, the capacity of the liver to metabolise the carcinogen is sufficient that the nitrosamine is effectively cleared in a 'first-pass' effect, leaving very little to interact with other organs (Pegg.A.E, 1980).

Cytochrome P450 (CYP) 2A5 is involved in the metabolism of NDEA, (Wastl.U.M *et al.*, 1998). Administration of NDEA increased the hepatic contents of cytochrome P450, cytochrome b5 and activities of NADPH-cytochrome c reductase and aryl hydrocarbon hydroxylase (AHH) (Habib.S.L *et al.*, 1998).

The expression of H-ras and N-ras was found to be increased in liver of rats fed with the carcinogen NDEA. N-ras appeared to be more aggressive than H-ras. This overexpression could be correlated with an inhibition in the functioning of GTPase activating protein (GAP). The activity of GAP in increasing the intrinsic GTPase activity of p21RAS was found to be much less in NDEA-treated rats as compared to that in control rats. It was observed that GAP isolated from NDEA-treated rats was extensively phosphorylated by protein kinase C, and this might be the reason for its decreased activity (Choudhury.S *et al.*, 1996).

Histological investigation of NDEA treated rats indicate mild hyperplasia and anisonucleosis at 30 days of treatment and prominent pathological features from 60 days onwards until the appearance of hepatocarcinoma at 120 days even without the development of any preneoplastic or neoplastic nodule. The results of the Northern blot hybridisation clearly indicate an increased expression of *c-jun* from 15 days onwards. This overexpression of *c-jun* at such an early stage indicates its association with the events earlier than the neoplastic changes. However, the persistent over expression of *c-jun* at all durations of treatment indicates its association with the events during the later stage of hepatocarcinogenesis, whereas *c-myc* over expression starts from 30 days of treatment and persists. The extent of increased expression of *c-myc* is less than that of *c-jun* expression until one month of treatment, after which the induction of *c-myc* exceeds

the expression of *c-jun*. Thus, the over expression of *c-myc* from two months onwards might be playing a critical role in maintenance of the malignant phenotype (Giri.R.K & Das.B.R, 1996).

Phosphatidyl inositol synthetase showed a transient increase in activity at 7 days after NDEA administration and thereafter the activity declined. The level of diacylglycerol (DAG), a key second messenger, showed a steady rise during the period of NDEA administration. A parallel increase in DAG kinase activity was also apparent. The observations suggest that alterations of enzymes central to second messenger system with resulting changes in phosphoinositide turnover are important events during hepatocarcinogenesis induced by NDEA (Choudhury.S *et al.*, 1995).

L-DOPA (L-dihydroxy phenyl acetic acid) administration prior to NDEA (influence on initiation) stimulated hepatocarcinogenesis considerably. A statistically significant decrease in hypothalamic noradrenaline content was identified during early stages of chemically induced neoplastic transformation of hepatic cellular elements (Gurkalo.V.K *et al.*, 1988).

The intracellular content of polyamines rises and the diaminooxidase activity falls in the liver of rats who were given NDEA in drinking water (Berdinskikh.N.K & Lialiushko.N.M, 1987). The rats treated with dopamine receptor agonist (apomorphine) showed statistically significant inhibition of carcinogenesis and neoplastic transformation of hepatocytes. Dopamine receptor antagonist (haloperidol) showed a tendency to stimulate the carcinogenesis. Also, the modifying effect of NDEA on behaviour stereotype and aggressiveness of experimental animals was found following apomorphine injection at various dosages (Gurkalo.V.K & Zabezhinski.M.A, 1984 & Gurkalo.V.K & Zabezhinskii.M.A, 1983).

Pharmacological activation of α adrenoreceptors or blocking of both β adrenoreceptors and cholinoreceptors has been revealed to stimulate hepatocarcinogenesis. On the contrary, the administration of α adrenoreceptors antagonist or β adrenoreceptor and cholinoreceptors agonists inhibited the process of carcinogenic transformation (Gurkalo.V.K & Zabezhinski.M.A, 1982).

PRIMARY HEPATOCYTE CULTURE

Important progress in defining the key factors in the hepatocyte proliferation was achieved using hepatocyte cultures in serum-free medium. Many hepatocytes can be produced by perfusing the rat liver with collagenase (Seglen.P.O, 1976). These hepatocytes can be placed in primary culture. These do not enter into DNA synthesis when kept in chemically defined media or media supplemented with fetal bovine serum. Insulin is a supplement required for all these media. In the absence of insulin, hepatocytes degenerate within 24-48 hours. Insulin, despite its strong trophic effects on hepatocytes, does not by itself stimulate DNA synthesis in chemically defined media. Two fundamental requirements need to be met for eliciting mitotic response in hepatocytes by growth stimuli. These are the presence of the amino acid proline in the medium (Houck.K.A & Michalopoulos.G, 1985) and plating of hepatocytes at low cell density (Michalopoulos.G.K *et al.*, 1982). Using the primary hepatocyte culture, studies were conducted which showed that few and specific polypeptide growth hormones can stimulate hepatocyte DNA synthesis. Other substances such as catecholamines do not stimulate DNA synthesis directly but can modulate DNA synthesis stimulated by other factors.

Classification of Growth Factors

Growth factors can be classified into three main groups based on their effect on DNA synthesis in primary cultures: complete mitogens, growth inhibitors and co-mitogenic growth factors.

Complete 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. The factors capable of this action include the following:

Epidermal growth factor

This prototype mitotic stimulator of most epithelial cells also stimulates DNA synthesis in hepatocytes (McGowan.J.A & Butcher.N.L.R, 1981). EGF was in fact the first substance to be shown to have this effect and is still the most frequently used polypeptide hormone to induce hepatocyte DNA synthesis in cultures. In suitable media,

EGF induces [^{3}H]thymidine labeling indices of 60-80%. Insulin is not essential for EGF-stimulated mitogenesis but is required for the full magnitude of the response. Transforming growth factor β1 (TGFβ1) suppresses the EGF-stimulated mitogenesis but not the EGF-stimulated increase in protein synthesis (Houck.K.A & Michalopoulos.G.K., 1989). Although EGF has been used as the prototype mitogen for hepatocytes, the significance of its role in liver regeneration is not very clear. A decline in the number of EGF receptors occurs rapidly, observed within 8 hours and reaching a low point approximately 40 hours after 2/3 PH. A parallel decline is also seen with EGF-dependent tyrosine kinase activity of the receptor (Rubin.R.A *et al.*, 1982).

Transforming growth factor α

Transforming growth factor α (TGFα) is mitogenic for hepatocytes and it shares the same receptor with EGF. This has been shown for mature and high molecular weight forms of TGFα extracted from hepatomas (Luetteke.N.C *et al.*, 1988) as well as for synthetic TGFα. It also appears that TGFα is a stronger mitogen than EGF (Mead.J.E & Fausto.N, 1989 & Brenner.D.A *et al.*, 1989). In the whole animal, the role of TGFα in liver regeneration has become a focus of attention as studies show that regenerating hepatocytes actively produce TGFα (Mead.J.E & Fausto.N, 1989). As TGFα is a complete hepatocyte mitogen, it is reasonable to hypothesise that secretion of TGFα by regenerating hepatocytes might constitute an autocrine loop resulting in stimulation of DNA synthesis. In this view, production of TGFα by hepatocytes may be the critical step that leads the cells towards DNA synthesis, whereas gene expression changes observed before this point might constitute a priming state in which hepatocytes prepare for DNA synthesis but are not committed to entering into it. Increased levels of TGFα were observed within 8 hours after two-thirds PH with a peak of expression at 24 hours followed by a decline and subsequent elevation with a peak at 72 hours.

Hepatocyte growth factor

Hepatocyte growth factor also known as scatter factor, stimulates division in hepatocytes, renal tubular epithelial cells, epidermal keratinocytes and melanocytes. The protein of 100,000 kDa was isolated from rat, rabbit, and human serum and plasma. It is more abundant in serum but it exists in plasma in measurable levels. It was originally

identified as Hepatopoietin A (HPTA) by Michalopoulos *et al* (Michalopoulos.G *et al.*, 1983) in 1983 as a fraction in chromatography of serum from hepatectomised rats. Haddad *et al.*, reported HGF mediated stimulation of the Met receptor tyrosine kinase resulting in pleiotropic cellular effects including proliferation, morphogenesis, motility and invasion in a variety of human cancers (Haddad.R *et al.*, 2001).

Heparin-binding growth factor - 1

Heparin-binding growth factor -1 (HBGF-1/acidic FGF) of 16,000kDa also stimulates DNA synthesis in hepatocytes. It appears however to act only on specific sub populations of hepatocytes, since only half of the cells respond to HBGF-1 [Michalopoulos.G.K, 1990 #51]. In contrast to HPTA, it requires heparin for its activity and is totally inactive in the absence of heparin.

Hepatopoietin B

Hepatopoietin B activity is also found in the serum of hepatectomised rats in addition to HPTA/HGF (Michalopoulos.G *et al.*, 1984). It is smaller than 500Da and does not contain amino acids in its molecule. Its properties are that of a glycolipid. It is a complete hepatocyte mitogen. It also interacts with both EGF and HPTA.

Hepatic Stimulatory Substance

In addition to the above four growth factors, which are complete hepatocyte mitogens, another substance called hepatic stimulatory substance (HSS) has also been described as mitogenic for hepatocytes *in vivo* (LaBrecque.D.R *et al.*, 1987, Francavilla.A *et al.*, 1987 & Fleig.W.E & Hoss.G, 1989). HSS is extracted from neonate and regenerating livers. Its molecular weight is approximately 16,000kDa. HSS increases DNA synthesis in livers subjected to 30% hepatectomy. It is not a complete mitogen for hepatocytes in culture. *In vitro,* it augments the effect of EGF and synergises with other substances extracted from hepatomas to stimulate hepatocyte DNA synthesis in culture. It also stimulates DNA synthesis in several hepatoma cell lines.

Growth Inhibitors

These substances have also been defined in primary culture based on their capability to inhibit EGF induced mitogenesis. Three main factors have so far been identified.

Transforming Growth Factor β1

More than three members of the TGFβ1 family of polypeptides have been identified. Their molecular weight is approximately 26,000kDa. TGFβ1 has been associated with a variety of *in vivo* functions, especially wound healing and proliferation of mesenchymal derived cells. TGFβ1 has been found to inhibit the growth of many epithelial cells in primary culture, including bronchial and mammary epithelial cells and keratinocytes (Sporn.M.B & Roberts.A.B, 1988). TGFβ1 inhibits mitogenesis induced by EGF or HPTA in hepatocyte cultures (Carr.B.I *et al.*, 1986). The ID_{50} for TGFβ1 varies with the dose of mitogenic stimulator and the culture condition. TGFβ1 may inhibit the G1 checkpoint, and serum TGFβ1 concentration may influence HGF to regulate liver regeneration and to maintain homeostasis of proliferation after PH anti- TGFβ1 antibody was injected immediately or 24 hours after 70% PH. Livers from treated animals contained an increased number of cells in S phase (Enami.Y *et al.*, 2001).

Interleukin 1β

It was recently shown that interleukin 1β (IL 1β), a form of IL1, inhibits hepatocyte proliferation. The degree of inhibition of DNA synthesis is not as complete as in the case of TGFβ1, with a residual 20% level of DNA synthesis still remaining after plateau effects are reached. Distinct but lesser inhibitory effects were also noticed with IL 6. This molecule is known for its strong effects in redirecting protein synthesis in the liver towards acute phase protein synthesis. Its effect on hepatocyte DNA synthesis might reflect an orchestrated reprogramming in gene expression in which synthesis of acute phase proteins takes precedence over the synthetic processes leading to hepatocyte replication (Nakamura.T *et al.*, 1988).

Hepatocyte proliferation inhibitor

This appears to be a protein of approximately 15,000kDa, which also inhibits hepatocyte proliferation in culture. Other epithelial cells derived from liver are also inhibited. The activity of hepatocyte proliferation inhibitor is not inhibited by anti-TGFβ1 antibodies.

Co-Mitogenic Growth Factors

This group is composed of substances that affect hepatocyte growth in a positive direction but in an indirect manner. These substances are best characterised as "growth triggers" that have the following properties:

1) Enhancement of the mitogenic effect of growth stimulators (EGF, HPTA etc.)

2) Decrease of inhibitory effect of growth inhibitors and

3) Absence of direct mitogenic effects in serum-free cultures

Hormones

Thyroid Hormone

The thyroid hormone (T_3) affects cell growth, differentiation, and regulates metabolic functions via its interaction with the thyroid hormone nuclear receptors (TRs). The mechanism by which TRs mediate cell growth is unknown. Studies showed that hepatocyte proliferation induced by a single administration of T_3 to Wistar rats occurred in the absence of activation of AP-1, NF-kappa B, and STAT3. Also, there was no change in the mRNA levels of the immediate early genes *c-fos*, *c-jun*, and *c-myc*. These genes are considered to be essential for liver regeneration after partial hepatectomy (PH). On the other hand, T_3 treatment caused an increase in cyclin D1 mRNA and protein levels that occurred much more rapidly compared to liver regeneration after 2/3 PH. The early increase in cyclin D1 expression was associated with accelerated onset of DNA synthesis. T_3 treatment also resulted in increased expression of cyclin E, E2F, and p107 and enhanced phosphorylation of pRb, the ultimate substrate in the pathway leading to transition from G1 to S phase (Pibiri.M *et al.*, 2001). 3,3',5-Triiodo-L-thyronine (200 microgram/100 g) is shown to be a potent liver parenchymal cell mitogen in the rat, and thyroidectomy is seen to severely inhibit hepatocyte proliferation in regenerating rat liver (Short.J *et al.*, 1980). Thyroid hormones can influence DNA synthesis during liver regeneration and they may regulate the activity of enzymes such as Thymidine kinase which are important for DNA synthesis and hence cell division (Maliekal.T.T *et al.*, 1997).

Vasopressin and angiotensin II

Liver regeneration following partial hepatectomy is significantly impaired in rats with hereditary vasopressin deficiency (Brattleboro strain), both in rate of DNA synthesis and in return of liver DNA content to normal. Vasopressin treatment at physiological doses ameliorates the defect and thus appears to be an important modulator of liver regeneration in response to partial hepatectomy in the rat (Russell.W.E & Bucher.N.L, 1983 & Metcalfe.A.M *et al.*, 1997). These hormones are acting through receptors that enhance PIP_2 turnover, and like norepinephrine stimulate glycogenolysis. Vasopressin stimulates DNA synthesis in cultured rat hepatocytes (Bhora.F.Y *et al.*, 1994). Vasopressin and angiotensin-II antagonised the effect of TGFβ1 and showed increased activity in regenerating hepatocytes (Houck.K.A & Michalopoulos.G.K., 1989). Vasopressin is secreted in the synapses of sympathetic nerves of the liver, however along with norepinephrine (Francavilla.A *et al.*, 1989). Thus both of these substances might be involved in mediation of the earlier observed effects of the sympathetic nervous system on liver regeneration.

Estrogens

Substantial evidence supports a role for estrogens in regeneration (Fisher.B *et al.*, 1984 & Francavilla.A *et al.*, 1984). Estrogens rise after 2/3 PH, reaching a peak at 24-48 hours. Conversely testosterone levels decrease. Tamoxifen, given soon after 2/3PH block hepatic DNA synthesis. Finally estrogens added to primary cultures with serum or EGF enhance mitogenesis (Francavilla.A *et al.*, 1989). Estrogen receptors and their retention time in the nucleus increase after 2/3 PH. Conversely, nuclear androgen receptors decrease. Hepatocytes from male rats are more responsive to EGF than of the female rats (Francavilla.A *et al.*, 1987). Ethinyl estradiol potentiates the effect of EGF in culture (Shi.Y.E & Yager.J.D, 1989). In the whole animal it acts as a promoter of hepatic tumourigenesis.

Insulin and Glucagon

Insulin does not stimulate hepatocyte proliferation in culture, despite strong trophic effects on hepatocyte physiology and viability. Hepatocytes in culture, in chemically defined media, degenerate and die in the absence of insulin. Pancreatectomy

results in diminished hepatic weight. Administration of insulin and glucagon partially reverses this atrophy. Despite strong evidence that insulin and glucagon play a permissive role for optimal DNA synthesis and liver regeneration, there is no evidence that these hormones have any mitogenic effects on the liver. Studies from Bucher *et al* (Bucher.N.L.R, 1982) have shown that injection of insulin and gulcagon in intact rats does not cause DNA synthesis.

Neurotransmitters

Norepinephrine

This is the well characterised neurotransmitter of the sympathetic nervous system. A role for sympathetic nervous system and its neurotransmitter in liver regeneration had been shown by several studies in the past (Cruise.J.L *et al.*, 1987). It was shown that blockade of α1 adrenergic receptor by prazosin abolishes the 24-hour peak of DNA synthesis seen during liver regeneration (Cruise.J.L *et al.*, 1987). DNA synthesis, however, reached normal levels at 72 hours after partial hepatectomy. This clearly shows that α1 adrenergic receptor has a function that is essential during the early stages of liver regeneration. Similar effects were seen with hepatic denervation. Addition of norepinephrine in culture has been shown to enhance the mitogenic effect of EGF and to cause a heterologous down-regulation of the EGF receptor. Norepinephrine alone is not mitogenic for hepatocytes (Cruise.J.L & Michalopoulos.G, 1985). Norepinephrine decreases the mitoinhibitory effect of TGFβ1 (Houck.K.A *et al.*, 1988). Hepatocyte from regenerating liver is particularly sensitive to this effect of norepinephrine on TGFβ1 (Houck.K.A & Michalopoulos.G.K., 1989). When regenerating liver hepatocytes from the above time points after 2/3 PH are kept in the presence of EGF and TGFβ1, addition of norepinephrine alone increases the labeling index from less than 4% to more than 70%. This clearly indicates the capability of norepinephrine, a non-mitogen *per se*, to tilt the balance between growth stimulators and inhibitors and to act as a trigger for hepatocyte mitogenesis. In view of the fact that inhibition of EGF stimulated mitogenesis by TGFβ1 is not mediated through effects on the EGF receptor (Russell.W.E, 1988), it appears that norepinephrine is acting independently on both the EGF and TGFβ1 mediated signaling pathways. Further evidence implicates α1 adrenergic receptor as

having a role in regeneration. The sensitivity of hepatocytes to norepinephrine changes at different times after hepatectomy. At 12-16 hours after 2/3 PH, regenerating hepatocytes are very sensitive to norepinephrine, especially in inhibiting the effects of TGFβ1 (Houck.K.A & Michalopoulos.G.K., 1989). Phenomena described as occurring very early after 2/3PH include membrane hyperpolarisation, glycogenolysis, and an increase in diacylglycerols (Houck.K.A & Michalopoulos.G.K., 1989). All of these effects are classically mediated by calcium mobilising receptors such as the $\alpha 1$ receptors. Catecholamines (NE and epinephrine) increase in the peripheral blood sharply after 2/3PH presumably due to removal of the bulk ($2/3^{rd}$) hepatic mono amino oxidase, the enzyme that degrades plasma catecholamines. Norepinephrine possess potent anti-apoptotic action in regenerating hepatocytes (Hamasaki.K *et al.*, 2001).

GABA

$GABA_A$ receptor agonist, muscimol, dose dependently inhibited EGF induced DNA synthesis and enhanced the TGFβ1 mediated DNA synthesis suppression in primary hepatocyte cultures. $GABA_A$ receptor act as an inhibitory signal for hepatic cell proliferation (Biju.M.P *et al.*, 2001). Serum GABA levels were increased in PH, LN treated and NDEA treated rats. GABA content decreased in the hypothalamus of PH and NDEA treated rats, while it increased in LN treated rats. $GABA_A$ receptor number and affinity in hypothalamic membrane preparations of rats showed a significant decrease in PH and NDEA treated rats, while in LN treated rats the affinity increased without any change in the receptor number. The $GABA_B$ receptor number increased in PH and NDEA treated rats, while it decreased in LN treated rats. The affinity of the receptor also increased in NDEA treated rats. Liver cell proliferation is influencing the hypothalamic GABAergic neurotransmission and these changes in turn regulate the hepatic proliferation (Biju.M.P *et al.*, 2001).

5-Hydroxytryptamine

5-HT may be tied to the evolution of life itself, particularly through the role of tryptophan, its precursor molecule. Tryptophan is an indole-based, essential amino acid, which is unique in its light absorbing properties. In plants, tryptophan-based compounds

capture light energy for use in metabolism of glucose, the generation of oxygen and reduced cofactors. Tryptophan, oxygen and reduced cofactors combine to form 5-HT. 5-HT-like molecules direct the growth of light-capturing structures towards the source of light. In plants, tryptophan produces receptor proteins, which harness light and thus produce biologically important molecules (Josefsson.L.G & Rask.L, 1997). Chlorophyll, for example, captures light because it contains tryptophan, and then generates ATP, reduced cofactors (NADH), and oxygen. This entire process is blocked if tryptophan is substituted with another amino acid (Mogi.T *et al.*, 1989). Furthermore, tryptophan itself is converted into the tropic factor auxin, by removing the amide group to make indole acetic acid. Auxin stimulates changes in cell shape and provides movement for plants. This morphogenic property also occurs in animal cells, in which 5-HT alters the cytoskeleton of cells and thus influences the formation of cell contacts. In addition, 5-HT regulates cell proliferation, migration and maturation in a variety of cell types, including lung, kidney, endothelial cells, mast cells, neurons and astrocytes (Azmitia.E.C, 2001).

5-HT is an endogenous amine involved in diverse biologic processes within the central and peripheral nervous system, cardiovascular, gastrointestinal and respiratory systems (Hindle.A.T, 1994). This diversity of actions is made possible because of the existence of specific 5-HT cell surface receptor subtypes and their coupling to distinct intracellular messenger systems or ion channels (Hoyer.D *et al.*, 1994). 5-HT is widely distributed in both the animal and the plant kingdoms and is found in such diverse locations as tunicates, mollusks, arthropods, fruits, nuts and venoms (Erspamer.V, 1996). In humans ~90% of the body's 5-10 mg of 5-HT is located in the intestines, and the rest is present in primarily in platelets and neural tissues (Garrison.J.C, 1990). The synthesis and degradation of 5-HT is a very active process, and it has been estimated that the total body pool of 5-HT is replaced every 24 hours. The synthesis of 5-HT occurs primarily by enzymatic hydroxylation of the benzene ring of tryptophan to form 5-hydroxytryptophan (5-HTP) and then through decarboxylation of the terminal carbon group of 5-HTP to form 5-HT. Once inside the cells, 5-HT is degraded by monoamine oxidase to form an aldehyde, which is then hydrolysed by aldehyde dehydrogenase to form 5-hydroxy indole acetic acid, the principal metabolite excreted in urine.

5-HT has been implicated more in behaviour, physiological mechanisms, and disease processes than any other brain neurotransmitter. The enormous range of this single brain chemical system may reflect the vast distribution of its fibers in brain, from a small group of large multipolar neurons. The neurons form a collection of clustered cells termed the raphe nuclei, located on the exact midline of the brainstem. Serotonergic fibers interact in complex ways with a variety of cell types—neurons, glial cells, endothelial cells, ependymal cells and others by binding to at least 14 distinct receptor proteins. Furthermore, 5-HT neurons are one of the first brainstem neurons to emerge during early development of the brain and spinal cord—present by the sixth week of gestation in humans. In rats, 5-HT neurons in the brainstem raphe are among the first neurons to differentiate in the brain and play a key role in regulating neurogenesis (Kligman.D & Marshak.D.R, 1985). The 5-HT neurons are the first neuronal system to innervate the primordial cortical plate. During development, 5-HT fibers arrive at the cortical plate during the peak period of mitosis and maturation (Dori.I *et al.*, 1996). Lauder and Krebs (Lauder.J.M & Krebs.H, 1978) reported that para-chlorophenylalanine (PCPA), a 5-HT synthesis inhibitor, retarded neuronal maturation. Since then, many other workers have shown a role for 5-HT in neuronal differentiation (Rodriguez.J, 1994 & Marois.R & Croll.R.P, 1992).

In addition to its role as a neurotransmitter, 5-HT has been implicated as a potential mitogen (Seuwen.K *et al.*, 1988 & Seuwen.K & J, 1990) and was shown to have effects on morphogenesis and neuronal development (Lauder.J.M, 1990). 5-HT mediates mitogenic effects in many cell types (Garnovskaya.M.N *et al.*, 1996 & Cowen.D.S *et al.*, 1996). The mitogenic action of 5-HT, first identified in bovine aortic smooth muscle cells by Nemeck *et al* (Nemeck.G.M *et al.*, 1986), may bear a relationship to the stimulatory effect of 5-HT on neuroembryogenesis. In cultured rat pulmonary artery smooth muscle cells (SMC), 5-HT induces DNA synthesis and potentiates the mitogenic effect of platelet-derived growth factor (Eddahibi.S *et al.*, 1999). 5-HT's effects on cell proliferation may involve the phosphorylation of GTPase-activating protein (GAP), an intermediate signal in 5-HT-induced mitogenesis of SMC (Lee.S.L *et al.*, 1997).

There is a synergistic effect of 5-HT with more traditional protein growth factors, such as platelet derived growth factor, fibroblast growth factor, and insulin like growth

factor and with ADP, ATP, thromboxaneA2 (Crowley.S.T *et al.*, 1994 & Stroebel.M & groppelt-Struebe.M, 1994).]. 5-HT in concentrations as low as 0.1–1 mM stimulates both proliferation and hypertrophy of SMC in culture (Lee.S.L *et al.*, 1994). Furthermore, the mitogenic action of 5-HT is synergistic with that of conventional peptide growth factors (Lee.S.L *et al.*, 1994 & Lee.S.L *et al.*, 1991). All agents that block transport of 5-HT block the proliferative response (Lee.S.L *et al.*, 1991).

The biological mechanism used by 5-HT to change cell morphology and induce proliferation may directly target the cytoskeleton. The main component of the cytoskeleton is microtubules which gives cells their shape. These microtubules consist of long polymers of tubulin, which spontaneously depolymerise if they are not actively polymerising (Mitchison.T & Kirschner.M, 1984). In 1975, Tan and Lagnado (Tan.L.P & Lagnado.J.R, 1975) found effects of 5-HT and related indole alkaloids on brain microtubular proteins. Several years later, it was found that 5-HT is taken up by endothelial cells and binds to stress fibers (Alexander.J.S *et al.*, 1987). Here 5-HT induces actin polymerisation and affects changes in the cytoskeleton. Thus, there is evidence that 5-HT has a direct role in regulating and maintaining microtubules and microfilaments. The changes reported in 5-HT induced cytoskeletal stability may be partially mediated by microtubule-associated proteins (MAPs). MAPs serve to stabilise the cytoskeleton by binding to tubulin polymers and inhibiting their depolymerisation. In undifferentiated human neuroblastoma cells (LAN-5), high levels of 5-HT (50 μM) induce a decrease while low levels of 5-HT (50 nM) induce an increase in the cytoplasmic tau protein, a MAP found in high concentrations in the axon.

The effects of 5-HT as a mitogen and/or growth factor have been documented as a part of neuromodulator substances acting via G protein coupled receptor signaling pathway (Seuwen.K *et al.*, 1988, Seuwen.K & J, 1990 & Julius.D, 1991). Specifically, 5-HT has been shown to increase DNA synthesis in rat pulmonary vascular smooth-muscle cells in culture (Pitt.B.R *et al.*, 1994). Introduction of functional 5-HT_{2A} receptor and 5-HT_{2C} receptor into NIH-3T3 cells results in generation of transformed foci at high frequency (Julius.D *et al.*, 1989). The long-term maintenance of the transformed state requires continued activation of these 5-HT receptors, indicating that they may represent conditional proto-oncogenes (Julius.D, 1991).

5-HT is mitogenic for bovine pulmonary artery SMC producing both hyperplasia and hypertrophy through its action on a 5-HT membrane transporter, with a rapid elevation in tyrosine phosphorylation (Tyr-P) of GTPase-activating protein (GAP) (Lee.S.L *et al.*, 1997) and early inductions of *c-myc* (Lee.S.L *et al.*, 1994). 5-HT, acting via the 5-HT_{2A} receptor, is a known activator of the ERK pathway in vascular smooth muscle cells (Watts.S.W, 1996 & Banes.A *et al.*, 1999).

Changes in cAMP and cGMP levels after X-irradiation and radioprotective treatment with 5-HT were studied using a model of impulse inhibition. Irradiation was shown to inhibit the cyclic nucleotide synthesis. 5-HT increased the contents of cAMP and cGMP maintaining their elevated level after X-irradiation (Aslamova.L.I *et al.*, 1985).

Mobilisation of 5-HT in intestine and its accumulation in liver and spleen tissues were observed at the initial periods after partial hepatectomy (Kulinskii.V.I *et al.*, 1983). 5-HT caused a dose-dependent increase in DNA synthesis in primary cultures of rat hepatocytes in the EGF and insulin, as measured by [^{3}H]thymidine incorporation (Sudha.B & Paulose.C.S, 1997).

5-HT and mono amine oxidase inhibitor o-chlorpargyline injected alone or combined increase the endogenous 5-HT level in the regenerating liver and stimulates mitotic activity. The tryptophan hydroxylase inhibitor p-chlorophenylalanine and reserpine decrease both the endogenous 5-HT level and the mitotic index. There is a close correlation between the endogenous 5-HT level and the mitotic index (Kulinskii.A.S *et al.*, 1983).

5-HT RECEPTOR CLASSIFICATION

Protein receptors that mediate the actions of 5-HT have existed in the membranes of a variety of animal cell types for millions of years, their ancestry being as old or older than that for the adrenoceptors and receptors for some peptide mediators (Venter.J.C *et al.*, 1988 & Hen.R, 1992). 5-HT receptors can be classified into seven classes from 5-HT_1 to 5-HT_7, based upon their pharmacological profiles, cDNA-deduced primary sequences and signal transduction mechanisms of receptors (Bradley.P.B *et al.*, 1986a, Zifa.E & Fillion.G, 1992 & Peroutka.S.J, 1993). All 5-HT receptors belong to the

superfamily of G-protein coupled receptors containing a seven transmembrane domain structure except 5-HT_3 receptor, which forms a ligand-gated ion channel.

5-HT_1 Receptor

At least five 5-HT_1 receptor subtypes have been recognised, 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E} and 5-HT_{1F}. All are seven transmembrane, G-protein coupled receptors (via Gi or Go), encoded by intronless genes, between 365 and 422 amino acids with an overall sequence homology of 40%. 5-HT_{1A} receptor subtype which is located on human chromosome 5cenq11 is widely distributed in the CNS, particularly hippocampus (Hoyer.D *et al.*, 1994). The 5-HT_{1B} receptor is located on human chromosome 6q13 and is concentrated in the basal ganglia, striatum and frontal cortex. The receptor is negatively coupled to adenylyl cyclase. The 5-HT_{1D} receptor has 63% overall structural homology to 5-HT_{1B} receptor and 77% amino acid sequence homology in the seven transmebrane domains. The receptor is located on human gene 1p36.3-p34.3 and is negatively linked to adenylyl cyclase. $5HT_{1D}$ receptor mRNA is found in the rat brain, predominantly in the caudate putamen, nucleus accumbens, hippocampus, cortex, dorsal raphe and locus ceoruleus (Hoyer.D *et al* ., 1994). The 5-HT_{1E} receptor was first characterised in man as a [^{3}H]5-HT binding site in the presence of 5-carboxyamidotryptamine (5-CT) to block binding to the 5-HT_{1A} and 5-HT_{1D} receptors. Human brain binding studies have reported that 5-HT_{1E} receptors are concentrated in the caudate putamen with lower levels in the amygdala, frontal cortex and globus pallidus (Hoyer.D *et al.*, 1994). This is consistent with the observed distribution of 5-HT_{1C} mRNA (Hoyer.D *et al.*, 1994). The receptor has been mapped to human chromosome 6q14-q15, is negatively linked to adenylyl cyclase and consists of a 365 amino acid protein with seven transmembrane domains. 5-HT_{1F} receptor subtype is most closely related to the 5-HT_{1E} receptor with 70% sequence homology across the 7 transmembrane domains. mRNA coding for the receptor is concentrated in the dorsal raphe, hippocampus and cortex of the rat and also in the striatum, thalamus and hypothalamus of the mouse (Hoyer.D *et al.*, 1994). The receptor is negatively linked to adenylyl cyclase.

5-HT_2 Receptor

The 5-HT_2 receptor family consists of three subtypes namely 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C}. 5-HT_{2C} was previously termed as 5-HT_{1C} before its structural similarity to the 5-HT_2 family members was recognised. All three are single protein molecules of 458-471 amino acids with an overall homology of approximately 50% rising to between 70-80% in the seven transmembrane domains. All three are thought to be linked to the phosphoinositol hydrolysis signal transduction system via the α subunit of Gq protein. In human pulmonary artery endothelial cells, 5-HT_{2C} receptor stimulation causes intracellular calcium release via a mechanism independent of phosphatidylinositol hydrolysis (Hagan.J.J *et al.*, 1995). 5-HT_{2A} receptor previously termed as $5HT_2$ receptor is located on human chromosome 13q14-q21 and is widely distributed in peripheral tissues. It mediates contractile responses of vascular, urinary, gastrointestinal and uterine smooth muscle preparations, platelet aggregation and increased capillary permeability in both rodent and human tissue (Hoyer.D *et al.*, 1994). The 5-HT_{2B} receptor located on chromosome 2q36-2q37.1 mediates contraction of the rat stomach fundus and endothelium dependent relaxation of the rat and cat jugular veins and possibly of the pig pulmonary artery, via nitric oxide release (Choi.D.S & Maroteaux.L., 1996). 5-HT_{2B} receptor mRNA has been detected throughout the mouse, rat and guinea pig colon and small intestine. 5-HT_{2C} specific antibodies have recently used to show the presence of the receptor protein in the choroid plexus, (highest density) and at a lower level in the cerebral cortex, hippocampus, striatum, and substantia nigra of rat and a similar distribution in man. The receptor has been mapped to human chromosome Xq24. No splice variants have been reported but the receptor is capable of post translational modification whereby adenosine residues can be represented as guanosine in the second loop to yield 4 variants.

5-HT_3 Receptor

The 5-HT_3 receptor binding site is widely distributed both centrally and peripherally and has been detected in a number of neuronally derived cells. The highest densities are found in the area postrema, nucleus tractus solitarius, substantia gelatinosa

and nuclei of the lower brainstem. It is also found in higher brain areas such as the cortex, hippocampus, amygdala and medial habenula, but at lower densities. Unlike other 5-HT receptors, 5-HT_3 receptor subunits form a pentameric cation channel that is selectively permeable to Na^+, K^+ and Ca^{++} ions causing depolarisation. The 5-HT_3 receptor is a member of a superfamily of ligand-gated ion channels, which includes the muscle and neuronal nicotinic acetylcholine receptor (AchR), the glycine receptor, and the γ aminobutyric acid type A receptor (Karlin.A & Akabas.M.H, 1995, Ortells.M.O & Lunt.G.G, 1995 & Unwin.N, 1993). Like the other members of the gene superfamily, the $5HT_3$ receptor exhibits a large degree of sequence similarity and thus presumably structural homology with the AchR (Maricq.A.V *et al.*, 1991).

5-HT_4 Receptor

Receptor binding studies have established that the 5-HT_4 receptor is highly concentrated in areas of the rat brain associated with dopamine function such as the striatum, basal ganglia and nucleus accumbens. These receptors are also located on GABAergic or cholinergic interneurons and/or on GABAergic projections to the substantia nigra (Patel.S *et al.*, 1995). The receptor is functionally coupled to the G protein.

5-HT_5 Receptor

Two 5-HT receptors identified from rat cDNA and cloned were found to have 88% overall sequence homology, yet were not closely related to any other 5-HT receptor family (Erlander.M.G *et al.*, 1993). These receptors have thus been classified as 5-HT_{5A} and 5-HT_{5B} and their mRNAs have been located in man (Grailhe.R *et al.*, 1994). In cells expressing the cloned rat 5-HT_{5A} site, the receptor was negatively linked to adenylyl cyclase and may act as terminal autoreceptors in the mouse frontal cortex (Wisden.W *et al.*, 1993).

5-HT_6 Receptor

Like the 5-HT_5 receptor, the 5-HT_6 receptor has been cloned from rat cDNA based on its homology to previously cloned G protein coupled receptors. The rat receptor consists of 438 amino acids with seven transmembrane domains and is positively coupled

to adenylyl cyclase via the Gs G protein. The human gene has been cloned and has 89% sequence homology with its rat equivalent and is coupled to adenylyl cyclase (Kohen.R *et al.*, 1996). Rat and human 5-HT_6 mRNA is located in the striatum, amygdala, nucleus accumbens, hippocampus, cortex and olfactory tubercle, but has not been found in peripheral organs studied (Kohen.R *et al.*, 1996).

5-HT_7 Receptor

5-HT_7 receptor has been cloned from rat, mouse, guinea pig and human cDNA and is located on human chromosome 10q23.3-q24.4. Despite a high degree of interspecies homology (95%) the receptor has low homology (<40%) with other 5-HT receptor subtypes. The human receptor has a sequence of 445 amino acids and appears to form a receptor with seven transmembrane domains.

RECEPTOR MEDIATED CELL PROLIFERATION

Receptors coupled to heterotrimeric guanine nucleotide-binding (G) proteins, the largest known family of cell surface receptors, mediate cellular responses to many extracellular stimuli, such as neurotransmitters, peptide hormones, odorants and photons (Hepler.J.R & Gilman.A.G., 1992). In addition to regulating the generation of soluble second messengers, many G-protein coupled receptors mediate proliferative or differentiative signals in various cultured cell lines and tissues via mitogen-activated protein (MAP) kinases (Biesen.T *et al.*, 1996 & Dhanasekaran.N *et al.*, 1995).

5-HT_{1A} Receptor and Cell Proliferation

5-HT_{1A} is a "transiently expressed" intronless receptor, i.e., at specific times in development or during stress, very high amounts are expressed quickly. 5-HT_{1A} receptor develops early and the receptor levels can then be reduced as the cells or animal ages. The decrease in receptor number is probably due to increased 5-HT brain levels, since the 5-HT_{1A} receptor expression is sensitive to autoinhibition (Nishi.M & Azmitia.E.C, 1999 & Whitaker-Azmitia.P.M *et al.*, 1987). The transduction action of the 5-HT_{1A} receptor is usually associated with a decrease in adenylyl cyclase activity. In cultures of hippocampal neurons, 5-HT_{1A} agonists block the forskolin-induced formation of p-CREB, an important transcription factor increased by cAMP (Nishi.M &

Azmitia.E.C, 1999). In adult neurons, the 5-HT_{1A} receptor also is associated with a hyperpolarisation of the membrane potential, attributed to opening a K^- current (Baskys.A *et al.*, 1989). The 5-HT_{1A} receptor uses these cellular mechanisms to differentiate its target cells. The 5-HT receptor 1A is found on serotonergic neurons and nonserotonergic neurons (Hamon.M, 1997). In the presence of a phosphodiesterase inhibitor 5-HT elevates bovine smooth muscle cellular cAMP and this elevation correlates with an inhibition of cellular proliferation (Assender.J.W *et al.*, 1992 & Lee.S.L *et al.*, 1991). Similarly other agents such as forskolin, histamine, isoproterenol and choleratoxin, that elevate cellular cAMP inhibit the proliferation. This activity of 5-HT is mimicked by 8-OH DPAT a reputed, 5-HT_{1A} agonist (Fanburg.B.L & Lee.S.L, 1997). 5-HT inhibits cellular growth of pulmonary artery smooth muscle cells (SMC) through its action on 5-HT_{1A} or 5-HT_4 receptors (Lee.S.L *et al.*, 1997).

The involvement of the 5-HT_{1A} receptor in cell proliferation is assumed to be inhibitory given its stimulatory effects on cell differentiation (Lauder.J.M *et al.*, 1983). However, some studies indicate a direct and indirect role for 5-HT_{1A} receptors in cell proliferation. 5-HT_{1A} agonists given in culture accelerate cell division, generate cell foci, and increase DNA synthesis in transfected NIH-3T3 cells (Varrault.A *et al.*, 1992). The early studies of 5-HT and cell proliferation in culture appear to argue that 5-HT may be important for cell differentiation and the inhibition of cell division in the CNS. The 5-HT_{1A} receptor is uniquely positioned during the early development of the brain to influence neuronal mitosis, in the maturation and the assembly of the spindle apparatus in the cell body which promotes cell division. In the 5-HT cell line, RN46A, the 5-HT_{1A} receptor is 20-fold higher in the undifferentiated cell than in the differentiated cell. It is suggested that the cell body 5-HT_{1A} receptors may mediate autoregulation of serotonergic neuron development (Eaton.M *et al.*, 1995).

The 5-HT_{1A} receptors in the adult brain have clearly been shown to be involved in maintaining the mature state of neurons in the mammalian brain (Azmitia.E.C, 1999 & Azmitia.E.C & Whitaker-Azmitia.P.M, 1997). Liu and Albert (Liu.Y.F *et al.*, 1991) have demonstrated with transfection of the rat 5HT_{1A} receptor into a variety of cells that the

receptor, acting through pertussis toxin (PTX) sensitive G proteins, can change its inhibitory signaled phenotype into a stimulatory one, depending on cell type, differentiation and culture medium.

5-HT_{1A} receptor is prominently expressed in neuronal cells (e.g. hippocampal CA-1, dorsal raphe nuclei) where it also opens potassium channels via activation of a PTX-sensitive G protein (Colino.A & Halliwell.J.V, 1987, Hoyer.D *et al.*, 1986, Ropert.N, 1988, Sprouse.J.S & Aghajanian.G.K, 1988 & Zgombick.J.M *et al.*, 1989) and closes calcium channels (Penington.N.J & Kelly.J.S, 1990 & Ropert.N, 1988). This results in hyperpolarisation of the membrane potential, closing of voltage-dependent calcium channels, and decrease in Ca^{2-}. The expression of the rat 5-HT_{1A} receptor in pituitary GH4C1 cells (GH4ZD10 cells) resulted in a 5-HT-induced inhibition of Ca^{2+}; and cAMP accumulation similar to that observed in neurons (Liu.Y.F *et al.*, 1991). Thus opposite effects occur with the use of 5-HT depending on whether or not cellular cAMP is elevated and this in turn, depends on the activity of cellular phosphodiesterase (Fanburg.B.L & Lee.S.L, 1997).

The cells exposed to apoptotic-inducing conditions may actually up-regulate 5-HT_{1A} receptors. Neuronal cell lines stably transfected with a promoter-less segment (G-21) of the human 5-HT_{1A} receptor gene (Singh.J.K *et al.*, 1996) show a 5 to15-fold increase in the receptor when deprived of nutrient. 5-HT_2 receptor drugs are not effective in these models of apoptosis. Conversely, reduced 5-HT levels in the hippocampus potentiate ischemic-induced neuronal damage (Nakata.N *et al.*, 1997).

5-HT_{2C} Receptor and Cell Proliferation

5-HT_{2C} receptors [formerly termed 5-HT_{1C}] are widely expressed in the brain and spinal cord, are particularly enriched in the choroid plexus, and appear to mediate many important effects of 5-HT (Blier.P *et al.*, 1990). Previous studies have shown that 5-HT_{2C} receptor undergoes RNA editing with the potential for producing 14 different receptor isoforms (Burns.C.M *et al.*, 1997, Fitzgerald.L.W *et al.*, 1999, Niswender.C.M *et al.*, 1998 & Niswender.C.M *et al.*, 1999). The rat 5-HT_{2C} receptor is one of the three 5-HT_2 subtype receptors linked to phospholipase C via G-protein coupling and is regulated by RNA editing (Burns.C.M *et al.*, 1997). Parrot *et al* (Parrot.D.P *et al.*, 1991)

and Pakala *et al* (Pakala.R *et al.*, 1994) proposed a 5-HT_2 receptor to be responsible for 5-HT induced proliferation of porcine smooth aortic muscle cells and canine and bovine aortic endothelial cells. Similarly, Pitt *et al* (Pitt.B.R *et al.*, 1994) and Corson *et al* (Corson.M.A *et al.*, 1992) suggested that a 5-HT_2 receptor is responsible for proliferation of rat vascular smooth muscle cells caused by 5-HT through an increase in intracellular Ca^{2+}. Crowley *et al* (Crowley.S.T *et al.*, 1994) also concluded that stimulation of proliferation of bovine aortic smooth muscle cells by 5-HT occurs through a 5-HT_2 receptor.

5-HT_2 receptor can be referred to as a programmable receptor i.e., events during development may affect the number, affinity, or function of these receptors in the adult brain (Meaney.M.J *et al.*, 1994). For example, both prenatal and postnatal stress to the mother significantly increases the number of 5-HT_2 receptors in the offspring, which leads to activation of protein kinase C (PKC) and the activation of several important transcription factors including c-Fos, Jak, and STAT. 5-HT stimulates the turnover of phosphoinositide in primary cultures of astroglia from the cerebral cortex, striatum, hippocampus, and brain stem. 5-HT_2 receptors in glioma cells appear to regulate proliferation, migration, and invasion. 5-HT was found to positively modulate these three processes *in vitro* (Merzak.A *et al.*, 1996).

5-HT_2 receptor antagonists, ketanserin (10^{-6}M) and spiperone (10^{-6}M), blocked stimulation of DNA synthesis by 5-HT. Displacement studies on [^{3}H]5-HT binding to crude membranes from control and regenerating liver tissue, using cold ketanserin and spiperone, showed an increased involvement of 5-HT_2 receptors of 5-HT in the regenerating liver during the DNA-synthetic phase. 5-HT enhanced the phosphorylation of a 40-kd substrate protein of protein kinase C (PKC) in the regenerating liver during the DNA synthetic phase of the hepatocyte cell cycle. This was blocked by ketanserin, indicating that 5-HT_2 receptor activates PKC, an important second messenger in cell growth and division, during rat liver regeneration. 5-HT can act as a potent hepatocyte co-mitogen and induce DNA synthesis in primary cultures of rat hepatocytes, which is suggested to be mediated through the 5-HT_2 receptors of hepatocytes (Sudha.B & Paulose.C.S, 1997).

The predominant 5-HT_2 receptor in the neonatal period is the 5-HT_{2C} receptor (Ike.J *et al.*, 1995). The 5-HT_{2C} receptors activate phospholipase C (Courcelles.D *et al.*, 1985 & Conn.P.J *et al.*, 1986), whereas 5-HT_{1A} receptors modulate adenylyl cyclase activity (Siegelbaum.S.A *et al.*, 1982, Hoyer.D & Schoeffter.P, 1988 & Vivo.M & Maayani.S, 1986). In neurons that express the 5-HT_{1C} receptor activation by 5-HT is likely to generate inositol polyphosphates that release intracellular Ca^{2+} (Courcelles.D *et al.*, 1985 & Conn.P.J *et al.*, 1986). NIH-3T3 cells that express high levels of 5-HT_{2C} receptor form foci in cell culture. Moreover the formation of foci is dependent on activation of the 5-HT_{2C} receptor by 5-HT. In addition the introduction of transformed foci into nude mice results in the rapid appearance of tumours. In fibroblasts this receptor alters the growth properties of cells and results in malignant transformation (Julius.D *et al.*, 1989).

5-HT AND SYMPATHETIC STIMULATION

A large amount of evidence has accumulated to indicate that central serotonergic neurons participate in the regulation of sympathetic nerve discharge. 5-HT neurons have an inhibitory influence on central sympathetic pathways (Kuhn.D.M *et al.*, 1980) while recent work indicates that 5-HT facilitates central sympathetic nerve activity. 5-HT_{1A} agonists act centrally to inhibit sympathetic nerve discharge (McCall.R.B *et al.*, 1987). Similar observations have been made by Fozard and coworkers (Fozard.R *et al.*, 1987 & Ramage.A.G & Fozard.J.R, 1987). A selective 5-HT_2 receptor agonist, 1-(2,5-di-methoxy-4-iodophenyl)-2-aminopropane (DOI) (Glennon.R.A, 1987) produced a tremendous increase in sympathetic nerve discharge (McCall.R.B & Harris.L.T, 1987). 5-HT_2 antagonists ketanserin and LY- 53587 reversed the massive increase in sympathetic discharge produced by DOI. Thus one subset of central nervous system 5-HT receptors (5-HT_{1A}) can inhibit sympathetic nerve discharge while a second subset of receptors (5-HT_2) can increase sympathetic nervous discharge (McCall.B.R & Harris.L.T, 1988).

SIGNAL TRANSDUCTION DURING HEPATOCYTE PROLIFERATION

Hepatocytes of normal adult liver divide infrequently. Immediately after partial hepatectomy, hepatocytes enter into a state of pre-replicative competence before they fully respond to growth factors. This priming step is an initiating event characterised by a transition from the G0 to the G1 phase and mediated by cytokines, including tumour necrosis factor-α and interleukin-6, and in part by changes in the extracellular matrix (Yamada.Y *et al.*, 1997 & Cressman.D.E *et al.*, 1996). However, progression of these initiated cells through the late G1 phase to the S phase is thought to require growth factors and involve activation of cyclin-cyclin dependent kinase (cdk) complexes. Therefore, a growth factor dependent restriction point is precisely localised in the mid late G1 phase in primary cultured rat hepatocytes (Loyer.P *et al.*, 1996), and it is mediated by TGFα, HB-EGF and mainly by HGF.

HGF is known to be one of the major agents promoting the proliferation of hepatocytes. It was originally purified from the plasma of patients with fulminant hepatic failure (Gohda.E *et al.*, 1986 & Gohda.E *et al.*, 1988) and rat platelets (Nakamura.T *et al.*, 1987). This protein is characterised as a pleiotropic factor acting as a mitogen, motogen and morphogen for a variety of cultured cells through binding to its receptor (c-Met) on the cell membrane (Weidner.K.M *et al.*, 1993). c-Met has a tyrosine kinase domain in its intracellular region. TGFα (Mead.J.E & Fausto.N, 1989) and HB-EGF (Ito.N *et al.*, 1994) also stimulate DNA synthesis in primary cultured rat hepatocytes through binding to EGF receptor (EGFR), which has a tyrosine kinase domain (Gentry.L.E & Lawton.A, 1986). These growth factors are induced in the liver during liver regeneration, and both c-Met and EGFR transmit signals into the nucleus, mainly through the Ras/mitogen activated protein kinase (MAPK) pathway (Wada.S *et al.*, 1998). Following Ras/MAPK activation, cyclin D1 protein is expressed in the nucleus and drives the cell cycle from the G1 to the S phase and initiates DNA synthesis (Talarmin.H *et al.*, 1999). These growth factors additively stimulate DNA synthesis in hepatocytes (Wada.S *et al.*, 1998, Hernandez.J *et al.*, 1992 & Webber.E.M *et al.*, 1993), and it is also thought that these factors act in concert during liver regeneration *in vivo*. In response to growth factors, Ras/MAPK, phosphatidyl inositol-3 kinase (PI3K) (Royal.I *et al.*, 1997) and Janus kinase (Jak)/signal transducer and activator of transcription (STAT)

(Runge.D.M *et al.*, 1999) are activated in hepatocytes. In those pathways, the Ras/MAPK system plays a central role and is mainly associated with cyclin D1 expression, which regulates the progression of the G1 phase in the cell cycle (Talarmin.H *et al.*, 1999). It is well established that, *in vivo*, normal hepatocytes are largely unresponsive to growth factors and become competent only after priming induced by specific treatments such as partial hepatectomy, necrosis following injury, metabolic stress, or any phenomenon leading to disruption of cell-cell contacts (Etienne.P.L *et al.*, 1988, Sawada.N, 1989 & Ikeda.T *et al.*, 1989) or digestion of the extracellular matrix (Liu.M.L *et al.*, 1994). These metabolic events would trigger the G0/G1 transition of hepatocytes *in vivo* and increase the expression of growth factors, which then induce DNA synthesis. This hypothesis is based on the fact that induction of immediate-early oncogenes such as c-*fos* or *c-jun* (Corral.M *et al.*, 1985, Thompson.N.L *et al.*, 1986, Sobczak.J *et al.*, 1989 & Morello.D *et al.*, 1990) takes place 20-30 minutes after PH, while HGF level rises around 2 hours post PH (Lindroos.P.M *et al.*, 1991).

The earliest signals after PH include increased JNK and ERK activity (Diehl.A.M *et al.*, 1994 & Westwick.J.K *et al.*, 1995). Inhibition of NF-κB induces apoptosis through a variety of cancer therapeutic agents and TNFα (Wang.C.Y *et al.*, 1996 & Antwerp.D.J *et al.*, 1996). An anti apoptotic role of NF-κB was suggested by the embryonic death of NFκB p65 knockout mice from extensive apoptosis in the liver (Beg.A.A *et al.*, 1995). After PH, there is rapid induction of DNA binding by NF-κB (Fitzgerald.M.J *et al.*, 1995).

Following a two-thirds partial hepatectomy, hepatocytes rapidly enter the cell cycle and begin their first round of DNA replication 18-20 hour later (Fabrikant.J.I, 1968). It has been known for some years that HGF and transforming growth factor α (TGFα) are primary mitogens during liver regeneration after partial hepatectomy or administration of CCL_4. (Mead.J.E & Fausto.N, 1989 & Michalopoulos.G.K, 1990). Mullhaupt *et al* (Mullhaupt.B *et al.*, 1994) reported a rapid increase of EGF levels in the immediate early phase of liver regeneration. Many attempts have been made to identify the proteins which control the progression of the cell cycle through G1 check points. Of the proteins characterised to date, the cyclin-dependent kinases (Cdks) and their cyclin

partners play a crucial role in cell cycle regulation (Sherr.C.J, 1993). Cyclins bound to Cdc2 or Cdk2 appear to be involved in regulating DNA initiation and/or synthesis (Pagano.M *et al.*, 1992 & Zindy.F *et al.*, 1992) and G2/M transition (Pagano.M *et al.*, 1992 & , Sherr.C.J, 1993). The cyclin E-Cdk2 complex is activated at the end of G1 and is considered to be a limiting step at the G1/S boundary (Koff.A *et al.*, 1991 & Dulic.V *et al.*, 1992 & Sherr.C.J, 1993). The D type cyclins also play a crucial role in G1 via their association with Cdk2, Cdk4 and Cdk6 (Xiong.Y *et al.*, 1992, Baldin.V *et al.*, 1993, Quelle.D.E *et al.*, 1993 & Meyerson.M & Harlow.E, 1994). Analysis of the expression and activation of Cdc2 and Cdk2 in regenerating liver revealed that Cdc2 was expressed and active in S, G2 and M phases but not in G1, whereas Cdk2 was constantly expressed during the cell cycle but inactive in G1 (Loyer.P *et al.*, 1994). The sequential activation of these complexes and their substrate specificities could be the key to their regulatory function throughout the G1 phase (Ajchenbaum.F *et al.*, 1993 & Sherr.C.J, 1993).

The molecular mechanisms that regulate these events include early signaling events such as increased production of HGF (Boros.P & Miller.C.M, 1995), TNFα, and IL-6 (Yamada.Y *et al.*, 1998), followed by induction of a number of immediate early genes (Taub.R, 1996). Subsequent changes occur in the activity of several transcription factors, including increased activity of NFkB, AP-1, signal transducer and activator of transcription-3 (STAT3), CREB, and CCAAT enhancer binding protein β (C/EBPβ) and decreased activity of C/EBPα (Cressman.D.E *et al.*, 1995, Cressman.D.E *et al.*, 1994, Heim.M.H *et al.*, 1997, Diehl.A.M, 1998, Greenbaum.L.E *et al.*, 1998 & Fazia.M.A *et al.*, 1997). The transcription factor STAT3 has been shown to be specifically activated after PH by gel shift analysis of hepatic nuclear extracts (Cressman.D.E *et al.*, 1995). STAT3 is not activated during the impaired regenerative response seen in the IL-6 null mouse and the TNFα receptor 1-null mouse (Yamada.Y *et al.*, 1997 & Cressman.D.E *et al.*, 1996). Two transcription factor complexes, nuclear factor kappa B (NF-κB) and STAT3, are rapidly activated by means of post translational modifications in the remnant liver within minutes to hours after partial hepatectomy and may provide clues to the initiating signals (Tewari.M *et al.*, 1992 & Cressman.D.E *et al.*, 1994).

TNF signaling through TNFR-I can initiate liver regeneration after PH and that IL-6 is a key target of TNF gene activation in the regenerating liver. Knockout mice that lack TNFR-I showed a severe defect in hepatocyte replication during the first 4 days after PH. Surviving animals (50% of the total) eventually regenerated their livers, but restoration of the normal liver weight-to-body weight ratio was still not completed 2 weeks after PH, a time at which the regenerative process was already terminated in wild-type mice. IL-6 reversed the deficiency in hepatocyte replication imposed by the lack of TNFR-I, corrected the defects in STAT3 and AP-1 binding, but did not reverse the almost complete inhibition of NF-kB binding after PH.

The mitogen-activated protein kinase (MAPK) family consists of three commonly recognised subgroups: the extracellular signal-regulated kinase (ERK), the c-jun-N-terminal kinase (JNK), also known as the stress activated protein kinase (SAPK) and the p38 kinase. Of the three MAPK pathways, activation of the ERK pathway and the intracellular signaling pathways associated with ERK activation are the best delineated. Known activators of ERK include reactive oxygen species (Lee.S *et al.*, 1999 & (Greene.E.L *et al.*, 2000), growth factors (Lorimer.I.A & Lavictorie.S.J, 2001), and agonists of G-protein coupled receptors (McDuffie.J.E *et al.*, 2000 & Watts.S.W *et al.*, 1998). The two other MAPK pathways, the JNK and p38 pathways, have been implicated in a variety of similar cellular functions. Known activators of the JNK and p38 pathways in vascular smooth muscle cells include reactive oxygen species (Kyaw.M *et al.*, 2001 & Yoshizumi.M *et al.*, 2000), mechanical strain (Li.C *et al.*, 2000 & Li.C *et al.*, 1999), hypoxia (Lin.Z *et al.*, 2000) and a variety of cytokines and growth factors. The mechanisms of many cellular functions of the JNK and p38 pathways are not yet clearly defined. The JNK pathway is involved in apoptosis (Soh.Y *et al.*, 2000). The p38 pathway has been implicated in apoptosis (Diep.Q.N *et al.*, 2000) and neointimal hyperplasia after vascular injury (Ohashi.N *et al.*, 2000). Activating transcription factor 3 (ATF3) is an early response gene that is induced rapidly during *in vivo* situations of cellular growth such as liver regeneration (Allan.A.L *et al.*, 2001).

Activin, which is expressed in normal rat liver, is another growth inhibitory factor for hepatocytes (Yasuda.H *et al.*, 1993) although details of the mechanism of action is not yet fully understood. After PH, activin expression transiently decreases but recovers

within 24 hours. A remarkable activation of p44/42 MAPK was seen but no such activation of p38 MAPK was observed at 48 hours after PH (Inui.T *et al.*, 2002). TGFβ1 is a growth inhibitory factor for hepatocytes; it completely inhibits the effect of HGF, TGFα and EGF on DNA synthesis in hepatocytes (Braun.L *et al.*, 1988). As is well known, TGFβ1 terminates liver regeneration in the late phase. Apoptosis is involved in the homeostatic control of organs. Fas showed an early decline by 24 hours, followed by a later peak from 3-5 days and then a constant expression thereafter. Meanwhile, the Fas ligand was also low until day 3, but showed a remarkable increase from days 5 to 7, followed by a gradual decrease. On the other hand, Bcl-2, an anti-apoptotic factor showed an early peak until 24 hour, followed by a decline from day 5. The coordinated interplay between these apoptosis-related proteins like Fas and hepatocyte apoptosis suggests the possible involvement of these proteins in the course of liver regeneration and termination of liver cell proliferation (Taira.K *et al.*, 2001).

INHIBITION OF APOPTOSIS AND TUMOURIGENESIS

Bcl-2 has anti apoptotic potential (Huang.D.C *et al.*, 1997). Bcl-2 levels are elevated in a broad range of human cancers including carcinomas of breast, prostrate, ovary, colon and lung, indicating that this molecule might have a role in raising the apoptotic threshold in a broad spectrum of cancerous disorders (Bruckheimer.E.M *et al.*, 2000). Since the discovery of *bcl*-2 as an oncogene that promotes cell survival it has been widely acknowledged that anti-apoptotic genetic lesions are necessary for tumours to arise. The net expansion of a clone of transformed cells is not only achieved by an increased proliferative index but also by a decreased apoptotic rate (Strasser.A *et al.*, 1997, Jaattela.M, 1999 & Hanahan.D & Weinberg.R.A, 2000). Enhanced cell survival is needed at several steps during tumourigenesis: deregulated oncogene expression not only leads to accelerated cell proliferation, but concomitantly induces apoptosis which needs to be suppressed for the transformed cell to survive and multiply. Having reached a certain tumour size, sufficient nutrition for every tumour cell becomes restricted. This selects for further apoptosis resistance and for angiogenesis. Finally the metastasising tumour cells, deprived of cell-cell contact and of their normal (growth factor)

environment, are prone to anoikis - further mutations can suppress this cell death which occurs when untransformed adherent cells detach from the extracellular matrix.

TNF receptor family members functional inactivation occur during tumourigenesis (Villunger.A & Strasser.A, 1998). Apoptosis is executed by a recently discovered family of cysteine proteases named caspases. Caspase-8 is frequently inactivated in neuroblastoma, a childhood tumour of the peripheral nervous system (Teitz.T *et al.*, 2000 & Juin.P & Evan.G, 2000). Decreased caspase-1 protein levels have been reported as a potential step in the loss of apoptotic control during prostrate tumourigenesis (Winter.R.N *et al.*, 2001). Inhibitors of Apoptosis Proteins (IAPs) plays an evolutionarily conserved role in regulating apoptosis in diverse species ranging from humans to insects (Lois.K.M, 1999). The central mechanism of IAP apoptotic suppression appear to be through direct caspase and procaspase inhibition and modulation of and by the transcription factor NF-κB. IAPs may contribute to cancer by facilitating the insurgence of mutations and by promoting resistance to therapy (LaCasse.E.C *et al.*, 1998). The IAP family member survivin, while undetectable in terminally differentiated adult tissues, becomes prominently expressed in transformed cell line and in almost common human cancers of lung, colon, pancreas, prostrate and breast (Ambrosini.G *et al.*, 1997). Germ-line mutation of one p53 allele in human confers a predisposition to develop various malignancies, the Li-Fraumeni syndrome (Strasser.A *et al.*, 1997). An impressive demonstration of p53's role as a tumour suppressor came from the p53 knockout mice which develop at broad spectrum of tumours with high incidence (Donehower.R.C, 1996). The oncoprotein MDM2 inhibits p53 and its anti-apoptotic and growth arresting activities (Ganguli.G *et al.*, 2000). Since Rb loss of function-induced apoptosis is p53 dependent, both genes, p53 and Rb, are frequently inactivated during cellular transformation. Studies on knockout mice have firmly established that germ-line mutations in p53 and Rb can cooperate in tumourigenesis (Morgenbesser.S.D *et al.*, 1994).

It has become clear that, together with deregulated growth, inhibition of programmed cell death plays a pivotal role in tumourigenesis. Impaired apoptosis is a prerequisite for tumourigenesis, as indicated by the fact that more and more neoplastic mutations appear to act by interfering with apoptosis (Zornig.M *et al.*, 2001).

Apart from its role as a neurotransmitter, 5-HT is known to function as a potential mitogen (Seuwen.K *et al.*, 1988 & Seuwen.K & J, 1990). 5-HT_{1A} receptor is suggested to be inhibitory for cell proliferation (Lauder.J.M *et al.*, 1983) while 5-HT_{2C} receptor can function as a protooncogene in NIH-3T3 cells (Julius.D *et al.*, 1989). In the present study, we analysed the involvement of 5-HT_{1A} and 5-HT_{2C} receptors in the regulation of hepatocyte proliferation and apoptosis *in vivo* using rat models. The central nervous system mediated hepatocyte proliferation and apoptosis was studied during controlled and uncontrolled cell proliferation and hepatic apoptosis. *In vitro* studies were also conducted to confirm the role of 5-HT_{1A} and 5-HT_{2C} receptors in the regulation of hepatocyte proliferation using specific ligands in primary cultures.

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

i) Biochemicals: (Sigma Chemical Co., USA.)

5-Hydroxytryptamine (5-HT), 8-Hydroxy dipropylaminotetraline (8-OH DPAT), Mesulergine, (±)Norepinephrine, Sodium octyl sulphonate, Ethylenediamine tetra acetic acid (EDTA), HEPES (N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulphonic acid. 2-Methane 2-propyl thiol]), Tris buffer, foetal calf serum (heat inactivated), collagenase type IV, Earl's Balanced Salt Solution (EBSS), Collagen from rat-tail, William's medium E, Epidermal Growth Factor (EGF), Transforming Growth Factorβ1 (TGFβ1), Insulin.

ii) Radiochemicals:

[N^6-methyl-^{3}H]Mesulergine (Sp. activity - 79.0 Ci/mmol) and [^{3}H] Thymidine (Sp.activity 25Ci/mmol), were purchased from Amersham Life Science, UK.

8-Hydroxy-DPAT [propyl-2,3-ring-1,2,3-^{3}H] (Sp. activity – 127.0 Ci/mmol), was purchased from NEN Life Sciences products, Inc., Boston, USA.

iii) Molecular biology chemicals

Random hexamers, Taq DNA polymerase, human placental RNAse inhibitor and DNA molecular weight markers were purchased from Bangalore Genei Pvt. Ltd., India. MuMLV, dNTPs were obtained from Amersham Life Science, UK. Tri-reagent kit was purchased from Sigma Chemical Co., USA. RT-PCR primers used in this study was synthesised by Sigma Chemical Co., USA.

ANIMALS

Adult male Wistar rats weighing 200-300g were obtained from Kerala Agricultural University, Mannuthy, Thrissur and used for all experiments. All animals were housed under conditions of controlled temperature and light with free access to food and water.

PARTIAL HEPATECTOMY AND SACRIFICE

Two-thirds of the liver constituting the median and left lateral lobes were surgically excised under light ether anaesthesia, following a 16-h fast. (Higgins.G.M & Anderson.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. The animals were sacrificed at various intervals after surgery by decapitation. Liver and brain were rapidly dissected into different regions (Glowinski.J & Iverson.L.L, 1966). The brain dissection was carried out on a chilled glass plate into hypothalamus (Hypo), brain stem (BS) and cerebral cortex (CC). These regions were immediately immersed into liquid nitrogen and stored at -70^{o}C for various experiments.

LEAD NITRATE ADMINISTRATION:

Experimental rats received a single intravenous injection of LN (100 μmol/kg body weight) (Kubo.Y *et al.*, 1996) while the control rats received distilled water only. The rats were kept for two different time periods, i.e., 48 hours and 5 days after LN treatment and sacrificed.

N-NITROSODIETHYLAMINE TREATMENT

Liver cancer was induced using NDEA (Narurkar.L.M. & Narurkar.M.V, 1989). Animals received 0.02% NDEA in distilled water (2.5 ml/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 22^{nd} week. Neoplasia was confirmed by histological techniques.

MEASUREMENT OF DNA SYNTHESIS IN LIVER

DNA synthesis was measured by thymidine incorporation. 5μCi of [^{3}H] thymidine (Sp. activity 25Ci/mmol) was injected intra-peritonially into PH/LN injected rats to study DNA synthesis at 12, 18, 24, 30, 48, 72 and 168 hours. Tritiated thymidine was injected 2 hrs 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 minutes. 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 50mM Tris HCl buffer pH 7.5. It was centrifuged at 36,000 *g* for 30 minutes. 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 disk (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 supernatant fraction (2.5µg protein). After incubation at 37°C for 15 minutes the reaction was stopped by placing the mixture in a boiling water bath for 3 minutes followed by immersing in an ice bath. Aliquots of 50µl were spotted on Whatman DE 81 paper discs, which were washed with 1mM 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 0.3ml of 0.2M KCl in 1M HCl. Radioactivity was measured in 10ml of liquid scintillation cocktail. The activity was calculated per mg protein. The activity of liver TK was measured by determining the conversion of [^{3}H]thymidine to [^{3}H]thymidine monophosphate and by the binding of monophosphate to DEAE cellulose disc.

HISTOLOGY

The liver was fixed in 10% formaldehyde, embedded in paraffin wax, and sections were prepared using microtome. The sections were stained with Giemsa stain and mounted on slides with DPX mountant. The slides were observed under light microscope.

GIEMSA STAINING

Hepatocytes isolated by collagenase perfusion method and prepared smears on glass slides. PBS : methanol mixture (1:1 ratio) was added, left for 2 minutes and it was discarded. Fresh methanol was added on to the slides and left for 10 minutes. The

methanol was discarded and the procedure was repeated. Added Giemsa stain, after 2 minutes, the stain was diluted with water and agitated gently for two minutes. Washed the cells in tap water, finally in deionised water and visualised under light microscope.

5-HT QUANTIFICATION BY HPLC

Brain 5-HT HPLC determinations were done by electrochemical detection (Paulose.C.S *et al.*, 1988). The tissues from brain regions were homogenised in 0.4N perchloric acid. The homogenate was centrifuged at 5000 x g for 10 minutes at 4°C (Kubota Refrigerated Centrifuge, Japan) and the clear supernatant was filtered through 0.22μm HPLC grade filters and used for HPLC analysis in Shimadzu HPLC system with electrochemical detector fitted with C18-CLC-ODS reverse phase column. Mobile phase was 75mM sodium dihydrogen orthophosphate buffer containing 1mM sodium octyl sulphonate, 50mM EDTA and 7% acetonitrile (pH 3.25), filtered through 0.22μm filter delivered at a flow rate of 1.0 ml/minute. Quantification was by electrochemical detection, using a glass carbon electrode set at +0.80 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 NOREPINEPHRINE

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

5-HT RECEPTOR STUDIES USING [^{3}H] RADIOLIGANDS

5-HT$_{1A}$ Receptor Binding Assays

5-HT$_{1A}$ receptor assay was done by using specific agonist [^{3}H]8-OH DPAT binding to the 5-HT$_{1A}$ receptors (Nenonene.E.K *et al.*, 1994). Brain tissues were homogenised in a polytron homogeniser with 50 volumes of 50mM Tris-HCl buffer, pH

7.4. After first centrifugation at 40,000 x g for 15 minutes, the pellets were resuspended in buffer and incubated at 37°C for 20 minutes to remove endogenous 5-HT. After incubation the homogenates were centrifuged and washed twice at 40,000 x g for 15 minutes and resuspended in appropriate volume of the buffer.

Binding assays were done using different concentrations i.e., 0.20nM - 100nM of [^{3}H]8-OH DPAT in 50mM Tris buffer, pH 7.4 in a total incubation volume of 250µl. Specific binding was determined using 100µM unlabelled 5-HT. Competition studies were carried out with 1.0nM [^{3}H]8-OH DPAT in each tube with unlabelled ligand concentrations varying from 10^{-12} – 10^{-4}M of 5-HT.

Tubes were incubated at 25°C for 60 minutes and filtered rapidly through GF/C filters (Whatman). The filters were washed quickly by three successive washing with 3ml of ice-cold 50mM Tris buffer, pH 7.4. Bound radioactivity was determined with cocktail-T in a Wallac 1409 liquid scintillation counter. Specific binding was determined by subtracting non-specific binding from total binding.

5-HT$_{2C}$ Receptor Binding Studies

Tritiated mesulergine binding to 5-HT$_{2C}$ receptor in the synaptic membrane preparations were assayed as previously described (Herrick-Davis.K *et al.*, 1999). Crude synaptic membrane preparation was suspended in 50mM Tris-HCl buffer (pH 7.4) and used for assay. In saturation binding experiments, 0.1nM - 6nM of [^{3}H]mesulergine was incubated with and without excess of unlabelled 5-HT (100µM) and in competition binding experiments the incubation mixture contained 1nM of [^{3}H]mesulergine with and without 5-HT at a concentration range of 10^{-12}M to 10^{-4}M. Tubes were incubated at 25°C for 60 minutes and filtered rapidly through GF/C filters (Whatman). The filters were washed quickly by three successive washing with 3ml of ice-cold 50mM Tris buffer, pH 7.4. Bound radioactivity was determined with cocktail-T in a Wallac 1409 liquid scintillation counter. Specific binding was determined by subtracting non-specific binding from total binding.

Protein Estimation

Protein concentrations were estimated (Lowry.O.H *et al.*, 1951) using bovine serum albumin as the standard.

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. The 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 analysed 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 K_i 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 are 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.

REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

Isolation of RNA

RNA was isolated from the liver and brain regions of control, PH, LN treated and NDEA treated rats using Tri reagent (Sigma Chemical Co., USA). Brain and liver tissues (25-50 mg) were homogenised in 0.5 ml Tri Reagent. The homogenate was centrifuged

at 12,000 *g* for 10 minutes at 4°C. 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 minutes. The tube was centrifuged at 12,000 *g* for 15 minutes at 4°C. 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 minutes. The tubes were centrifuged at 12,000 *g* for 10 minutes at 4°C. 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 minutes at 4°C. The pellet was semi dried and dissolved in minimum volume of DEPC-treated water. 2µl of RNA was made up to 1ml and optical density was measured at 260nm and 280nm. For pure RNA preparation the ratio of optical density at 260/280 was ≥ 1.7. The concentration of RNA was calculated as one optical density$_{260}$ = 42µg.

RT PCR Primers

The following primers were used for 5-HT$_{1A}$, 5-HT$_{2C}$ receptors and *β*-actin mRNA expression studies.

5'- TGG CTT TCT CAT CTC CAT CC -3' 5'- CTC ACT GCC CCA TTA GTG C –3' PRODUCT SIZE: 357bp	5-HT$_{1A}$
5'- CCA ACG AAC ACC TTC TTT CC -3' 5'- GCA TTG TGC AGT TTC TTC TCC -3' PRODUCT SIZE: 252bp	5-HT$_{2C}$
5'- CAA CTT TAC CTT GGC CAC TAC C -3' 5'- TAC GAC TGC AAA CAC TCT ACA CC -3' PRODUCT SIZE: 150bp	*β*-ACTIN

RT-PCR Of 5-HT$_{1A}$, 5-HT$_{2C}$ Receptor and *β*-Actin

RT-PCR was carried out in a total reaction volume of 20µl in 0.2ml tubes. RT-PCR was performed on an Eppendorf Personal thermocycler. cDNA synthesis of 2µg RNA was performed in a reaction mixture containing MuMLV reverse transcriptase

(40units/reaction), 2mM dithiothreitol, 4 units of human placental RNAse inhibitor, 0.5μg of random hexamer and 0.25mM dNTPs (dATP, dCTP, dGTP and dTTP). The tubes were then incubated at 42°C for one hour. After incubation the reverse transcriptase enzyme, MuMLV, was inactivated by heating at a temperature of 95°C.

Polymerase Chain Reaction (PCR)

PCR was carried out in a 20μl volume reaction mixture containing 4μl of cDNA, 0.25mM dNTPs (dATP, dCTP, dGTP and dTTP), 0.5units of Taq DNA polymerase and 10 picomoles of specific primer. The three primers used have the same annealing temperature

Following is the thermocycling profile used for PCR

94°C -- 5 minutes --- Initial Denaturation

94°C -- 30 seconds --- Denaturation

56°C -- 30 seconds --- Annealing 30 cycles

72°C -- 30 seconds --- Extension

72°C -- 5 minutes --- Final Extension

Analysis of RT-PCR product

After completion of RT-PCR reaction 5μl of bromophenol blue gel-loading buffer was added to 10μl reaction mixture and the total volume was applied to a 2.0% 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 VDS gel documentation system (Pharmacia Biotech) and densitometrically analysed using Imagemaster ID software to quantitate the 5-HT_{1A} receptor, 5-HT_{2C} receptor mRNA expression in control, PH, LN treated and NDEA treated experimental rats.

ISOLATION OF RAT HEPATOCYTES AND PRIMARY CULTURE

Buffers used for perfusion

Ca^{2+}-Free Perfusion Buffer

This buffer contained 142mM NaCl, 6.7mM KCl, 10mM HEPES and 5.5mM 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 67mM NaCl, 6.7mM KCl, 100mM HEPES, 4.76mM $CaCl_2.2H_2O$, and 66mM 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 hours, the unattached collagen is aspirated out and the dishes are 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 #70]. The liver was perfused *in situ* with the calcium -free HEPES buffer (pH 7.4) and then with calcium 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 three hours 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 and appropriate concentrations of 5-HT ligands. 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 hours of incubation and cold 10% TCA was added. The hepatocytes were digested by incubation at 37°C for overnight 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).

Statistical Analysis

Statistical comparisons were performed by Students *t*-test using GraphPad InStat software.

RESULTS

RESULTS

DNA Synthesis in Rat the Liver after Partial Hepatectomy

Tritiated thymidine incorporation into replicating DNA was used as a biochemical index for quantifying DNA synthesis during liver regeneration after PH. Hepatic DNA synthesis increased significantly ($p<0.001$) at 18 hours and reached a maximum at 24 hours. It reversed to the near normal value by 72 hours and reached the basal level by 7 days after PH (Figure - 1).

DNA Synthesis in the Rat Liver after Lead Nitrate Treatment

Tritiated thymidine incorporation increased significantly ($p<0.001$) at 36 hours after LN (100μmol/kg body weight) injection. Hepatic DNA synthesis reached a maximum at 48 hours and reversed to the basal level by 72 hours after LN treatment (Figure - 2).

DNA Synthesis in the Rat Liver after N-Nitrosodiethylamine Treatment

Thymidine kinase enzyme specific activity, a biochemical index for DNA synthesis increased significantly ($p<0.001$) in the NDEA treated rat liver compared to the corresponding control (Figure - 3).

Morphological and Histological Analysis of Rat Livers

Morphological analysis after 20 weeks of NDEA administration showed development of hepatocellular carcinoma (Figure - 4). Histological analysis revealed enlarged nuclei in NDEA treated rats and apoptotic bodies in 5 days after LN treated rats (Figure - 5).

Giemsa Staining of Hepatocytes

Giemsa staining of isolated hepatocytes revealed condensed nuclei in 5 days after LN treatment (Figure - 6).

5-HT Content in the Brain Regions of Rats

Brain Stem

There was a significant increase in 5-HT content in PH ($p<0.05$), 48 hours after LN treatment ($p<0.05$) and NDEA treated rats ($p<0.001$) without any change in 5 days after LN treatment compared with control (Table - 1).

Cerebral Cortex

The 5-HT content increased significantly in PH ($p<0.01$), 48 hours after LN treatment ($p<0.05$) and NDEA treated rats ($p<0.01$) while it remained unchanged in 5 days after LN treatment compared with control (Table - 1).

Hypothalamus

There was an increase in the 5-HT content in PH and NDEA treated rats ($p<0.05$) compared with control (Table - 1).

5-HT Content in the Liver of Rats

There was a significant increase in the hepatic 5-HT content during active cell proliferation i.e., in PH ($p<0.01$), 48 hours after LN treatment ($p<0.01$) and NDEA treated rats ($p<0.001$). During hepatic apoptosis, i.e., in 5 days after LN treatment there was a significant decrease ($p<0.05$) in hepatic 5-HT content compared with control (Table - 2).

Circulating NE Levels in Rats

There was a significant increase in the plasma NE levels in PH ($p<0.01$), 48 hours after LN treatment ($p<0.05$) and NDEA treated ($p<0.001$) rats compared with control (Table - 3).

Receptor Alterations in the Brain Regions of Rats

Brain Stem

5-HT$_{1A}$ Receptor Analysis

[^{3}H]8-OH DPAT Binding Parameters

The B_{max} of the high affinity [^{3}H]8-OH DPAT receptor binding significantly decreased ($p<0.01$) in NDEA treated rats. The K_d value of the high affinity receptor binding increased significantly in PH ($p<0.01$) and NDEA treated rats ($p<0.001$) (Figure - 7 & Table - 4). B_{max} of the high affinity [^{3}H]8-OH DPAT receptor binding decreased significantly in 48 hours ($p<0.05$) and increased significantly in 5 days ($p<0.001$) after LN treatment when compared with control. The K_d value of the receptor binding increased significantly in 48 hours ($p<0.05$) and 5 days ($p<0.01$) after LN treatment compared with control (Figure - 8 & Table - 5).

There was a significant decrease in the B_{max} of the low affinity receptor binding in PH ($p<0.01$) and NDEA treated rats ($p<0.001$). The K_d of the receptor binding increased significantly in NDEA treated rats ($p<0.001$) compared with control (Figure - 9 & Table - 6). There was a significant increase ($p<0.001$) in the B_{max} of low affinity [^{3}H]8-OH DPAT binding in 5 days after LN treatment compared with control. The K_d value of the receptor increased significantly in rats 48 hours after LN treatment ($p<0.001$) while it decreased significantly in 5 days after LN treatment ($p<0.01$) compared with control (Figure - 10 & Table - 7).

Displacement Analysis of [^{3}H]8-OH DPAT by 5-HT

The competition curve for 5-HT against [^{3}H]8-OH DPAT fitted for two-sited model in all the groups with hill slope value away from Unity. The $Ki_{(H)}$ increased in PH and NDEA treated group with an increased log (EC_{50})-1. This indicates a shift of high affinity towards low affinity. $Ki_{(L)}$ increased in PH and NDEA treated group with an increase in the log (EC_{50})-2 indicating a shift in low affinity towards much lower affinity (Figure - 11 & Table - 8). $Ki_{(H)}$ and log (EC_{50})-1 remained unaltered in LN treated rats indicating no affinity shift in the high affinity site in these rats. $Ki_{(L)}$ increased in 48 hours after LN treatment with an increase in the log (EC_{50})-2 indicating a shift in the low affinity site towards much lower affinity. There was no change in the $Ki_{(L)}$ and log (EC_{50})-2 in 5 days after LN treatment compared with control indicating no shift in affinity of the low affinity receptor in this group (Figure - 12 & Table - 9).

RT-PCR Analysis of 5-HT_{1A} Receptor

RT-PCR analysis revealed a decreased expression of 5-HT_{1A} receptor mRNA in PH and NDEA treated rats (Figure - 13) while it increased in LN treated rats (Figure - 14).

5-HT_{2C} Receptor Analysis

[^{3}H]Mesulergine Binding Parameters

There was a significant increase ($p<0.001$) in the B_{max} of [^{3}H]mesulergine binding in PH and NDEA treated rats . The K_d of the receptor binding showed a

significant decrease ($p<0.001$) in NDEA treated rats (Figure - 15 & Table - 10). B_{max} of the receptor showed a significant increase ($p<0.001$) in 48 hours after LN treatment with a significant decrease ($p<0.05$) in the K_d of the receptor binding. In 5 days after LN treatment the receptor binding parameters remained unchanged (Figure - 16 & Table - 11).

Displacement Analysis of [^{3}H]Mesulergine by 5-HT

The competition curve for 5-HT against [^{3}H]mesulergine fitted for one-sited model in all the groups with Unity as hill slope value. There was a decrease in Ki and log (EC_{50}) in PH and NDEA treated rats compared with control indicating a shift in affinity towards high affinity (Figure - 17 & Table - 12). Ki and log (EC_{50}) showed a decrease in 48 hours after LN treatment indicating a shift in affinity towards high affinity. In 5 days after LN treatment there was no significant change in the Ki and log (EC_{50}) compared with control indicating no shift in affinity in this group (Figure - 18 & Table - 13).

RT-PCR Analysis of 5-HT$_{2C}$ Receptor

5-HT_{2C} receptor mRNA was found to be increased in PH and NDEA treated rats (Figure - 19) while the increased 5-HT_{2C} receptor in 48 hours after LN treatment was coming to the control level in 5 days after LN treatment (Figure - 20).

Cerebral Cortex

5-HT$_{1A}$ Receptor Analysis

[^{3}H]8-OH DPAT Binding Parameters

A significant increase was observed in the B_{max} of [^{3}H]8-OH DPAT high affinity receptor binding to the membrane preparation of PH ($p<0.05$) and NDEA treated ($p<0.001$) rats. The K_d value of the high affinity receptor significantly increased ($p<0.001$) in PH and NDEA treated rats (Figure - 21 & Table - 14). There was a significant decrease in the B_{max} of high affinity receptor in 48 hours after LN treatment ($p<0.01$) and 5 days after LN treatment ($p<0.05$) compared with control. The K_d of the receptor increased significantly ($p<0.05$) in 48 hours after LN treatment (Figure - 22 & Table - 15).

The B_{max} of low affinity [^{3}H]8-OH DPAT receptor binding decreased significantly in NDEA treated rats ($p<0.001$). The K_d of the receptor binding increased significantly in PH ($p<0.001$) and NDEA treated rats ($p<0.01$) compared with control rats (Figure - 23 & Table - 16). The B_{max} of the low affinity [^{3}H]8-OH DPAT receptor binding increased significantly in 48 hours ($p<0.01$) and 5 days ($p<0.001$) after LN treatment compared with control. The K_d of the receptor binding increased significantly ($p<0.001$) in 48 hours after LN treatment compared with control (Figure - 24 & Table - 17).

Displacement Analysis of [^{3}H] 8-OH DPAT by 5-HT

The competition curve for 5-HT against [^{3}H]8-OH DPAT fitted for two-sited model in all the groups with hill slope value away from Unity. The $Ki_{(H)}$ increased in PH and NDEA treated rats along with an increase in the log (EC_{50})-1 indicating a shift in high affinity towards low affinity. $Ki_{(L)}$ also showed an increase in PH and NDEA treated rats with an increase in log (EC_{50})-2 denoting a shift in the low affinity site towards much lower affinity (Figure - 25 & Table - 18). $Ki_{(H)}$ showed an increase in 48 hours after LN treatment indicating a shift in affinity of the low affinity site towards much lower affinity in this group (Figure - 26 & Table - 19).

RT-PCR analysis of 5-HT$_{1A}$ receptor

In PH 5-HT$_{1A}$ receptor mRNA decreased while in NDEA treated rats it increased when compared with control (Figure - 27). 5-HT$_{1A}$ receptor mRNA expression increased in LN treated rats with a very high increase in 5 days after LN treatment (Figure - 28).

5-HT$_{2C}$ Receptor Analysis

[^{3}H]Mesulergine Binding Parameters

The B_{max} of the [^{3}H]mesulergine binding increased significantly ($p<0.001$) in NDEA treated rats. The K_d of the receptor binding decreased significantly ($p<0.001$) in PH and NDEA treated rats (Figure - 29 & Table - 20). The B_{max} of the receptor binding increased significantly ($p<0.01$) in 48 hours after LN treatment with a significant

decrease ($p<0.001$) in the K_d of the receptor compared with control (Figure - 30 & Table - 21).

Displacement Analysis of [^{3}H]Mesulergine by 5-HT

The competition curve for 5-HT against [^{3}H]mesulergine fitted for one-sited model in all the groups with Unity as hill slope value. There was a decrease in the Ki and log (EC_{50}) in PH and NDEA treated rats indicating a shift in affinity of the receptor towards high affinity (Figure - 31 & Table - 22). The Ki in 48 hours after LN treatment decreased with a decrease in log (EC_{50}) indicating a shift in affinity towards high affinity compared with control. There was no significant change in the Ki and log (EC_{50}) in 5 days after LN treatment indicating no shift in the affinity of the receptor (Figure - 32 & Table - 23).

RT-PCR analysis of 5-HT$_{2C}$ receptor

RT-PCR analysis revealed an increased mRNA in PH and NDEA treated rats (Figure - 33). In 48 hours after LN treatment the mRNA status increased while in 5 days after LN treatment it decreased (Figure - 34).

Hypothalamus

5-HT$_{1A}$ Receptor Analysis

[^{3}H]8-OH DPAT binding parameters

There was a significant decrease ($p<0.01$) in the B_{max} of the high affinity [^{3}H]8-OH DPAT receptor binding in PH and NDEA treated rats compared with control. The K_d of the receptor binding increased significantly in NDEA ($p<0.05$) treated rats (Figure - 35 & Table - 24). The B_{max} of the high affinity [^{3}H]8-OH DPAT receptor binding increased significantly in 5 days after LN treatment ($p<0.001$) without any change in the K_d of the receptor (Figure - 36 & Table - 25).

There was a significant decrease ($p<0.001$) in the B_{max} of the low affinity [^{3}H]8-OH DPAT receptor binding in PH and NDEA treated rats. The K_d value of the receptor binding showed a significant increase ($p<0.01$) in NDEA treated rats compared with control (Figure - 37 & Table - 26). The low affinity [^{3}H]8-OH DPAT receptor binding decreased significantly in 48 hours ($p<0.05$) after LN treatment while it increased very

significantly in 5 days ($p<0.001$) after LN treatment. The K_d value of the low affinity receptor binding increased significantly ($p<0.001$) in 48 hours after LN treatment compared with control (Figure - 38 & Table - 27).

Displacement Analysis of [^{3}H]8-OH DPAT by 5-HT

The competition curve for 5-HT against [^{3}H]8-OH DPAT fitted for two-sited model in all the groups with hill slope value away from unity. The $Ki_{(H)}$ and log (EC_{50})-1 increased in NDEA treated rats indicating a shift in affinity of the high affinity receptor binding site towards low affinity. $Ki_{(L)}$ and log (EC_{50})-2 increased in NDEA treated rats indicating a decrease in the low affinity site towards much lower affinity (Figure - 39 & Table - 28). There was an increase in the $Ki_{(H)}$, log (EC_{50})-1, $Ki_{(L)}$ and log (EC_{50})-2 in 48 hours after LN treatment indicating a shift in affinity of the receptor towards the respective lower affinity sites (Figure - 40 & Table - 29).

RT-PCR Analysis of 5-HT$_{1A}$ Receptor

During liver regeneration after PH the mRNA expression decreased while it was almost nil in NDEA treated rats (Figure - 41). It showed a decreased in 48 hours after LN treatment while it increased during hepatic apoptosis i.e., 5 days after LN treatment (Figure - 42).

5-HT$_{2C}$ receptor analysis

[^{3}H]Mesulergine Binding Parameters

There was a significant increase in the B_{max} of the [^{3}H]mesulergine binding to the membrane preparation of hypothalamus in PH ($p<0.01$) and NDEA treated ($p<0.05$) rats. The K_d of the receptor binding showed a significant decrease ($p<0.001$) in both PH and NDEA treated rats (Figure - 43 & Table - 30). B_{max} of the receptor showed a significant increase ($p<0.05$) in 48 hours after LN treatment with a significant decrease ($p<0.001$) in the K_d of the receptor binding. In 5 days after LN treatment the receptor binding parameters remained unchanged compared to the control (Figure - 44 & Table - 31).

Displacement Analysis of [^{3}H]Mesulergine by 5-HT

The competition curve for 5-HT against [^{3}H]mesulergine fitted for one-sited model in all the groups with Unity as the hill slope value. There was decrease in the Ki and log (EC_{50}) in PH and NDEA treated rats compared with control indicating a shift in affinity of the receptor towards high affinity (Figure - 45 & Table - 32). In 48 hours after LN treatment there was a decrease in the Ki and log (EC_{50}) compared with control indicating a shift in affinity of the receptor towards high affinity site (Figure - 46 & Table - 33).

RT-PCR Analysis of 5-HT$_{2C}$ Receptor

5-HT$_{2C}$ receptor mRNA increased in PH and NDEA treated rats (Figure - 47). The receptor mRNA increased in 48 hours after LN treatment while it decreased in 5 days after LN treatment (Figure - 48).

Receptor Alterations in the Liver of Rats

5-HT$_{1A}$ Receptor Analysis

[^{3}H]8-OH DPAT binding parameters

B_{max} of the high affinity [^{3}H]8-OHDPT receptor binding decreased significantly ($p<0.001$) in PH and NDEA treated rats with no alteration in the K_d of the receptor (Figure - 49 & Table - 34). There was a significant decrease ($p<0.001$) in the B_{max} of the high affinity receptor in 48 hours after LN treatment. The K_d of the receptor showed a significant decrease in both LN treated rats ($p<0.05$) (Figure - 50 & Table - 35).

There was a significant decrease ($p<0.001$) in the B_{max} of the low affinity [^{3}H]8-OH DPAT receptor binding in PH and NDEA treated rats. The K_d of the receptor showed a significant increase ($p<0.05$) in PH rats while it decreased significantly in NDEA treated rats ($p<0.001$) (Figure - 51 & Table - 36). B_{max} of the low affinity receptor binding decreased significantly in the liver of rats 48 hours after LN treatment ($p<0.001$) while it increased significantly in rats after 5 days of LN treatment ($p<0.01$). The K_d of the receptor increased significantly ($p<0.05$) in rats after 5 days of LN treatment when compared with control (Figure - 52 & Table - 37).

Displacement Analysis of [^{3}H]8-OH DPAT by 5-HT

The competition curve for 5-HT against [^{3}H]8-OH DPAT fitted for two-sited model in all the groups with hill slope value away from Unity. $Ki_{(H)}$ decreased in NDEA treated rats indicating a shift in high affinity receptor binding towards high affinity. $Ki_{(L)}$ also decreased in NDEA treated rats indicating a shift in affinity of the low affinity receptor towards high affinity (Figure - 53 & Table - 38). $Ki_{(H)}$ decreased in 5 days after LN treatment indicating a shift in affinity of the high affinity receptor towards high affinity. In 5 days after LN treatment $Ki_{(L)}$ increased along with an increase in the log (EC_{50})-2 denoting a shift in low affinity of the receptor towards much lower affinity (Figure - 54 & Table - 39).

RT-PCR Analysis of 5-HT$_{1A}$ Receptor

5-HT$_{1A}$ receptor mRNA decreased in PH and NDEA treated rats (Figure - 55). It increased in LN treated rats with a very high increase in 5 days after LN treatment (Figure - 56).

5-HT$_{2C}$ Receptor Analysis

[^{3}H]Mesulergine Binding Parameters

[^{3}H]Mesulergine binding was observed in the liver of NDEA treated rats alone with a B_{max} of 71 fmoles/mg protein and an affinity of 35 nM, while the binding was absent in all the other groups (Figure - 57 & Table - 40).

Displacement Analysis of [^{3}H]Mesulergine by 5-HT

The competition curve for 5-HT against [^{3}H]mesulergine fitted for one-sited model in NDEA treated rat hepatic membrane preparations with unity as the hill slope value. Ki value of the receptor is 3.91×10^{-8} and log (EC_{50}) is -7.192 (Figure - 58 & Table - 41).

RT-PCR analysis of 5-HT$_{2C}$ receptor

5-HT$_{2C}$ receptor mRNA expression was found in NDEA treated rats only (Figure - 59) while it was absent in other groups (Figure - 60).

Effect of 5-HT on Cultured Hepatocytes

Isolated hepatocytes in serum-free culture medium exhibited very low levels of [^{3}H]thymidine incorporation into DNA. Addition of EGF (10ng) caused a significant increase ($p<0.001$) in the hepatocyte DNA synthesis. When 5-HT (100μM) alone was added to cultured hepatocytes there was no significant change in the DNA synthesis from basal level. However addition of 5-HT (100μM) along with EGF caused a significant increase in DNA synthesis when compared with EGF alone group ($p<0.001$). Addition of TGFβ1 (1ng) caused a significant decrease ($p<0.05$) in the basal level of DNA synthesis, while addition of 5-HT (100μM) brought back this decreased DNA synthesis to the basal level (Figure - 61).

Effect of 8-OH DPAT on Hepatocyte DNA Synthesis

Addition of 8-OH DPAT (100μM) caused a significant decrease ($p<0.001$) in the basal and EGF mediated DNA synthesis. TGFβ1 mediated hepatocyte DNA synthesis suppression increased significantly ($p<0.001$) by the addition of 8-OH DPAT to the primary hepatocyte culture (Figure - 62).

Dose-dependent Response of Hepatocyte DNA Synthesis to 8-OH DPAT

8-OH DPAT inhibited significantly ($p<0.001$) the DNA synthesis of primary hepatocytes in culture dose-dependently from 10^{-6}M to 10^{-4}M. 10^{-8}M 8-OH DPAT also induced a significant ($p<0.001$) DNA synthesis suppression (Figure - 63).

Dose-dependent Response of EGF Induced Hepatocyte DNA synthesis to 8-OH DPAT

Addition of 8-OH DPAT at a concentration from 10^{-8}M to 10^{-4}M significantly ($p<0.001$) suppressed the EGF mediated DNA synthesis of cultured hepatocytes (Figure - 64).

Dose-dependent Response of TGFβ1 Induced Hepatocyte DNA Synthesis to 8-OH DPAT

8-OH DPAT increased significantly ($p<0.001$) TGFβ1 mediated DNA synthesis suppression in primary hepatocyte culture dose-dependently from 10^{-6}M to 10^{-4}M. A significant ($p<0.001$) DNA synthesis suppression was also found at 10^{-8}M 8-OH DPAT which is comparable to 10^{-4}M concentration of 8-OH DPAT (Figure - 65).

Effect of Mesulergine on Hepatocyte DNA Synthesis

Addition of mesulergine (100μM) caused a significant decrease ($p<0.001$) in the basal and EGF mediated DNA synthesis. TGFβ1 mediated hepatocyte DNA synthesis suppression increased significantly ($p<0.05$) by the addition of mesulergine to the primary hepatocyte culture (Figure - 66).

Dose-dependent Response of Hepatocyte DNA Synthesis to Mesulergine

Mesulergine inhibited significantly the DNA synthesis of primary hepatocytes in culture dose-dependently from 10^{-8}M ($p<0.01$) to 10^{-4}M ($p<0.001$) (Figure - 67).

Dose-dependent Response of EGF Induced Hepatocyte DNA Synthesis to Mesulergine

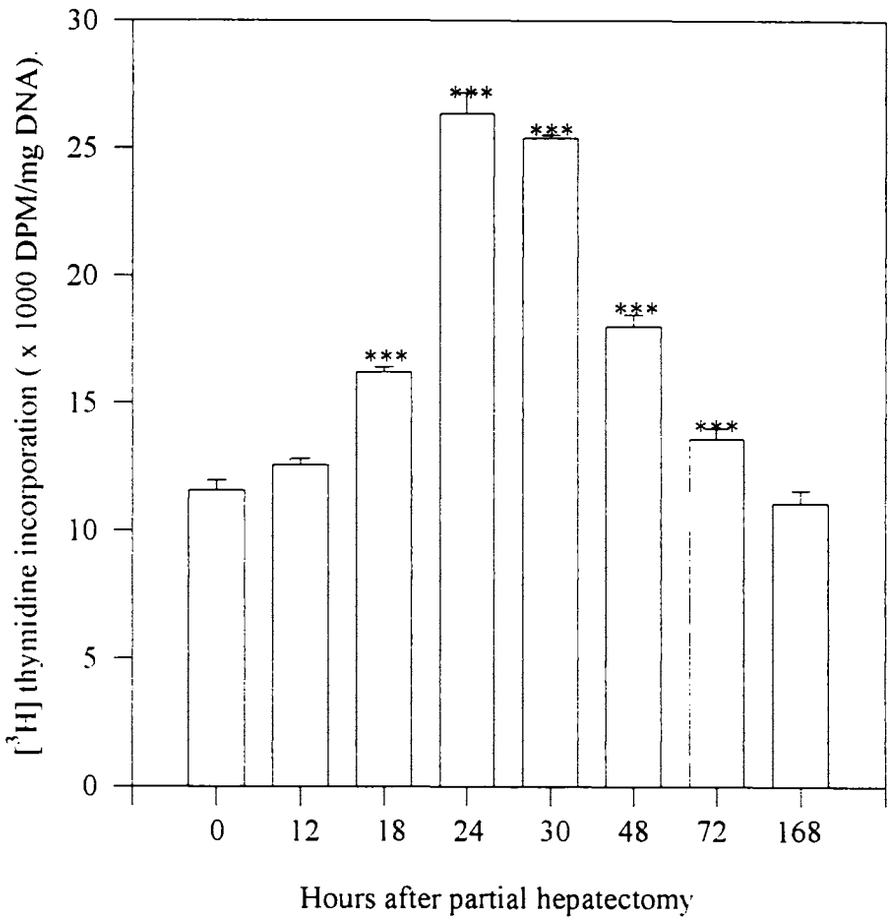
Addition of mesulergine at a concentration from 10^{-8}M to 10^{-4}M significantly ($p<0.001$) suppressed the EGF mediated DNA synthesis of cultured hepatocytes (Figure - 68).

Dose-dependent Response of TGFβ1 Induced Hepatocyte DNA Synthesis to Mesulergine

Mesulergine at a concentration of 10^{-4}M significantly ($p<0.01$) enhanced TGFβ1 mediated DNA synthesis suppression in primary hepatocyte culture (Figure - 69).

Figure - 1

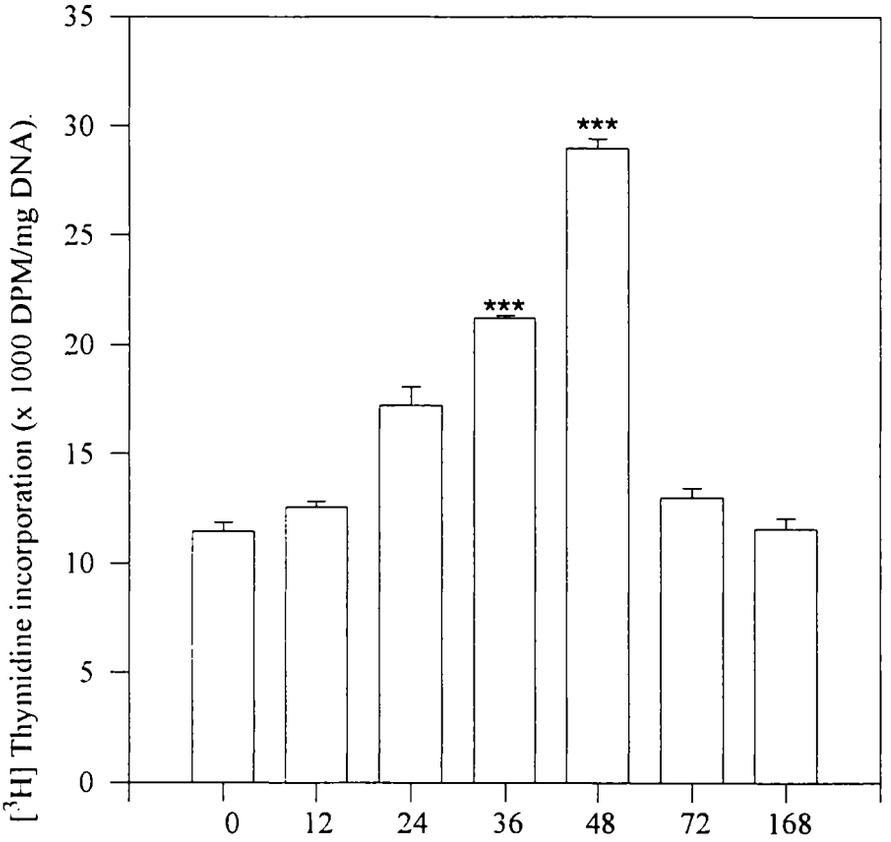
DNA synthesis in the liver of rats after partial hepatectomy



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

Figure-2

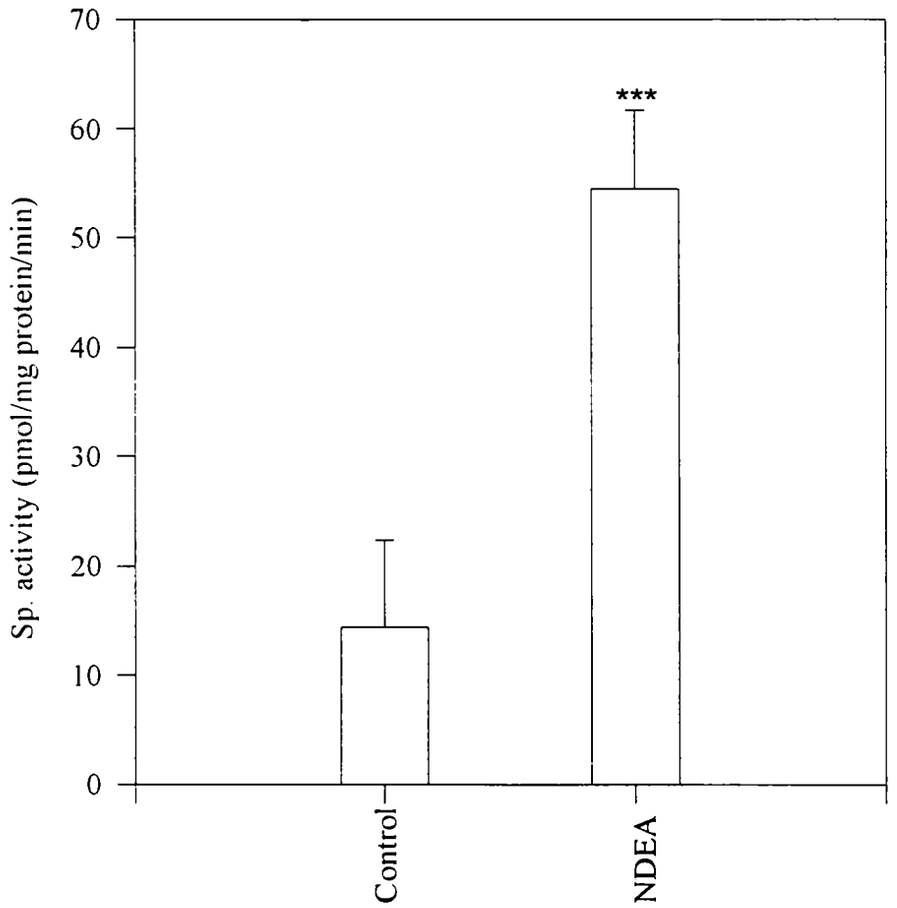
Effect of lead nitrate on liver DNA synthesis



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

Figure - 3

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



NDEA- *N*-nitrosodiethylamine treated

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

Figure - 4

Morphology of Control and *N*-nitrosodiethylamine treated Rat livers

a)

b)

a). Control
b). *N*-nitrosodiethylamine treated

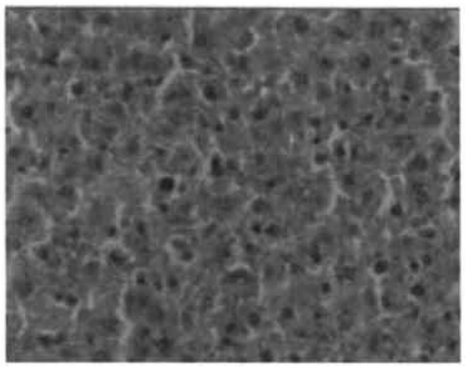
Figure - 5

Slides were prepared from processed liver tissues, stained with giemsa and observed under light microscope (x100)

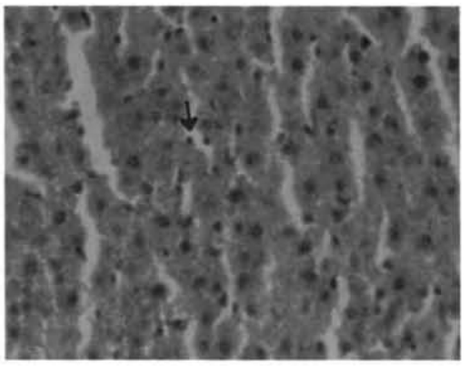
a) Control liver
b) 5 days after lead nitrate treatment showing condensed nuclei
c) *N*-nitrosodiethylamine induced hepatocellular carcinoma showing enlarged nuclei.

Figure - 5
Histological Sections of Rat Liver

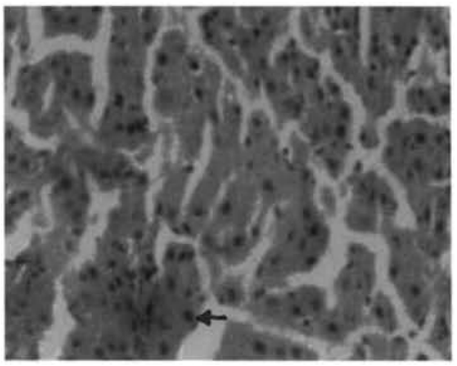
a)



b)



c)



a). Control
b). 5 days after lead nitrate treatment
c). *N*-nitrosodiethylamine treated

Figure - 6

Hepatocytes isolated by collagenase perfusion is stained with giemsa and observed under light microscope (x200).

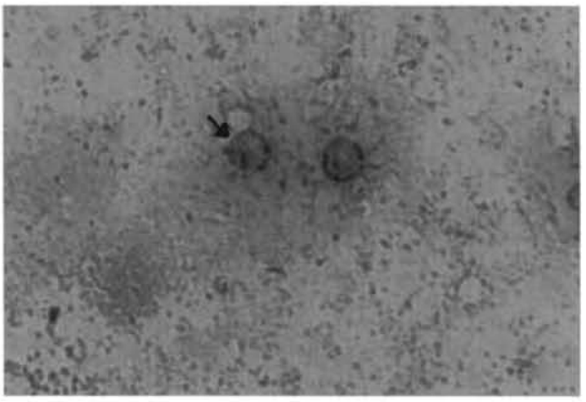
a). Control hepatocytes

b). Hepatocytes isolated from 5 days after lead nitrate treatment showing condensed darkly stained nuclei, an indication of apoptosis

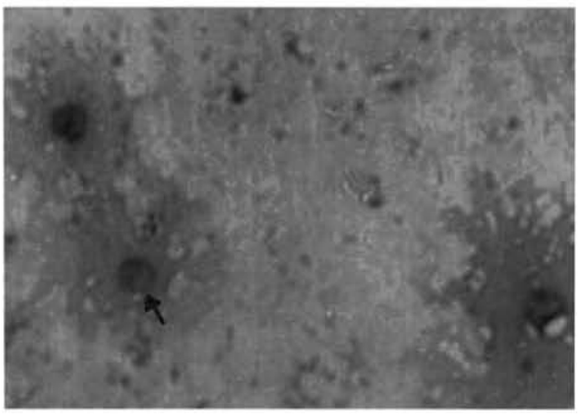
Figure -6

Giemsa Stained Hepatocytes

a)



b)



a). Control
b). 5 days after lead nitrate treatment

Table-1

5-HT Content in the brain regions of rats

(nanomoles/gm wet weight of the tissue)

	Brain Stem	Cerebral Cortex	Hypothalamus
Control[¶]	1.28 ± 0.02	0.99 ± 0.03	1.30 ± 0.24
Partially Hepatectomised	1.89 ± 0.08*	2.15 ± 0.28**	3.12 ± 0.51*
Lead Nitrate 48 hours	1.84 ± 0.36*	1.79 ± 0.30*	1.68 ± 0.24
Lead Nitrate 5days	1.20 ± 0.08	0.87 ± 0.06	2.06 ± 0.29
N-Nitrosodiethylamine Treated	3.22 ± 0.03***	2.26 + 0.16**	3.32 ± 0.15*

*** $p<0.001$, ** $p<0.01$, * $p<0.05$ compared with the control

Values are ± SEM 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-2

5-HT Content in the liver of rats

(nanomoles/gm wet weight of the tissue)

Control[¶]	1.76 ± 0.17
Partially Hepatectomised	2.91 ± 0.09**
Lead Nitrate 48 hours	3.14 ± 0.07**
Lead Nitrate 5days	0.83 ± 0.18*
N-Nitrosodiethylamine Treated	5.33 + 0.05***

*** $p<0.001$, ** $p<0.01$, * $p<0.05$ compared with the control

Values are ± SEM 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-3

Levels of Plasma Norepinephrine in Rats

(nanomoles/ml)

Control[¶]	0.53 ± 0.01
Partially Hepatectomised	1.88 ± 0.02**
Lead Nitrate 48 hours	1.64 ± 0.04*
Lead Nitrate 5days	0.77 ± 0.03
N-Nitrosodiethylamine Treated	8.77 ± 0.65***

*** $p<0.001$, ** $p<0.01$, * $p<0.05$ compared with the control

Values are ± SEM 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 - 7

Scatchard analysis of high affinity [^{3}H]8-OH DPAT receptor binding against 5-HT in the brain stem of rats

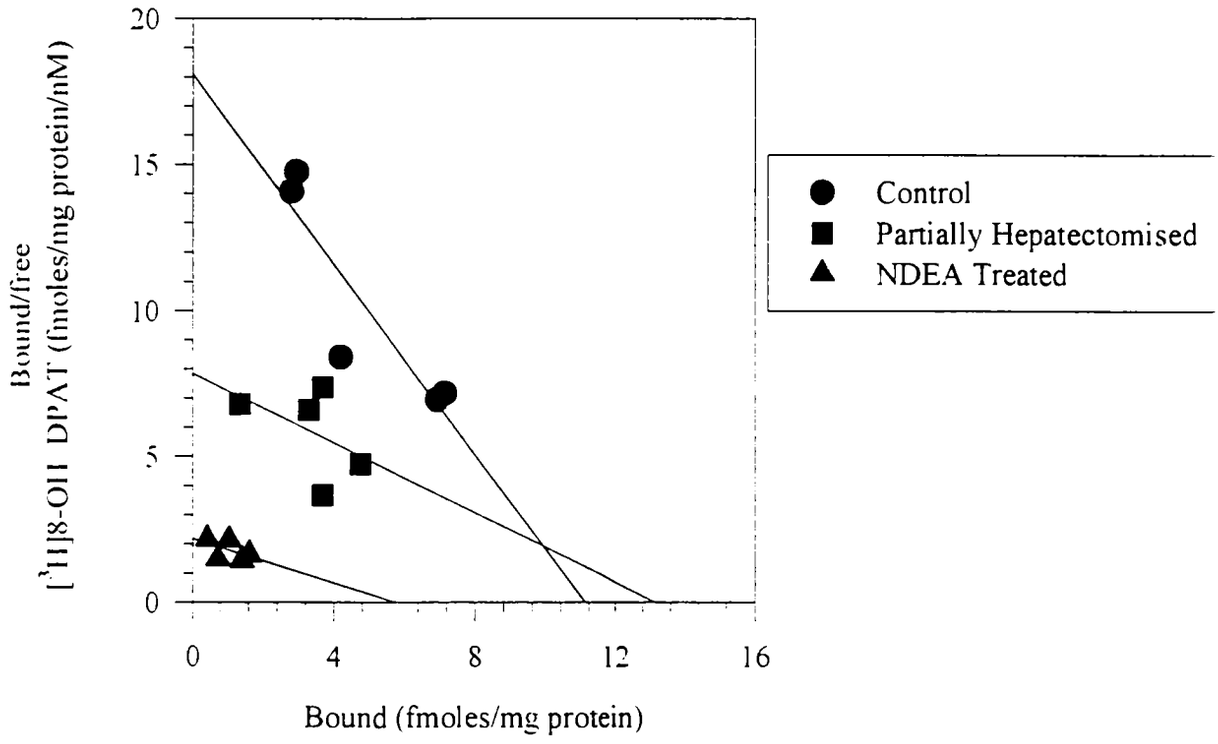


Table - 4

[^{3}H]8-OH DPAT high affinity receptor binding parameters in the brain stem of rats

Experimental Group	B_{max} (fmoles/mg protein)	K_d (nM)
Control[¶]	11.46 ± 0.58	0.63 ± 0.13
Partially Hepatectomised	13.06 ± 0.69	1.67 ± 0.14**
NDEA Treated	5.65 ± 0.55**	2.61 ± 0.19***

***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.

Figure - 8

Scatchard analysis of high affinity [^{3}H]8-OH DPAT receptor binding against 5-HT in the brain stem of rats

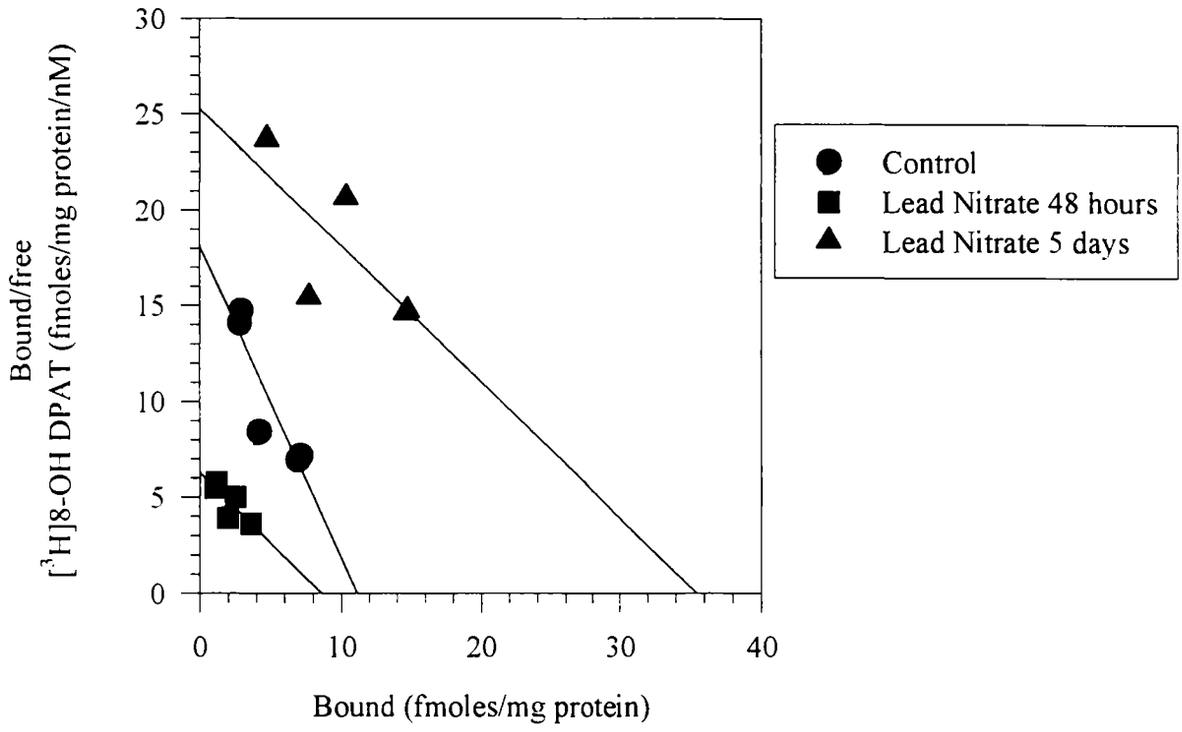


Table - 5

[^{3}H]8-OH DPAT high affinity receptor binding parameters in the brain stem of rats

Experimental Group	B_{max} (fmoles/mg protein)	K_d (nM)
Control[¶]	11.46 ± 0.58	0.63 ± 0.13
Lead Nitrate 48 hours	8.46 ± 0.59*	1.34 ± 0.17*
Lead Nitrate 5 days	36.73 ± 4.39***	1.44 ± 0.09**

*p<0.05, **p<0.01, ***p<0.001, 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-9

Scatchard analysis of low affinity [^{3}H]8-OH DPAT receptor binding against 5-HT in the brain stem of rats

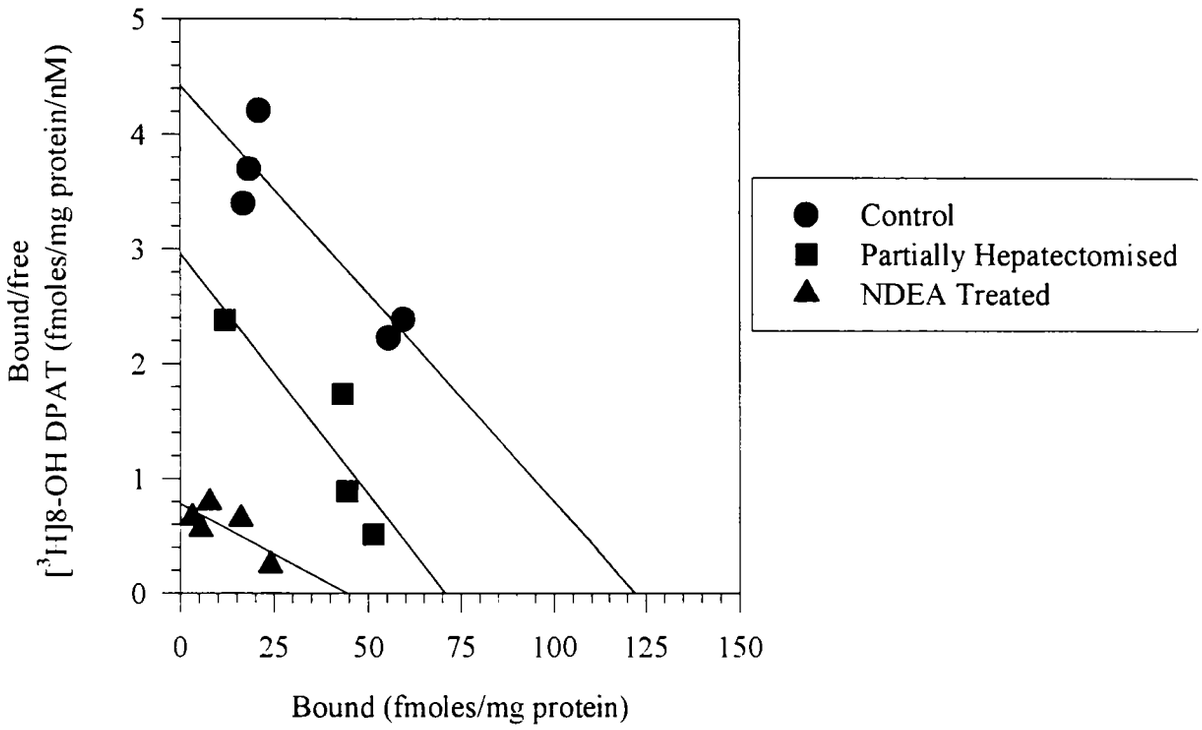


Table-6

[^{3}H]8-OH DPAT low affinity receptor binding parameters in the brain stem of rats

Experimental Group	B_{max} (fmoles/mg protein)	K_d (nM)
Control¶	122.67 ± 1.05	28.15 ± 1.08
Partially Hepatectomised	70.66 ± 9.33**	24.01 ± 1.45
NDEA Treated	43.05 ± 0.05***	54.49 ± 3.58***

***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.

Figure-10

Scatchard analysis of low affinity [^{3}H]8-OH DPAT receptor binding against 5-HT in the brain stem of rats

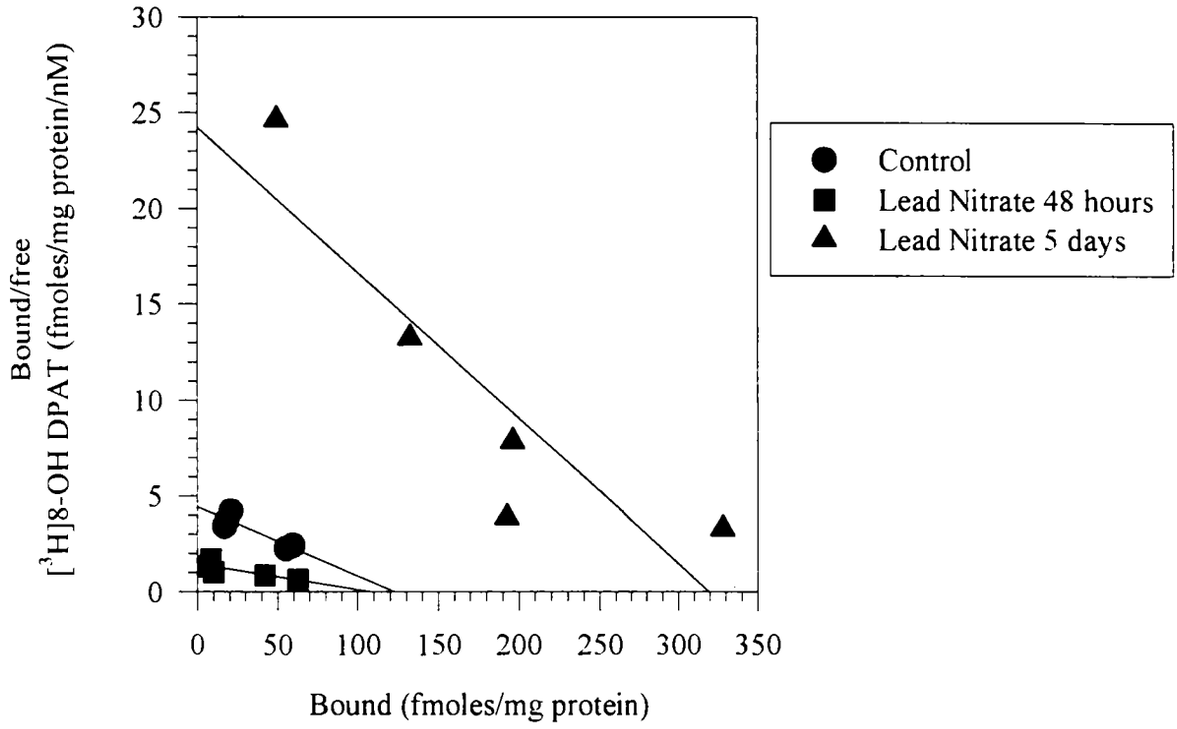


Table-7

[^{3}H]8-OH DPAT low affinity receptor binding parameters in the brain stem of rats

Experimental Group	B_{max} (fmoles/mg protein)	K_d (nM)
Control[¶]	122.67 ± 1.05	28.15 ± 1.08
Lead Nitrate 48 hours	112.67 ± 5.89	79.82 ± 4.62***
Lead Nitrate 5 days	328.33 ± 21.60***	13.14 ± 0.08**

***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.

Table - 8

Binding parameters of [^{3}H]8-OH DPAT against 5-HT in the brain stem of rats

Experimental Group	Best-fit model	log (EC_{50})-1	log (EC_{50})-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Control¶	Two-site	-8.58	-5.69	1.02×10^{-9}	7.74×10^{-7}	-0.39
Partially Hepatectomised	Two-site	-7.58	-5.38	1.02×10^{-8}	1.59×10^{-6}	-0.49
N-Nitrosodiethylamine Treated	Two-site	-7.32	-4.91	1.85×10^{-8}	4.76×10^{-6}	-0.48

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.

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

PH – Partially Hepatectomised
NDEA Treated – *N*-Nitrosodiethylamine Treated

Figure - 11

Displacement of [^{3}H]8-OH DPAT with 5-HT in the brain stem of rats

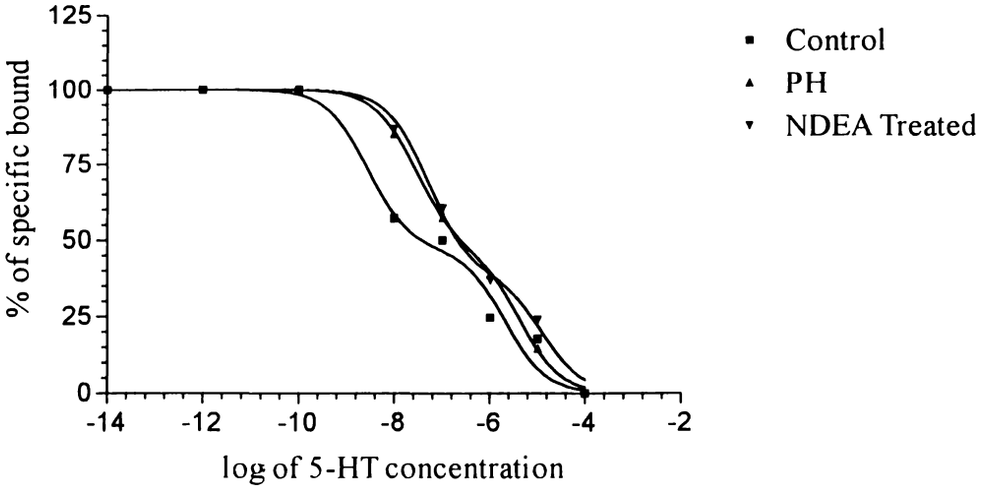


Table - 9

Binding parameters of [^{3}H]8-OH DPAT against 5-HT in the brain stem of rats

Experimental Group	Best-fit model	log (EC_{50})-1	log (EC_{50})-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Control¶	Two-site	-8.58	-5.69	1.02×10^{-9}	7.74×10^{-7}	-0.39
Lead Nitrate 48 hours	Two-site	-8.20	-5.14	2.43×10^{-9}	2.53×10^{-6}	-0.41
Lead Nitrate 5 days	Two-site	-8.52	-5.88	1.16×10^{-9}	5.05×10^{-7}	-0.43

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. 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.

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

LN 48hrs – Lead Nitrate 48 hours
LN 5days – Lead Nitrate 5 days

Figure - 12

Displacement of [^{3}H]8-OH DPAT with 5-HT in the brain stem of rats

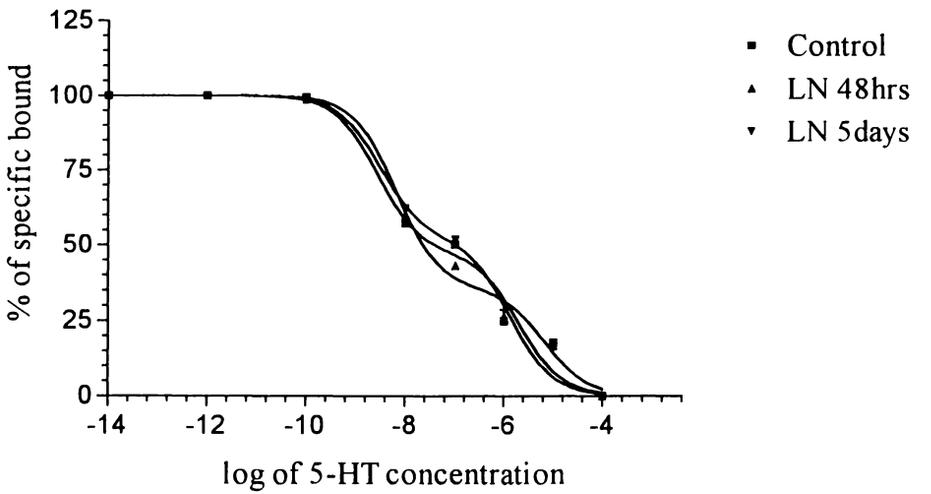
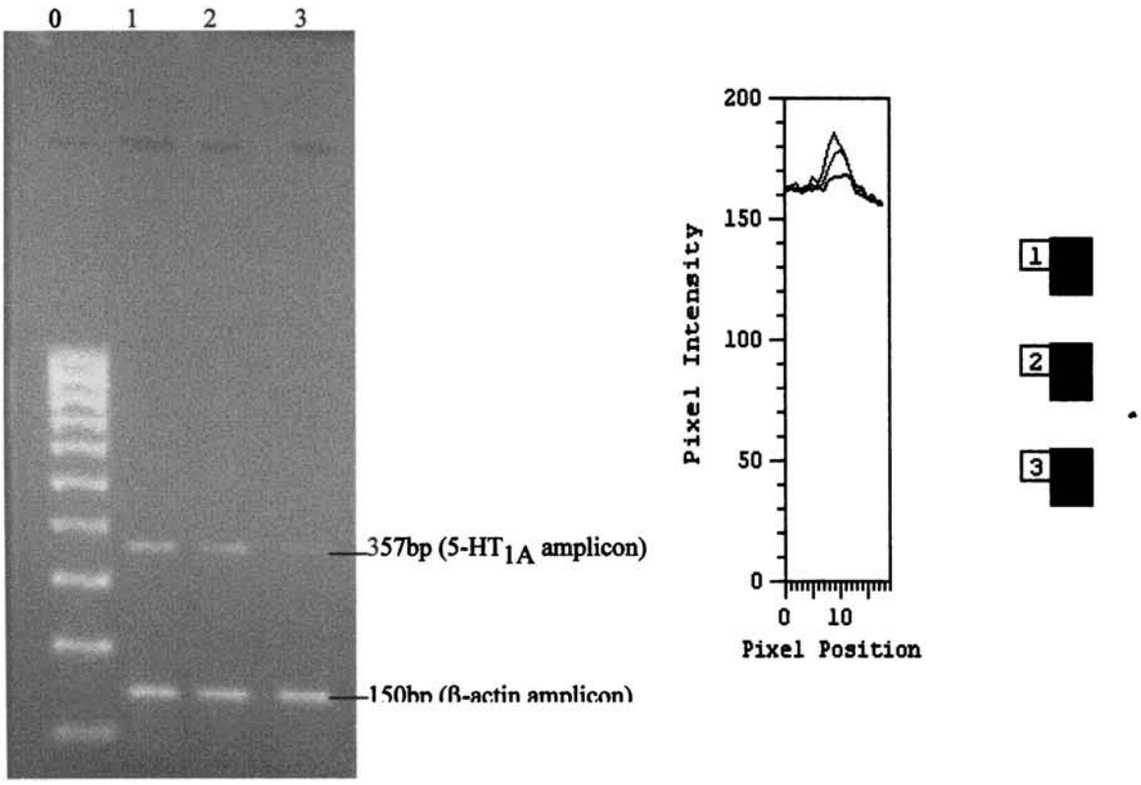


Figure - 13
RT-PCR amplification product of 5-HT_{1A} receptor mRNA from the brain stem of rats



Band properties of 5-HT_{1A} receptor mRNA RT-PCR amplicon

Lane No	Raw Volume	Area	Peak
1	48734	286	186.46
2	39534	234	180.00
3	34240	208	171.23

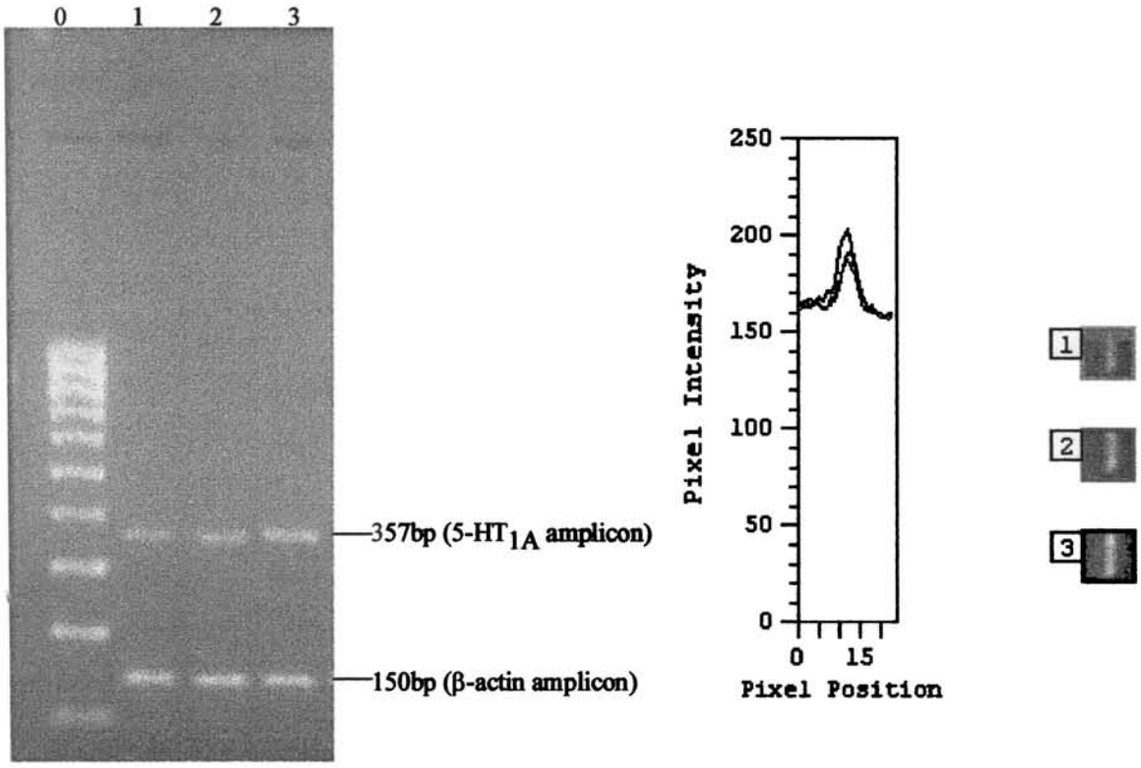
0 – 100bp ladder

1 – Control

2 – Partially Hepatectomised

3 – *N*-Nitrosodiethylamine Treated

Figure - 14
RT-PCR amplification product of 5-HT_{1A} receptor mRNA from the brain stem of rats



Band properties of 5-HT_{1A} receptor mRNA RT-PCR amplicon

Lane No.	Raw Volume	Area	Peak
1	48734	286	186.46
2	53210	234	188.96
3	55018	208	196.81

0 - 100bp ladder

1 - Control

2 - Lead Nitrate 48 hours

3 - Lead Nitrate 5 days

Figure-15

Scatchard analysis of [^{3}H]Mesulergine binding against 5-HT in the brain stem of rats

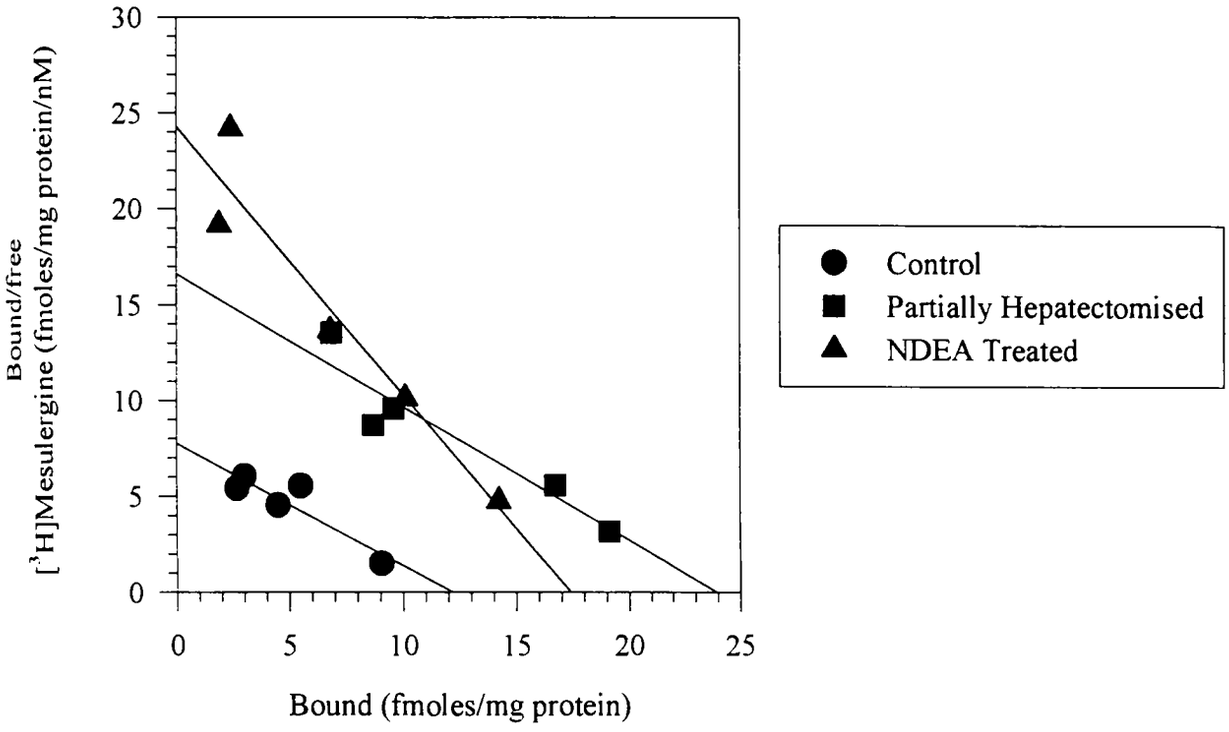


Table-10

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

Experimental Group	B_{max} (fmoles/mg protein)	K_d (nM)
Control[¶]	12.29 ± 0.32	1.59 ± 0.13
Partially Hepatectomised	24.1 ± 0.93***	1.45 ± 0.13
NDEA Treated	17.25 ± 0.59***	0.69 ± 0.04***

***$p<0.001$ 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-16

Scatchard analysis of [^{3}H]Mesulergine binding against 5-HT in the brain stem of rats

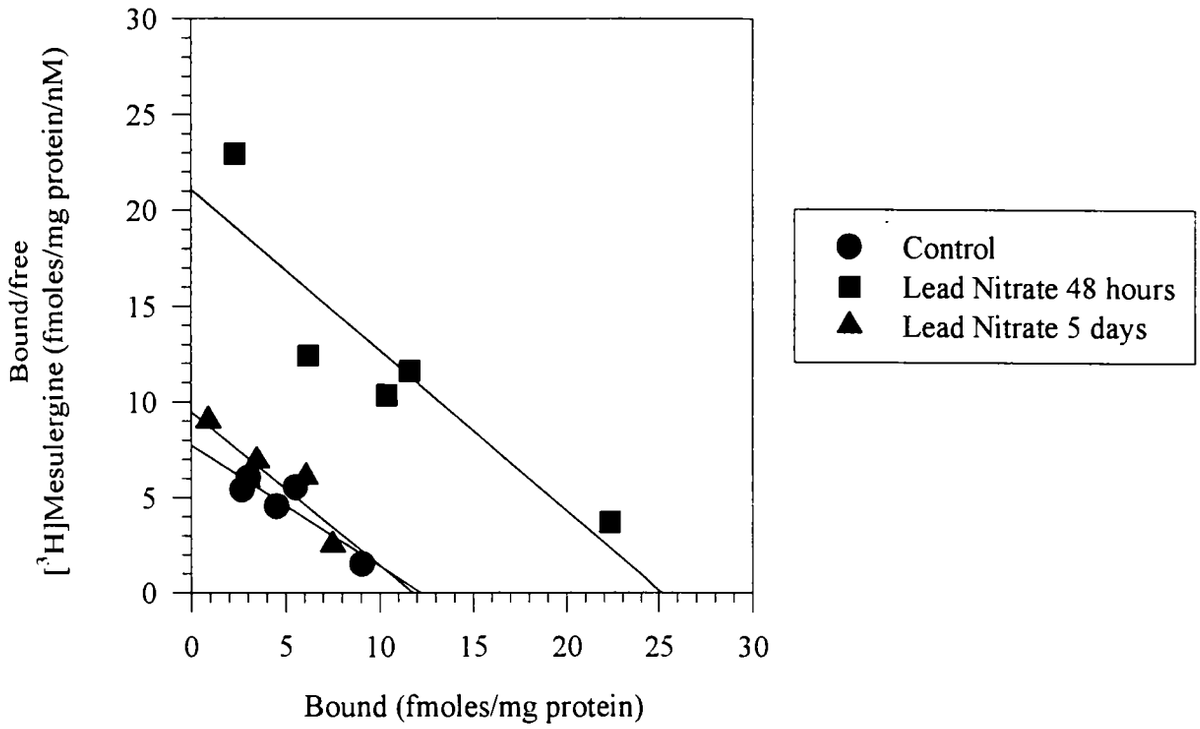


Table-11

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

Experimental Group	B_{max} (fmoles/mg protein)	K_d (nM)
Control[¶]	12.29 ± 0.32	1.59 ± 0.13
Lead Nitrate 48 hours	25.13 ± 1.76***	1.21 ± 0.07*
Lead Nitrate 5 days	11.60 ± 0.43	1.28 ± 0.09

***$p<0.001$, *$p<0.05$ 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]mesulergine in the brain stem of rats

Experimental Group	Best-fit model	Log (EC_{50})	Ki	Hill slope
Control	One-site	-5.58	1.61×10^{-6}	-1.00
Partially Hepatectomised	One-site	-5.72	1.16×10^{-6}	-1.00
NDEA Treated	One-site	-6.11	4.78×10^{-7}	-1.00

Values are mean of 4-6 experiments

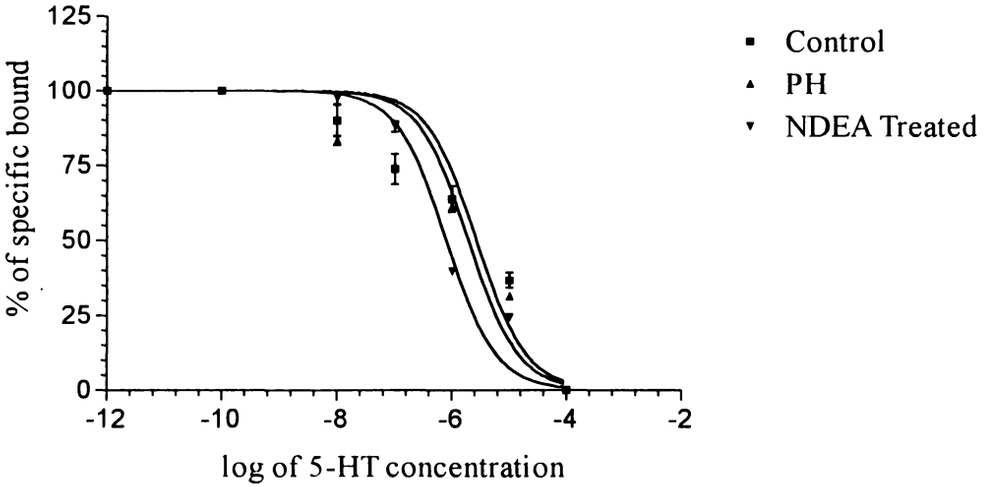
Data were fitted with an iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki – The affinity f the receptor for the competing drug. EC_{50} is the concentration of the competitor that competes for the half the specific binding

PH – Partially Hepatectomised

NDEA – *N*-Nitrosodiethylamine Treated

Figure - 17

Displacement of [^{3}H]mesulergine with 5-HT in the brain stem of rats



Binding parameters of [^{3}H]mesulergine in the brain stem of rats

Experimental Group	Best-fit model	Log (EC_{50})	Ki	Hill slope
Control	One-site	-5.58	1.61×10^{-6}	-1.00
Lead Nitrate 48 hours	One-site	-7.75	1.09×10^{-8}	-1.00
Lead Nitrate 5 days	One-site	-5.72	1.18×10^{-6}	-1.00

Values are mean of 4-6 experiments

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

LN 48hrs – Lead Nitrate 48 hours

LN 5days – Lead Nitrate 5 days

Figure - 18

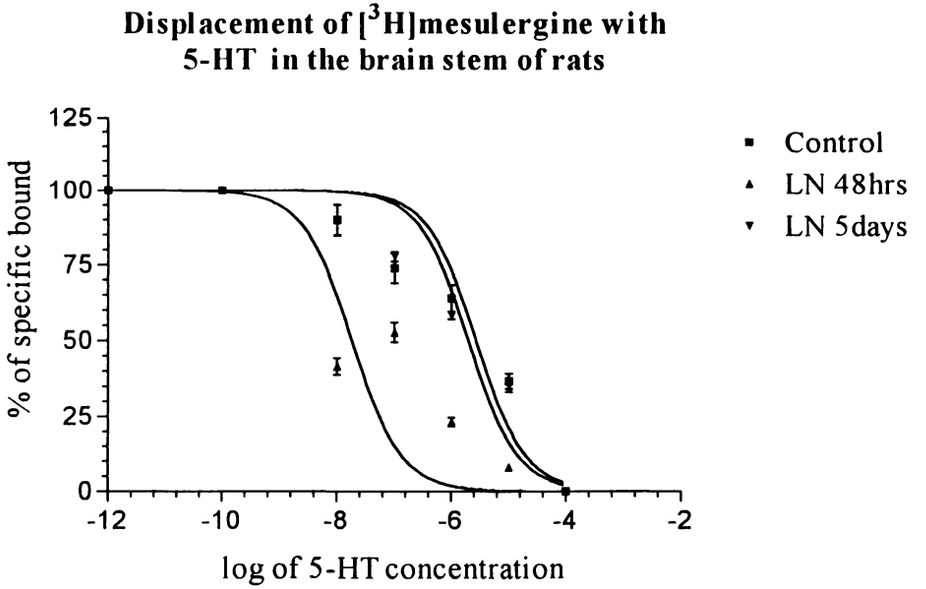
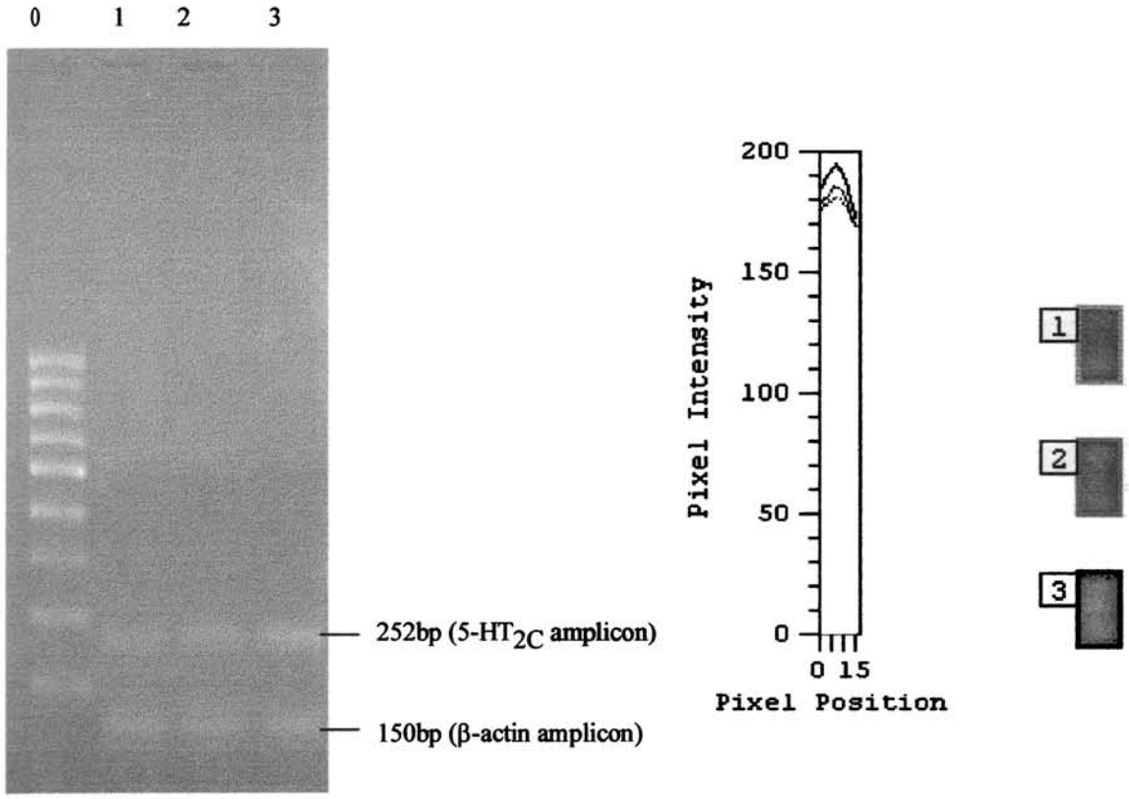


Figure - 19
RT-PCR amplification product of 5-HT_{2C} receptor mRNA from the brain stem of rats



Band properties of 5-HT_{2C} receptor mRNA RT-PCR amplicon

Lane No	Raw Volume	Area	Peak
1	45842	256	181.44
2	52671	288	185.16
3	54922	288	194.41

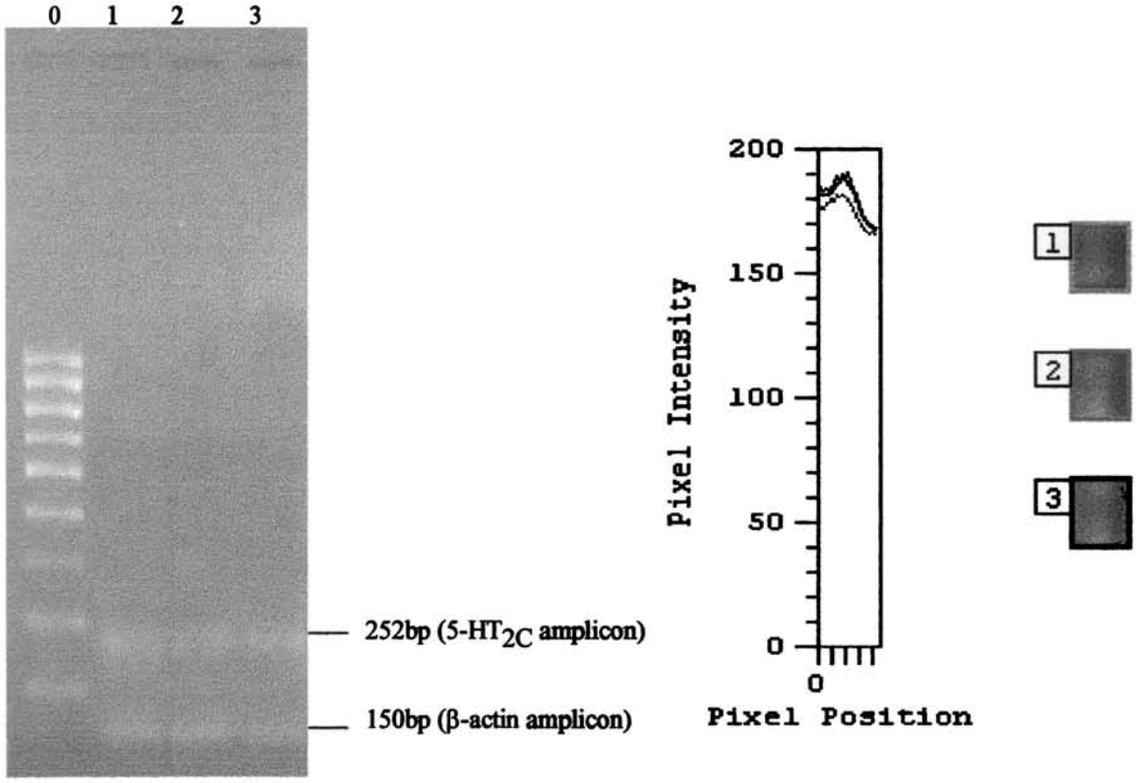
0 – 100bp ladder

1 – Control

2 – Partially Hepatectomised

3 – *N*-nitrosodiethylamine Treated

Figure - 20
RT-PCR amplification product of 5-HT_{2C} receptor mRNA from the brain stem of rats



Band properties of 5-HT_{2C} receptor mRNA RT-PCR amplicon

Lane No	Raw Volume	Area	Peak
1	45842	256	181.44
2	48793	256	190.54
3	48130	256	187.96

0 – 100bp ladder

1 – Control

2 – Lead Nitrate 48 hours

3 – Lead Nitrate 5 days

Figure-21

Scatchard analysis of high affinity [^{3}H]8-OH DPAT receptor binding against 5-HT in the cerebral cortex of rats

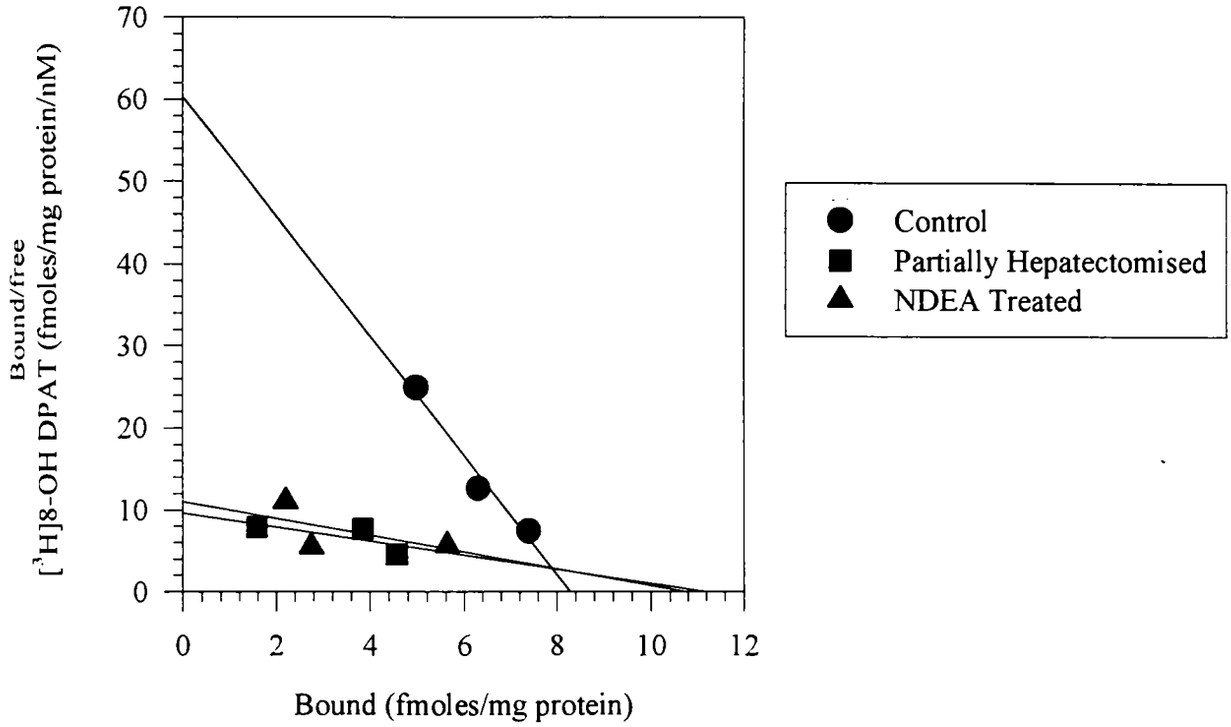


Table-14

[^{3}H]8-OHDPAT high affinity receptor binding parameters in the cerebral cortex of rats

Experimental Group	B_{max} (fmoles/mg protein)	K_d (nM)
Control[¶]	8.40 ± 0.10	0.14 ± 0.03
Partially Hepatectomised	9.10 ± 0.15*	0.93 ± 0.09***
NDEA Treated	10.70 ± 0.10***	0.97 ± 0.01***

***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.

Figure-22

Scatchard analysis of high affinity [^{3}H]8-OH DPAT receptor binding against 5-HT in the cerebral cortex of rats

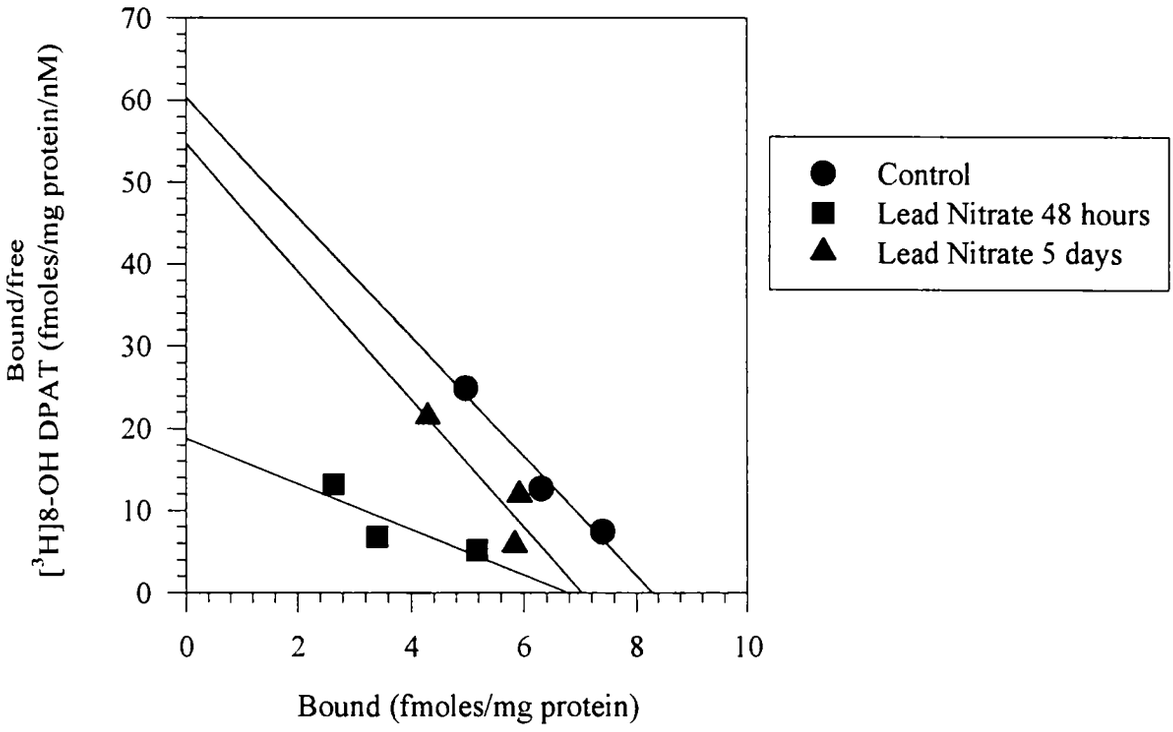


Table-15

[^{3}H]8-OH DPAT high affinity receptor binding parameters in the cerebral cortex of rats

Experimental Group	B_{max} (fmoles/mg protein)	K_d (nM)
Control[¶]	8.40 ± 0.10	0.14 ± 0.03
Lead Nitrate 48 hours	6.85 ± 0.15**	0.38 ± 0.07*
Lead Nitrate 5 days	7.10 ± 0.30*	0.13 ± 0.01

*p<0.05, **p<0.001, 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 low affinity [^{3}H]8-OH DPAT receptor binding against 5-HT in the cerebral cortex of rats

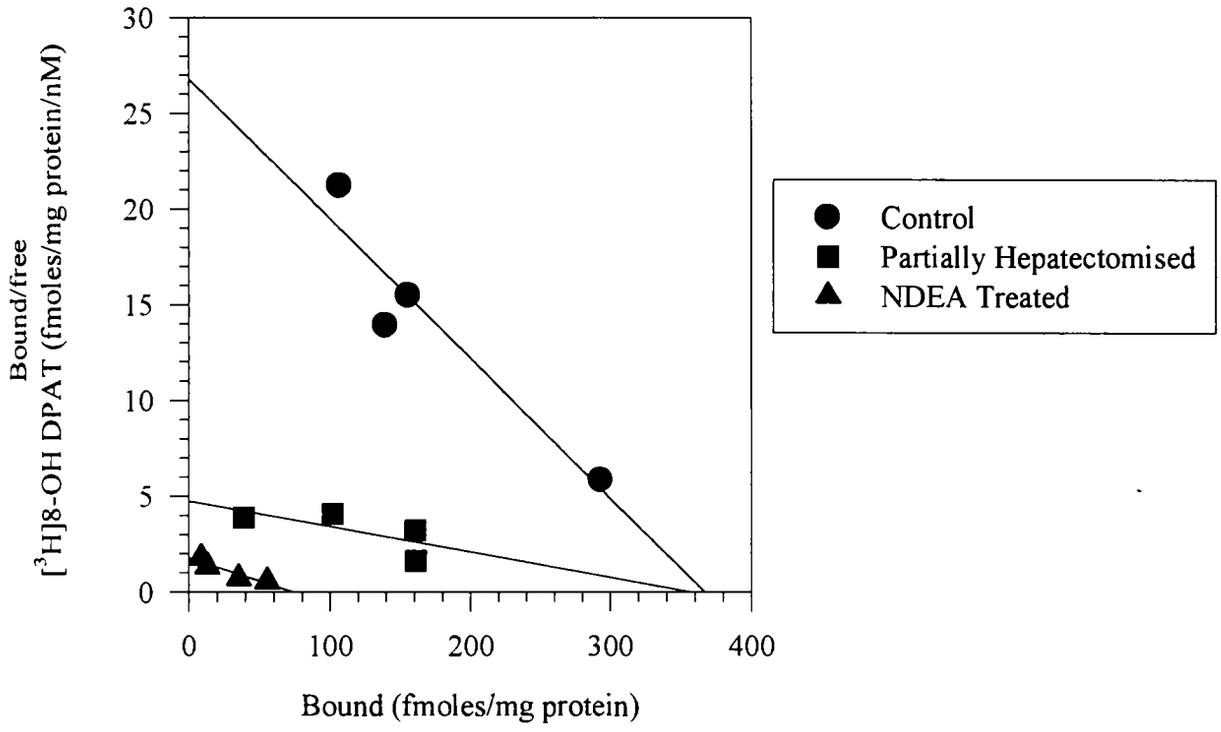


Table-16

[^{3}H]8-OH DPAT low affinity receptor binding parameters in the cerebral cortex of rats

Experimental Group	B_{max} (fmoles/mg protein)	K_d (nM)
Control[¶]	366.67 ± 3.33	13.67 ± 1.17
Partially Hepatectomised	361.5 ± 23.50	77.73± 5.85***
NDEA Treated	72.5 ± 4.99***	41.19 ± 0.81**

***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.

Figure-24

Scatchard analysis of low affinity [^{3}H]8-OH DPAT receptor binding against 5-HT in the cerebral cortex of rats

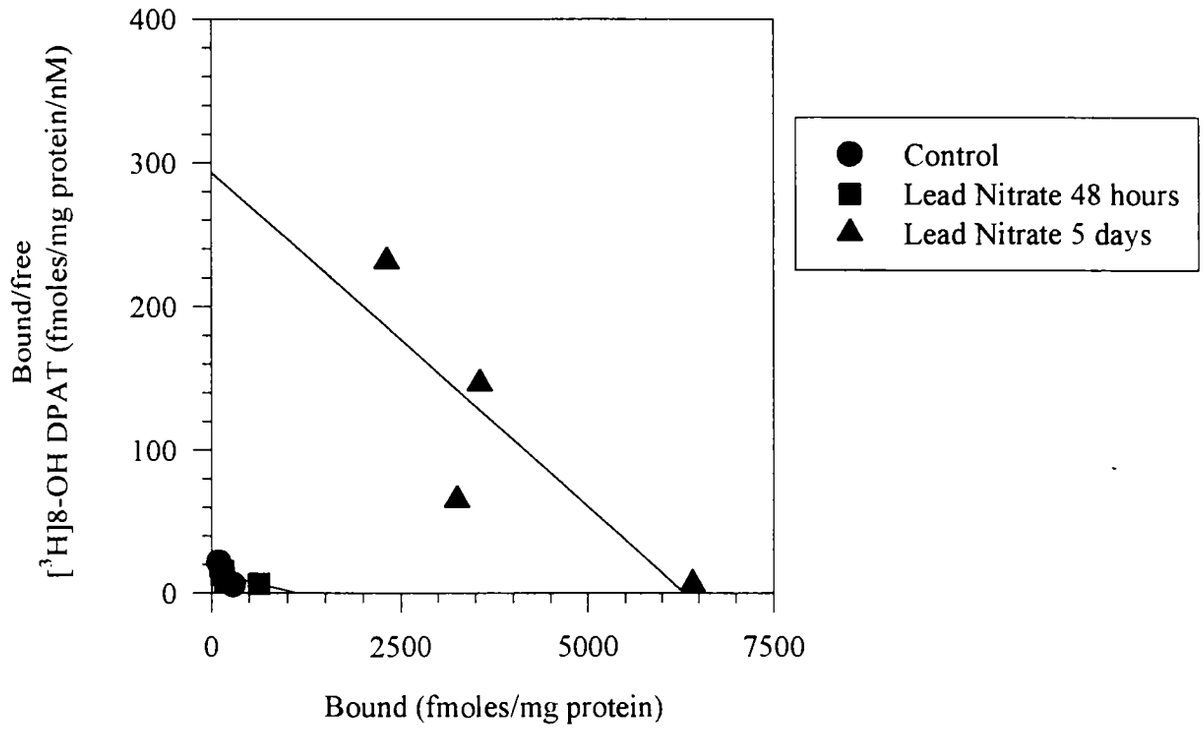


Table-17

[^{3}H]8-OH DPAT low affinity receptor binding parameters in the cerebral cortex of rats

Experimental Group	B_{max} (fmoles/mg protein)	K_d (nM)
Control[¶]	366.67 ± 3.33	13.67 ± 1.17
Lead Nitrate 48 hours	1100.00 ± 20.00**	77.94 ± 5.94***
Lead Nitrate 5 days	6400.00 ± 200.00***	21.56 ± 5.11

***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]8-OH DPAT against 5-HT in the cerebral cortex of rats

Experimental Group	Best-fit model	log (EC_{50})-1	log (EC_{50})-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Control[¶]	Two-site	-9.21	-6.32	7.65×10^{-11}	5.84×10^{-8}	-0.41
Partially Hepatectomised	Two-site	-8.06	-5.08	1.06×10^{-10}	1.03×10^{-6}	-0.69
N-Nitrosodiethylamine Treated	Two-site	-8.68	-4.72	2.54×10^{-10}	2.37×10^{-6}	-0.30

Values are mean of 4-6 separate experiments

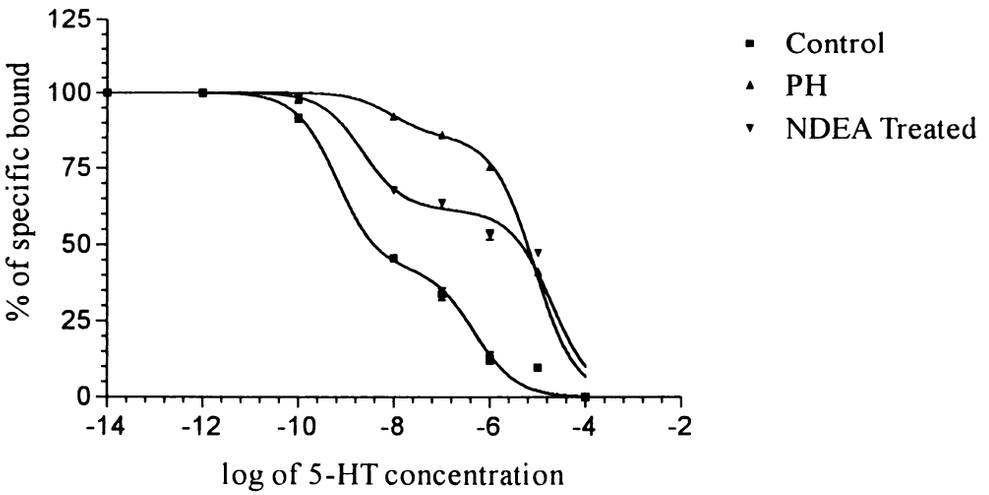
Data were fitted with an iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki - The affinity of the receptor for the competing drug. The affinity for the first and second site of the competing drug are designated as $Ki_{(H)}$ (for high affinity) and $Ki_{(L)}$ (for low affinity).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

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

PH – Partially Hepatectomised
NDEA Treated – *N*-Nitrosodiethylamine Treated

Figure - 25

Displacement of [^{3}H]8-OH DPAT with 5-HT in the cerebral cortex of rats



Binding parameters of [^{3}H]8-OH DPAT against 5-HT in the cerebral cortex of rats

Experimental Group	Best-fit model	log (EC_{50})-1	log (EC_{50})-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Control¶	Two-site	-9.21	-6.32	7.65×10^{-11}	5.84×10^{-8}	-0.41
Lead Nitrate 48 hours	Two-site	-9.16	-6.80	8.53×10^{-11}	1.93×10^{-8}	-0.52
Lead Nitrate 5 days	Two-site	-9.39	-6.59	5.03×10^{-11}	3.16×10^{-8}	-1.22

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.

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

LN 48hrs – Lead Nitrate 48 hours
LN 5days – Lead Nitrate 5 days

Figure - 26

Displacement of [^{3}H]8-OHDPAT with 5-HT in the cerebral cortex of rats

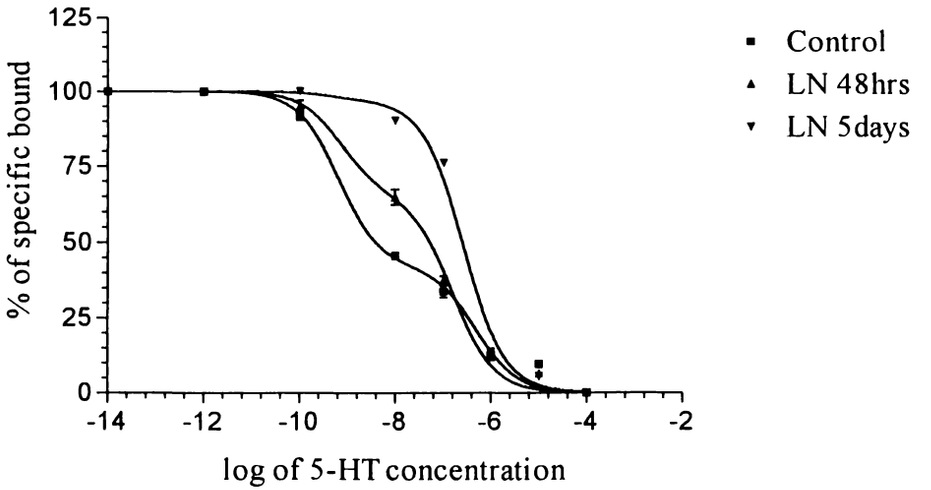
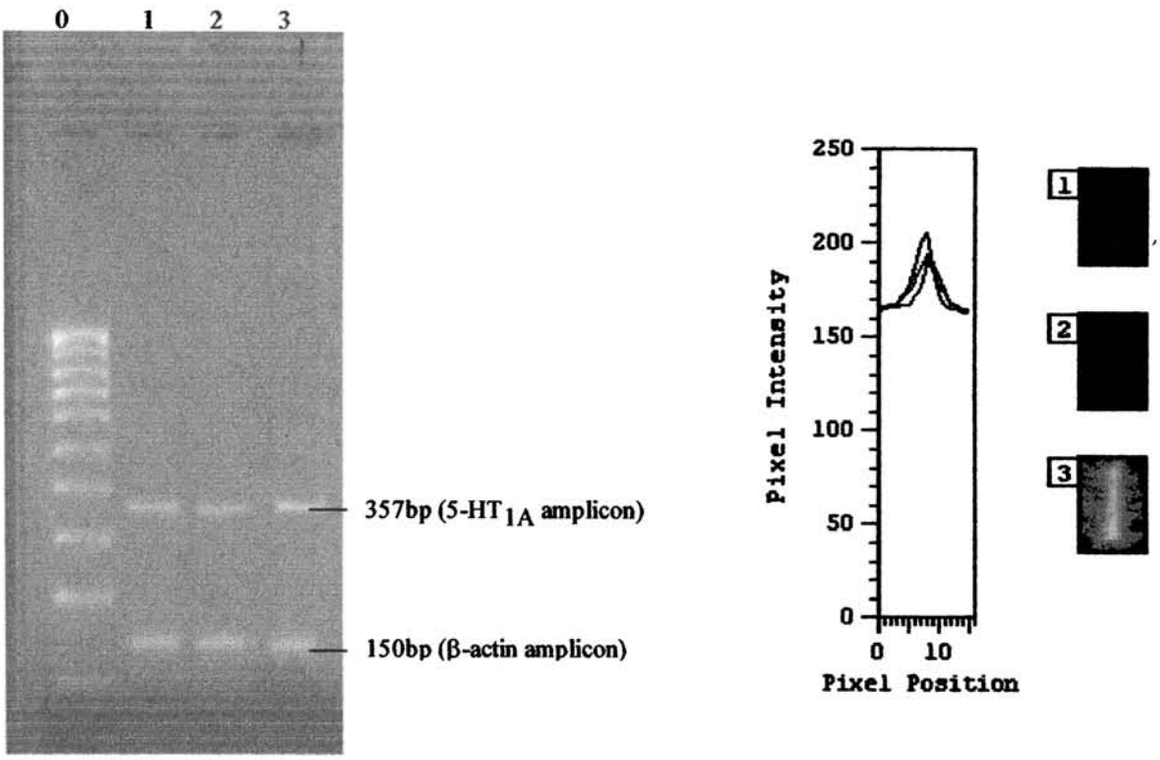


Figure - 27
RT-PCR amplification product of 5-HT_{1A} receptor mRNA from the cerebral cortex of rats



Band properties of 5-HT_{1A} receptor mRNA RT-PCR amplicon

Lane No	Raw Volume	Area	Peak
1	34498	192	192.71
2	33868	192	189.38
3	35104	192	204.96

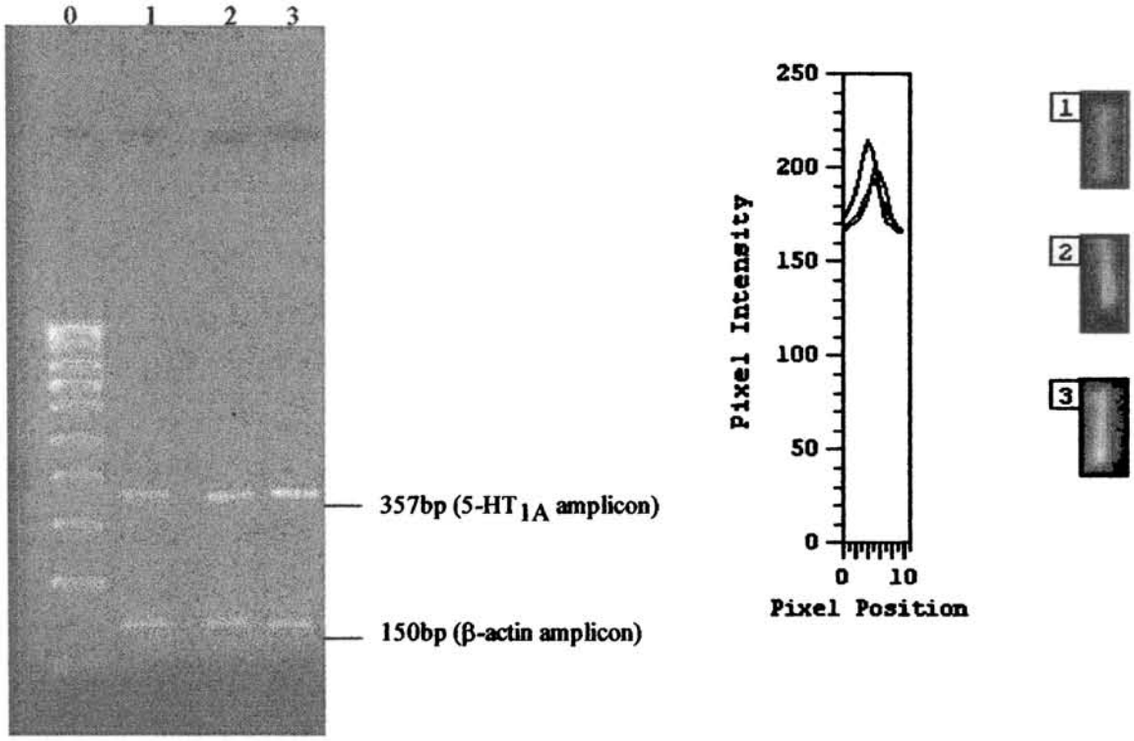
0 – 100bp ladder

1 – Control

2 – Partially Hepatectomised

3 – *N*-Nitrosodiethylamine Treated

Figure - 28
RT-PCR amplification product of 5-HT_{1A} receptor mRNA from cerebral cortex of rats



Band properties of 5-HT_{1A} receptor mRNA RT-PCR amplicon

Lane No	Raw Volume	Area	Peak
1	34498	192	192.71
2	35180	192	189.38
3	49297	192	204.96

0 – 100bp ladder

1 – Control

2 – Lead Nitrate 48 hours

3 – Lead Nitrate 5 days

Figure-29

Scatchard analysis of [^{3}H]Mesulergine binding against 5-HT in the cerebral cortex of rats

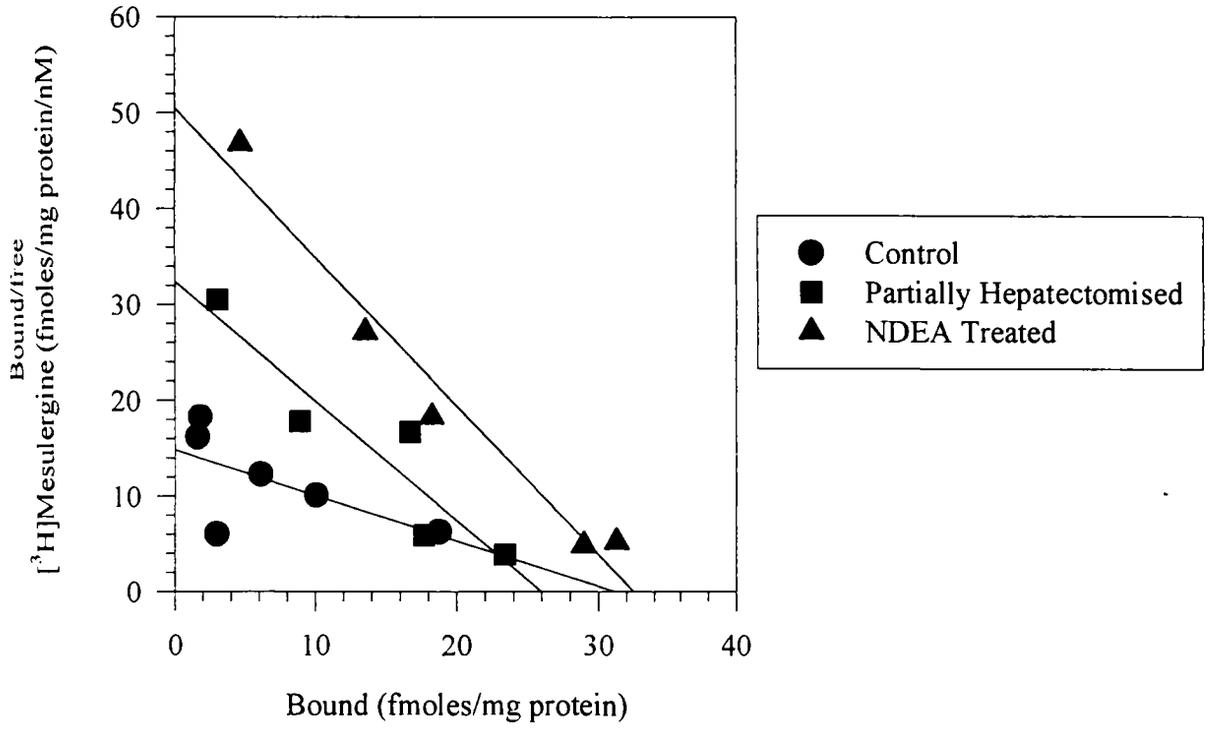


Table-20

[^{3}H]Mesulergine binding parameters in the cerebral cortex of rats

Experimental Group	B_{max} (fmoles/mg protein)	K_d (nM)
Control[¶]	27.33 ± 0.57	1.55 ± 0.08
Partially Hepatectomised	26.13 ± 1.18	0.77 ± 0.03***
NDEA Treated	33.10 ± 0.96***	0.66 ± 0.02***

***p<0.001 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-30

Scatchard analysis of [^{3}H]Mesulergine binding against 5-HT in the cerebral cortex of rats

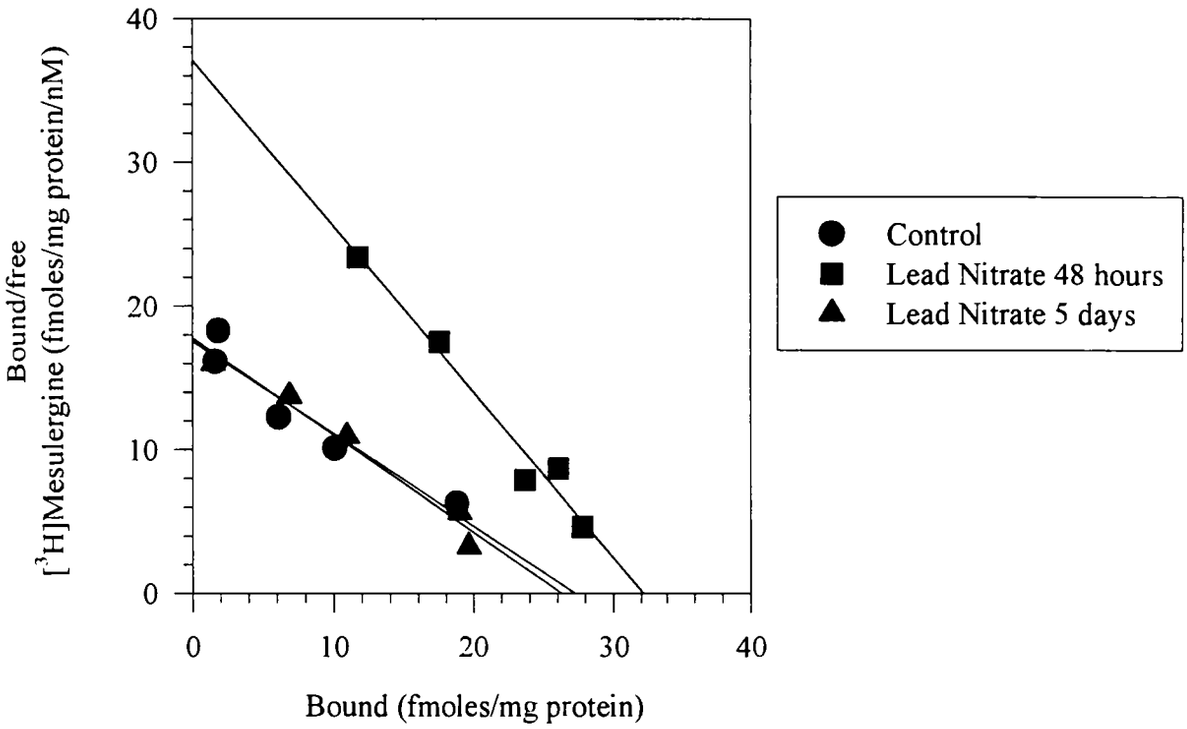


Table-21

[^{3}H]Mesulergine binding parameters in the cerebral cortex of rats

Experimental Group	B_{max} (fmoles/mg protein)	K_d (nM)
Control[¶]	27.33 ± 0.57	1.55 ± 0.08
Lead Nitrate 48 hours	31.63 ± 0.75**	0.84 ± 0.01***
Lead Nitrate 5 days	26.00 ± 0.71	1.54 ± 0.11

***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]mesulergine in the cerebral cortex of rats

Experimental Group	Best-fit model	Log (EC_{50})	Ki	Hill slope
Control	One-site	-5.98	6.43×10^{-7}	-1.00
Partially Hepatectomised	One-site	-6.80	9.75×10^{-8}	-1.00
NDEA Treated	One-site	-6.21	3.73×10^{-7}	-1.00

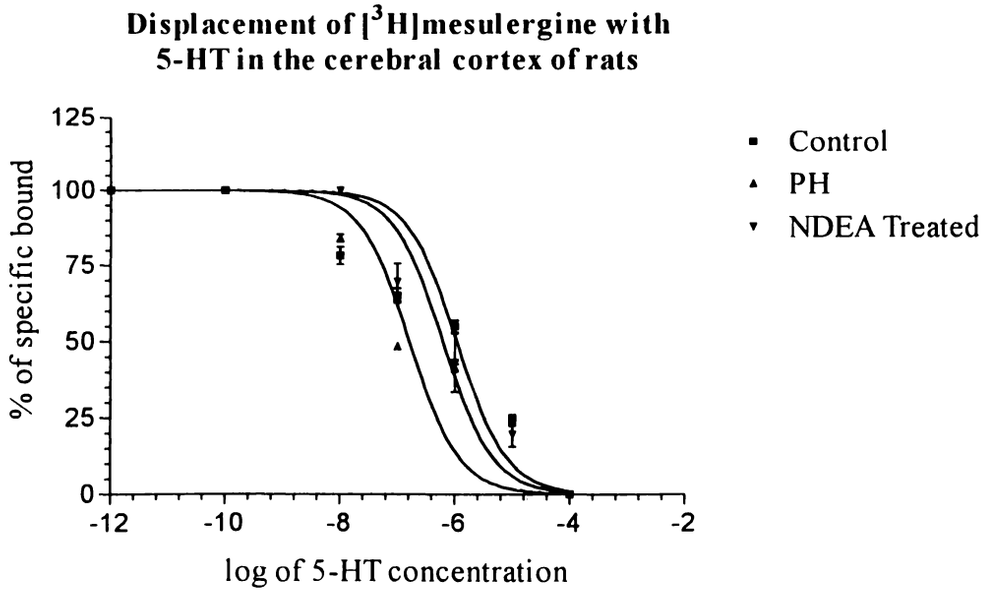
Values are mean of 4-6 experiments

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

PH – Partially Hepatectomised

NDEA – *N*-Nitrosodiethylamine Treated

Figure - 31



Binding parameters of [^{3}H]mesulergine in the cerebral cortex of rats

Experimental Group	Best-fit model	Log (EC_{50})	Ki	Hill slope
Control	One-site	-5.98	6.43×10^{-7}	-1.00
Lead Nitrate 48 hours	One-site	-7.15	4.28×10^{-8}	-1.00
Lead Nitrate 5 days	One-site	-6.02	5.84×10^{-7}	-1.00

Values are mean of 4-6 experiments

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

LN 48hrs – Lead Nitrate 48 hours

LN 5days – Lead Nitrate 5 days

Figure - 32

Displacement of [^{3}H]mesulergine with 5-HT in the cerebral cortex of rats

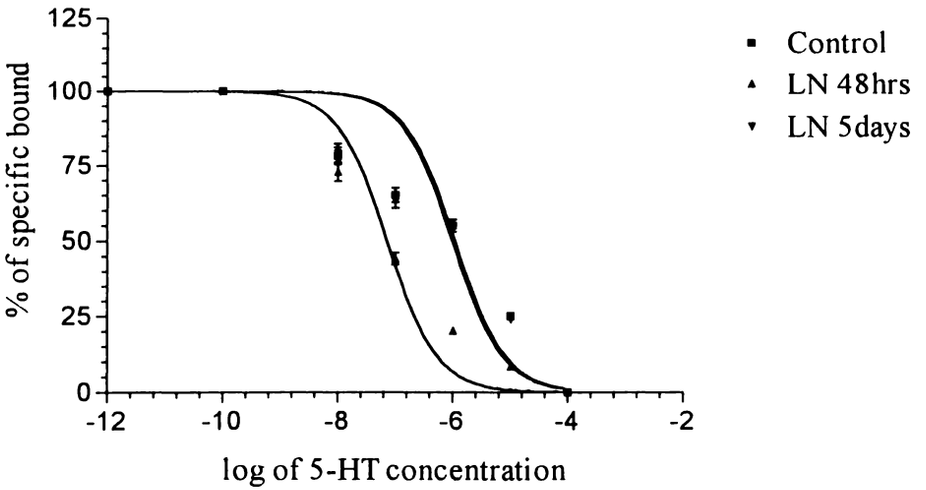
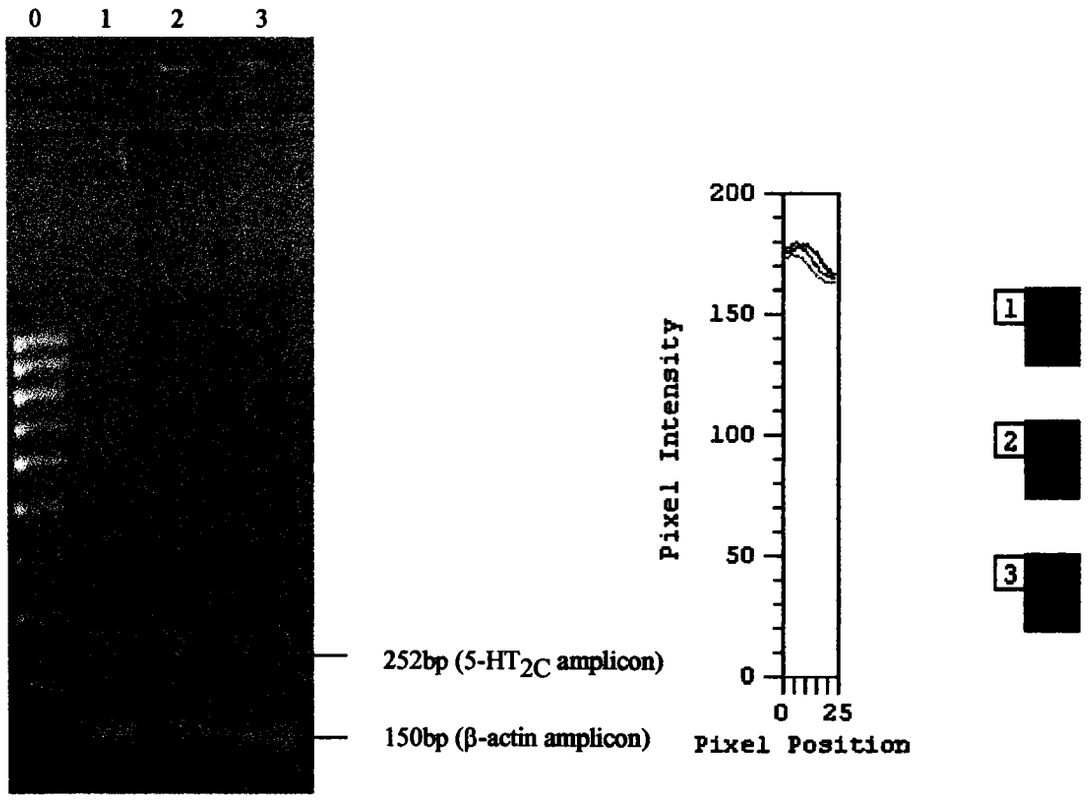


Figure - 33
RT-PCR amplification product of 5-HT_{2C} receptor mRNA from the cerebral cortex of rats



Band properties of 5-HT_{2C} receptor mRNA RT-PCR amplicon

Lane No	Raw Volume	Area	Peak
1	59855	352	173.56
2	78183	448	179.63
3	84242	480	178.91

0 – 100bp ladder

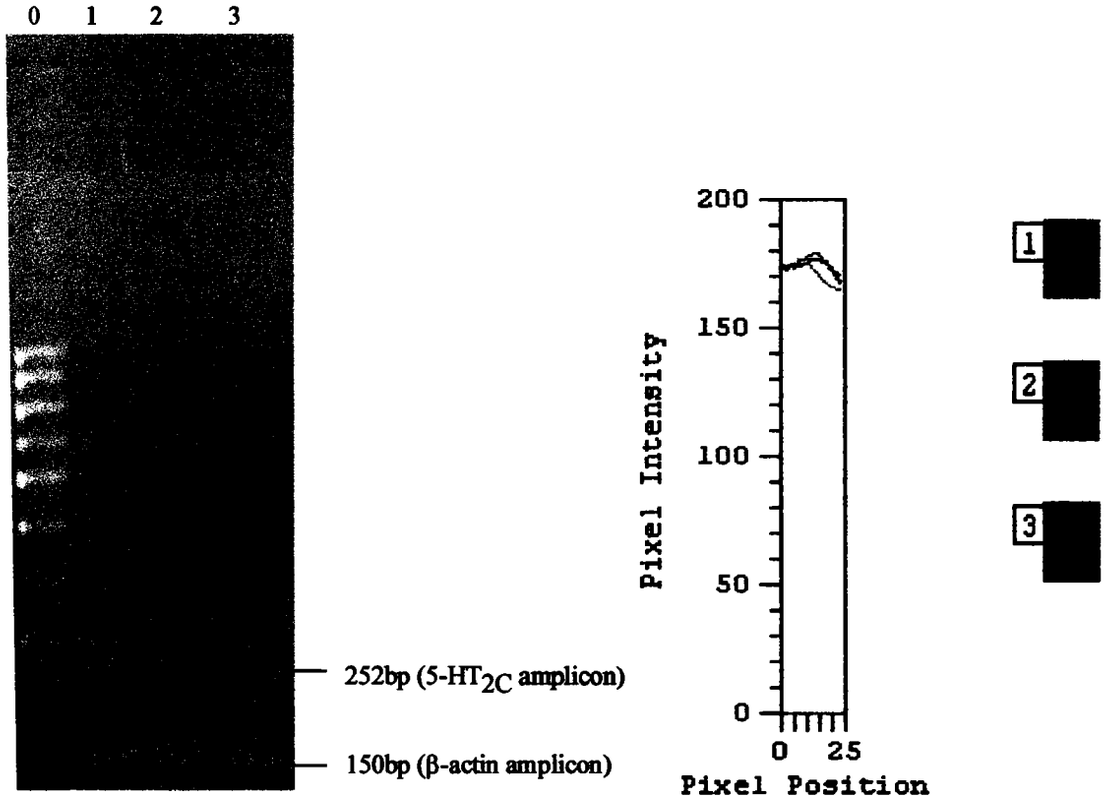
1 – Control

2 – Partially Hepatectomised

3 – *N*-nitrosodiethylamine Treated

Figure - 34

RT-PCR amplification product of 5-HT_{2C} receptor mRNA from the cerebral cortex of rats



Band properties of 5-HT_{2C} receptor mRNA RT-PCR amplicon

Lane No	Raw Volume	Area	Peak
1	59855	352	173.56
2	61744	420	178.75
3	56186	392	176.11

0 - 100bp ladder

1 - Control

2 - Lead Nitrate 48 hours

3 - Lead Nitrate 5 days

Figure-35

Scatchard analysis of high affinity [^{3}H]8-OH DPAT receptor binding against 5-HT in the hypothalamus of rats

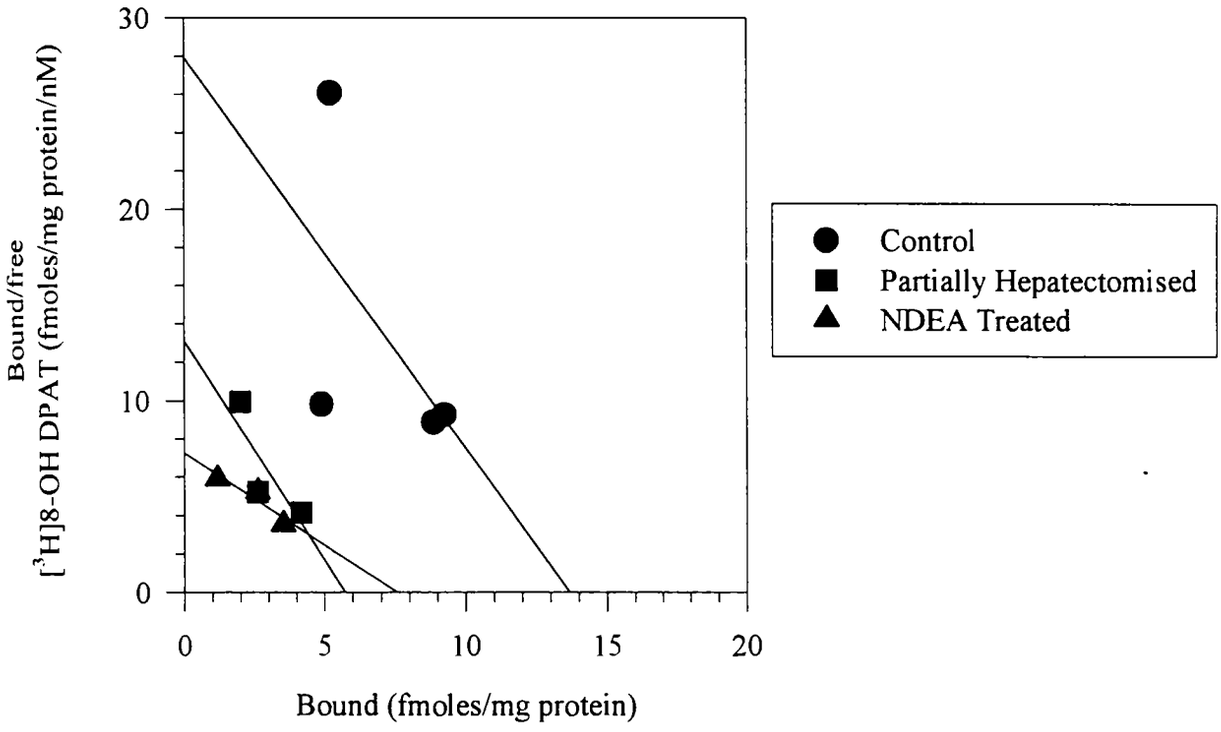


Table-24

[^{3}H]8-OH DPAT high affinity receptor binding parameters in the hypothalamus of rats

Experimental Group	B_{max} (fmoles/mg protein)	K_d (nM)
Control[¶]	13.93 ± 1.95	0.50 ± 0.16
Partially Hepatectomised	5.80 ± 0.08**	0.43 ± 0.12
NDEA Treated	7.50 ± 1.30**	1.02 ± 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-36

Scatchard analysis of high affinity [^{3}H]8-OH DPAT receptor binding against 5-HT in the hypothalamus of rats

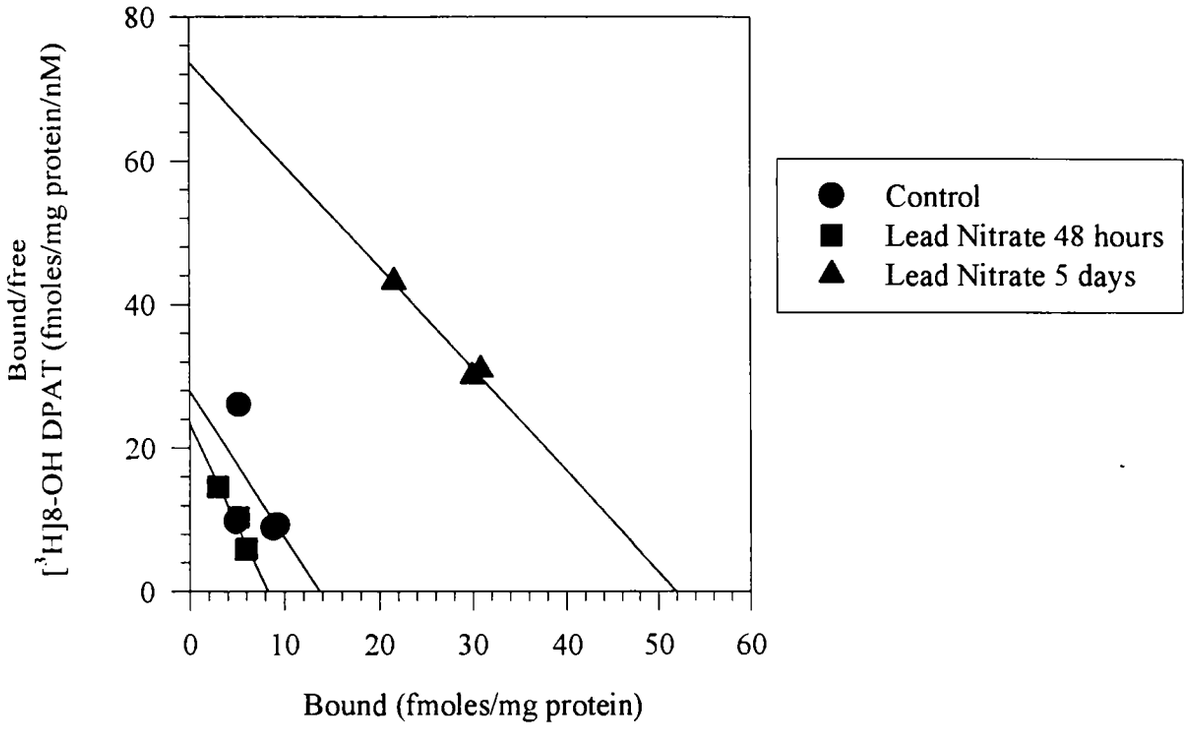


Table-25

[^{3}H]8-OH DPAT high affinity receptor binding parameters in the hypothalamus of rats

Experimental Group	B_{max} (fmoles/mg protein)	K_d (nM)
Control[¶]	13.93 ± 1.95	0.50 ± 0.16
Lead Nitrate 48 hours	8.25 ± 0.19	0.35 ± 0.07
Lead Nitrate 5 days	52.67 ± 3.70***	0.73 ± 0.16

***p<0.001 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-37

Scatchard analysis of low affinity [^{3}H]8-OH DPAT receptor binding against 5-HT in the hypothalamus of rats

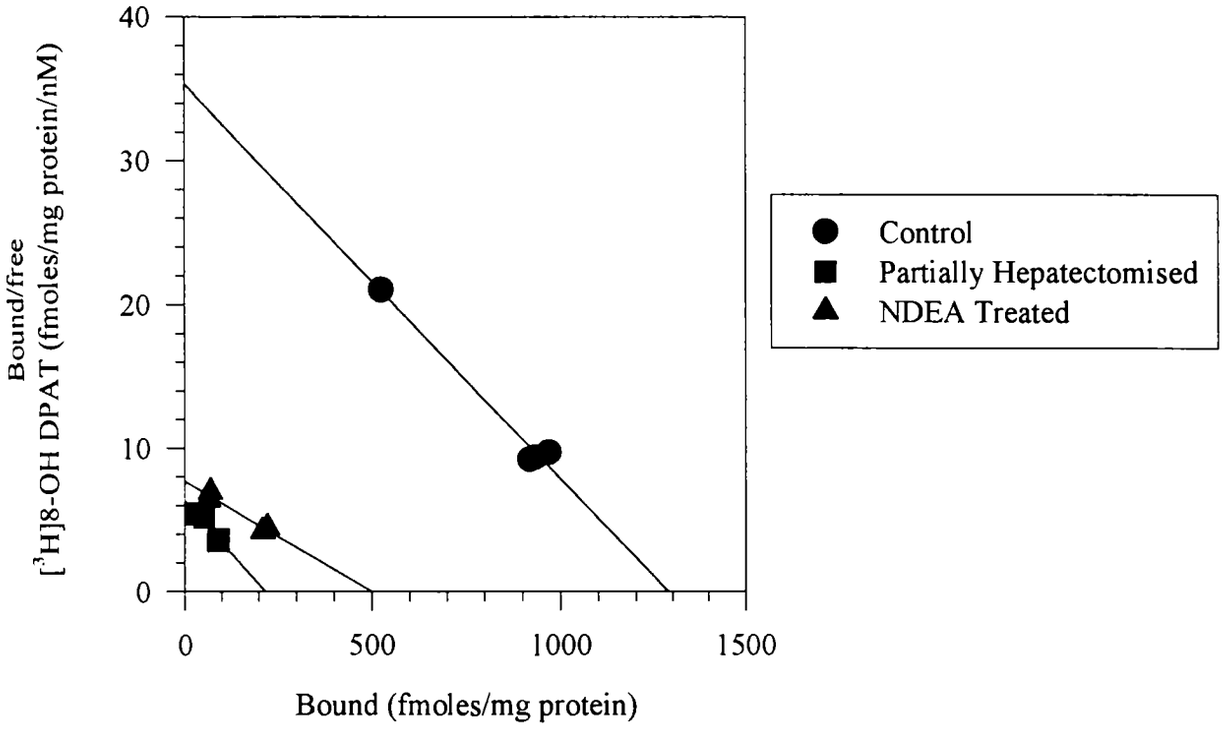


Table-26

[^{3}H]8-OH DPAT low affinity receptor binding parameters in the hypothalamus of rats

Experimental Group	B_{max} (fmoles/mg protein)	K_d (nM)
Control[¶]	1291.00 ± 36.32	36.50 ± 3.50
Partially Hepatectomised	213.33 ± 19.65***	34.75 ± 0.75
NDEA Treated	503.75 ± 46.25***	65.99 ± 0.21**

***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.

Figure-38

Scatchard analysis of low affinity [^{3}H]8-OH DPAT receptor binding against 5-HT in the hypothalamus of rats

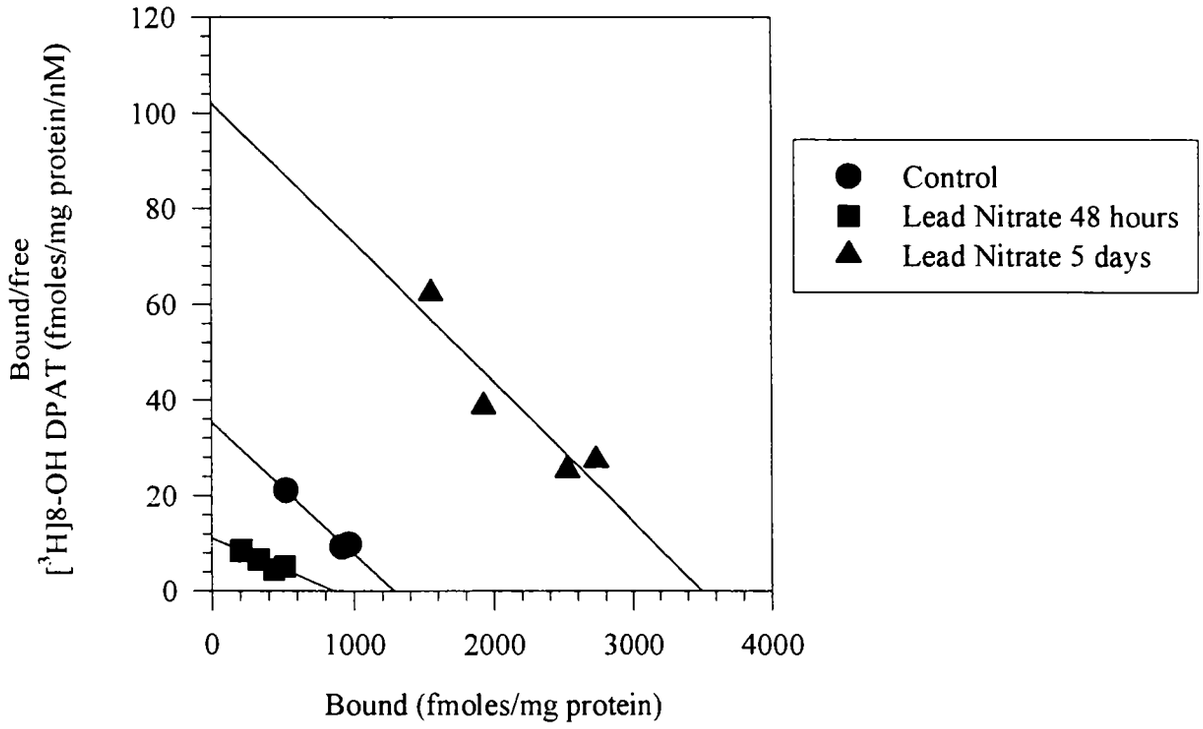


Table-27

[^{3}H]8-OHDPAT low affinity receptor binding parameters in the hypothalamus of rats

Experimental Group	B_{max} (fmoles/mg protein)	K_d (nM)
Control[¶]	1291.00 ± 36.32	36.50 ± 3.50
Lead Nitrate 48 hours	847.50 ± 27.50*	76.31 ± 3.39***
Lead Nitrate 5 days	3500.00 ± 200.00***	33.85 ± 3.37

***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]8-OH DPAT against 5-HT in the hypothalamus of rats

Experimental Group	Best-fit model	log (EC_{50})-1	log (EC_{50})-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Control[¶]	Two-site	-10.08	-4.85	2.75×10^{-11}	4.72×10^{-6}	-0.20
Partially Hepatectomised	Two-site	-10.17	-4.93	2.26×10^{-11}	3.92×10^{-6}	-0.20
N-Nitrosodiethylamine Treated	Two-site	-7.68	-4.64	6.97×10^{-9}	7.67×10^{-6}	-0.35

Values are mean of 4-6 separate experiments

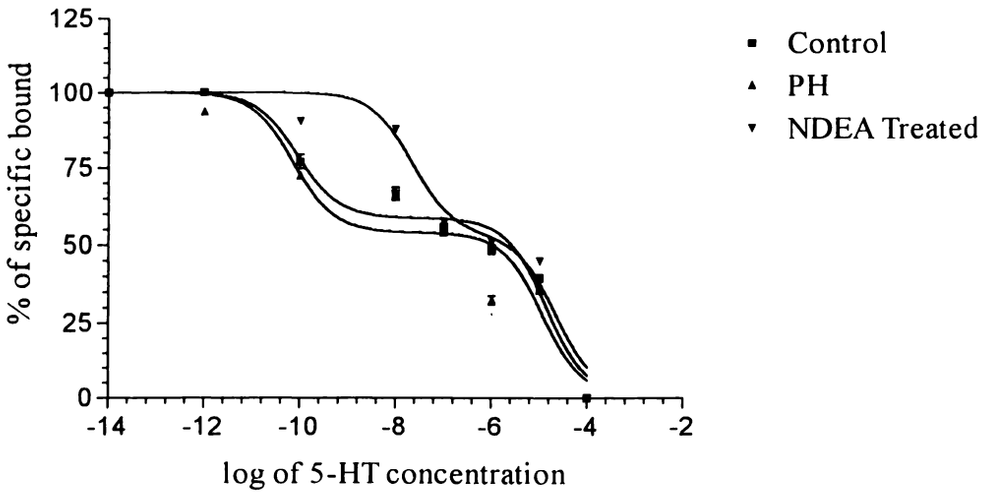
Data were fitted with an iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki - The affinity of the receptor for the competing drug. The affinity for the first and second site of the competing drug are designated as $Ki_{(H)}$ (for high affinity) and $Ki_{(L)}$ (for low affinity).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

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

PH – Partially Hepatectomised
NDEA Treated – *N*-Nitrosodiethylamine Treated

Figure - 39

Displacement of [^{3}H]8-OH DPAT with 5-HT in the hypothalamus of rats



Binding parameters of [^{3}H]8-OH DPAT against 5-HT in the hypothalamus of rats

Experimental Group	Best-fit model	log (EC_{50})-1	log (EC_{50})-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Control¶	Two-site	-10.08	-4.85	2.75×10^{-11}	4.72×10^{-6}	-0.20
Lead Nitrate 48 hours	Two-site	-9.59	-4.75	8.63×10^{-11}	5.82×10^{-6}	-0.23
Lead Nitrate 5 days	Two-site	-10.05	-4.83	2.97×10^{-11}	4.91×10^{-6}	-0.20

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.

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

LN 48hrs – Lead Nitrate 48 hours
LN-5days – Lead Nitrate 5 days

Figure - 40

Displacement of [^{3}H]8-OH DPAT with 5-HT in the hypothalamus of rats

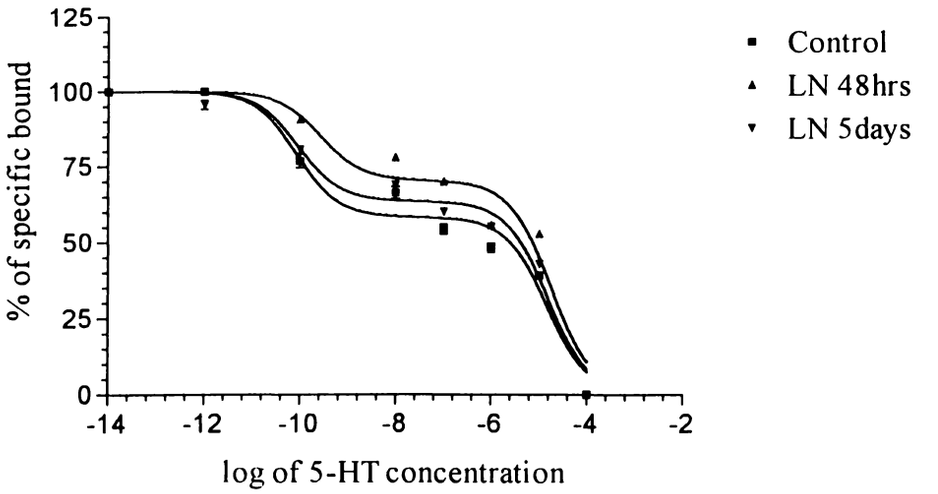
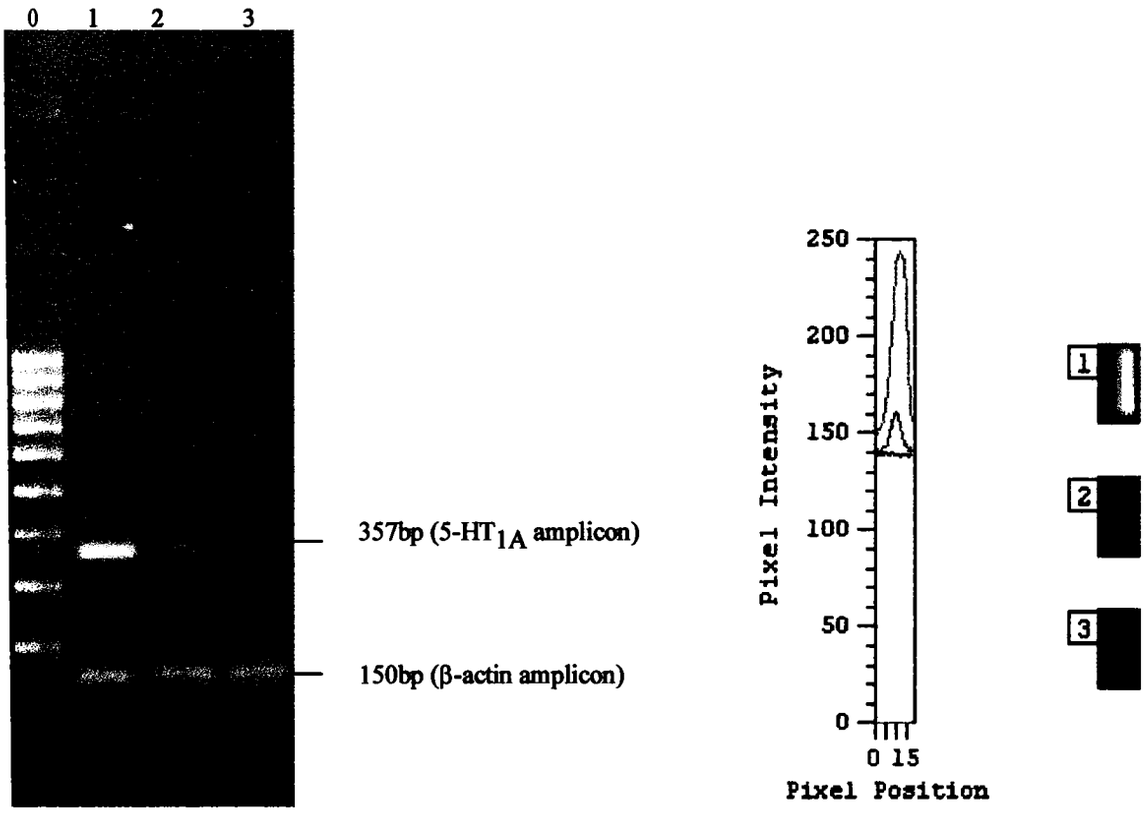


Figure - 41
RT-PCR amplification product of 5-HT_{1A} receptor mRNA from the hypothalamus of rats



Band properties of 5-HT_{1A} receptor mRNA RT-PCR amplicon

Lane No	Raw Volume	Area	Peak
1	78048	374	243.00
2	46481	306	159.65
3	-	-	-

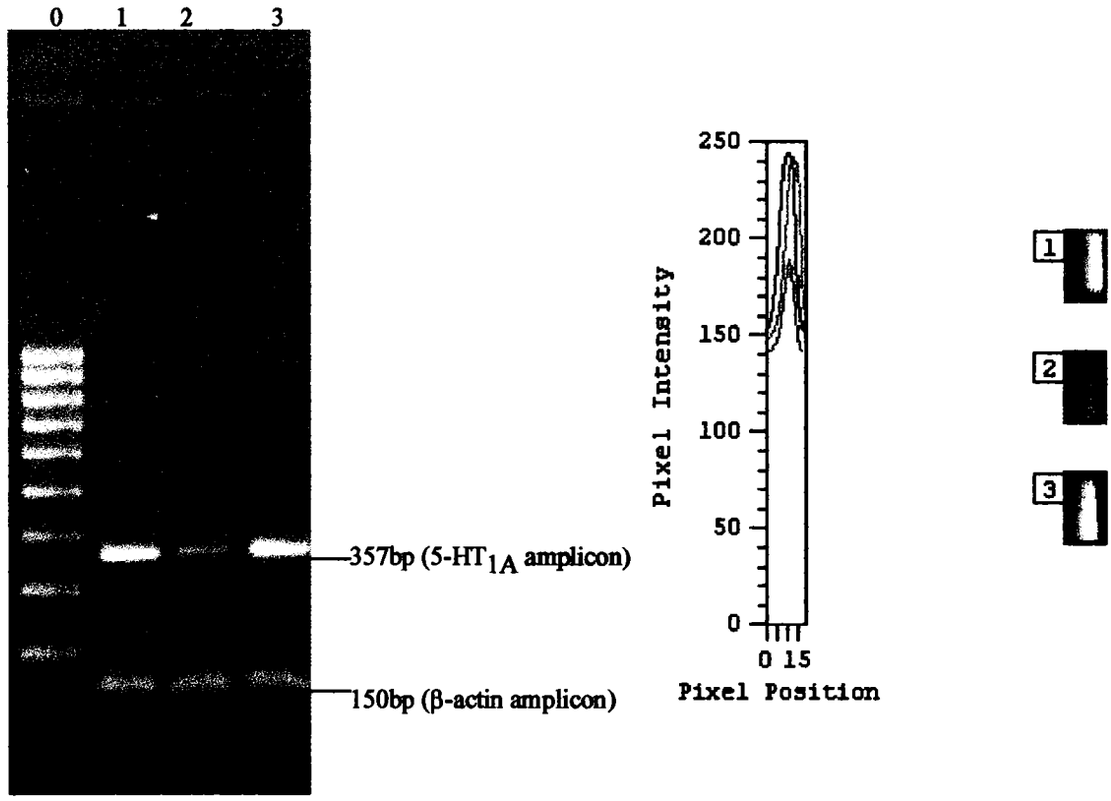
0 - 100bp ladder

1 - Control

2 - Partially Hepatectmised

3 - *N*-nitrosoditheylamine Treated

Figure - 42
RT-PCR amplification product of 5-HT_{1A} receptor mRNA from the hypothalamus of rats



Band properties of 5-HT_{1A} receptor mRNA RT-PCR amplicon

Lane No	Raw Volume	Area	Peak
1	78048	374	243.00
2	57692	374	187.53
3	93699	374	243.68

0 – 100bp ladder

1 – Control

2 – Lead Nitrate 48 hours

3 – Lead Nitrate 5 days

Figure-43

Scatchard analysis of [^{3}H]Mesulergine binding against 5-HT in the hypothalamus of rats

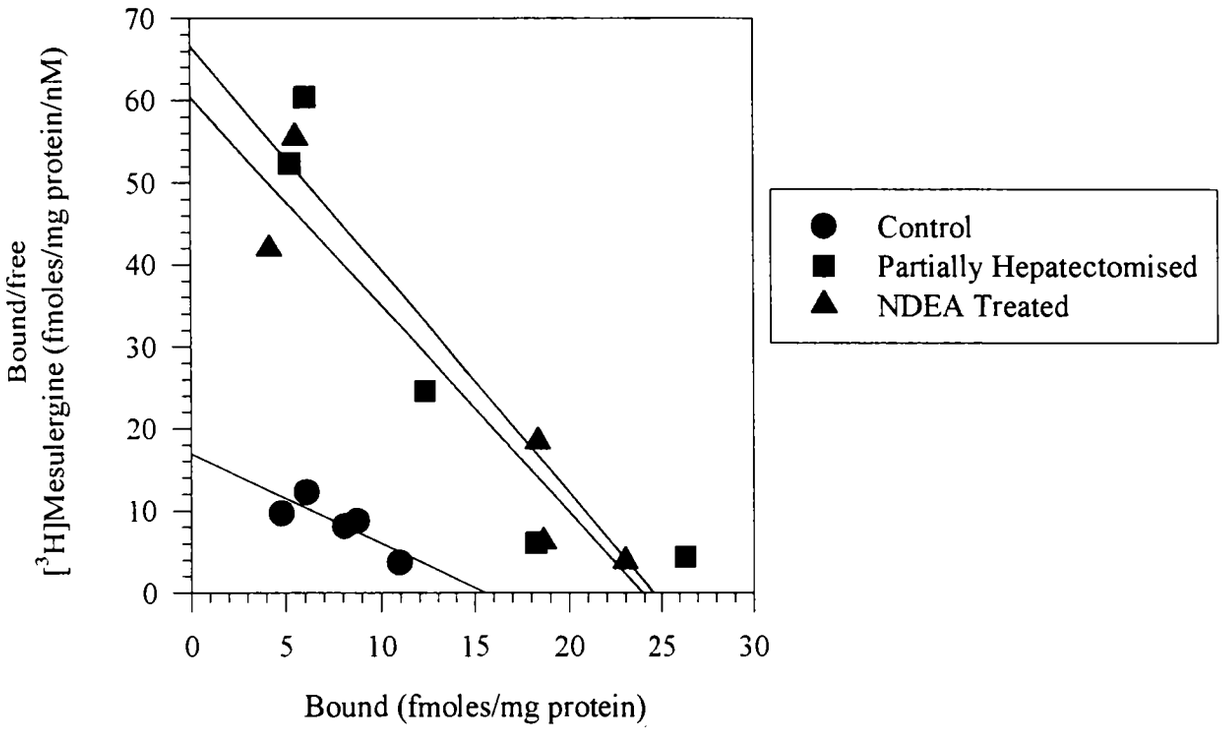


Table-30

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

Experimental Group	B_{max} (fmoles/mg protein)	K_d (nM)
Control	15.00 ± 1.50	0.87 ± 0.07
Partially Hepatectomised	25.33 ± 1.36**	0.38 ± 0.66***
NDEA Treated	24.10 ± 0.56*	0.39 ± 0.01***

***p<0.001, **p<0.01, *p<0.05 with respect to control.
Values are mean ± S.E.M of 4-6 separate experiments.

Figure-44

Scatchard analysis of [^{3}H]Mesulergine binding against 5-HT in the hypothalamus of rats

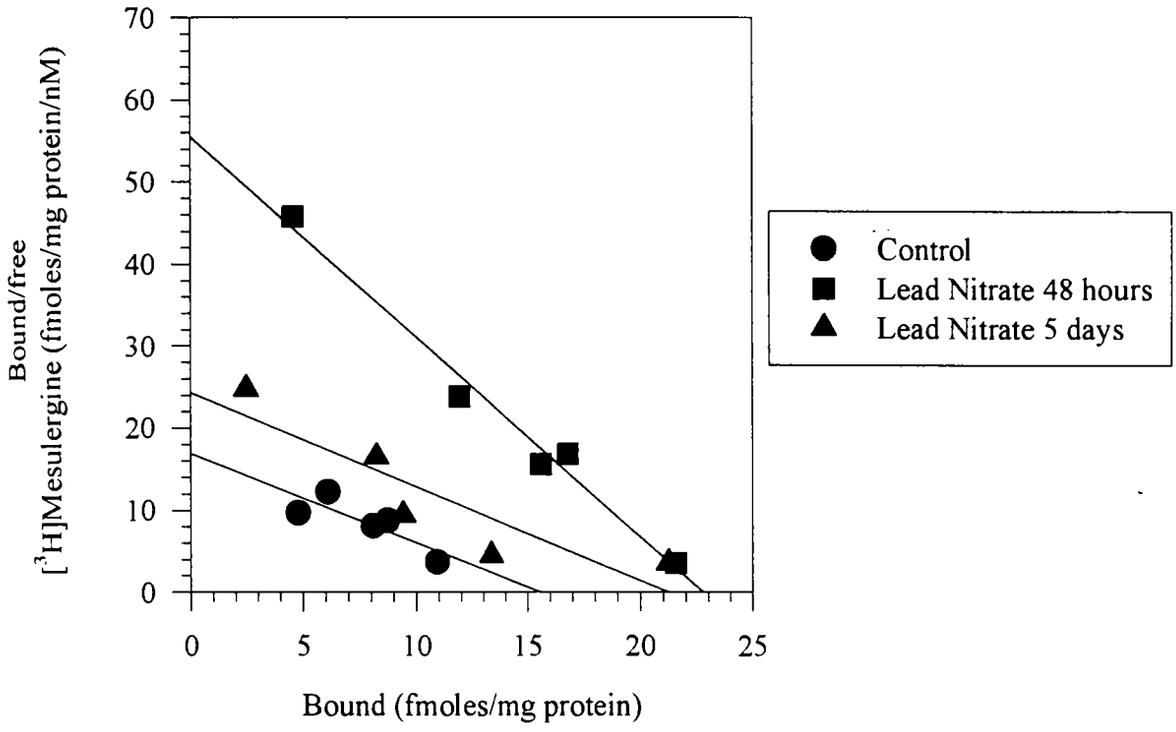


Table-31

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

Experimental Group	B_{max} (fmoles/mg protein)	K_d (nM)
Control[¶]	15.00 ± 1.50	0.87 ± 0.07
Lead Nitrate 48 hours	23.66 ± 2.24*	0.43 ± 0.04***
Lead Nitrate 5 days	20.43 ± 0.53	0.86 ± 0.06

***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]mesulergine in the hypothalamus of rats

Experimental Group	Best-fit model	Log (EC_{50})	Ki	Hill slope
Control	One-site	-5.58	1.22×10^{-6}	-1.00
Partially Hepatectomised	One-site	-5.79	7.56×10^{-7}	-1.00
NDEA Treated	One-site	-6.08	3.86×10^{-7}	-1.00

Values are mean of 4-6 experiments

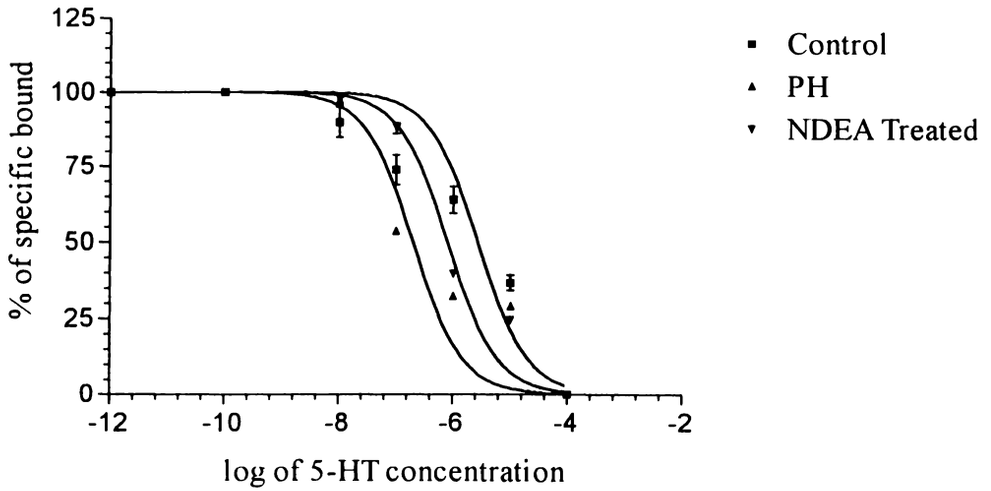
Data were fitted with an iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki – The affinity f the receptor for the competing drug. EC_{50} is the concentration of the competitor that competes for the half the specific binding

PH – Partially Hepatectomised

NDEA – *N*-Nitrosodiethylamine Treated

Figure - 45

Displacement of [^{3}H]mesulergine with 5-HT in the hypothalamus of rats



Binding parameters of [^{3}H]mesulergine in the hypothalamus of rats

Experimental Group	Best-fit model	Log (EC_{50})	Ki	Hill slope
Control	One-site	-5.58	1.22×10^{-6}	-1.00
Lead Nitrate 48 hours	One-site	-6.91	5.76×10^{-8}	-1.00
Lead Nitrate 5 days	One-site	-5.67	1.00×10^{-6}	-1.00

Values are mean of 4-6 experiments

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

LN 48hrs – Lead Nitrate 48 hours

LN 5days – Lead Nitrate 5 days

Figure - 46

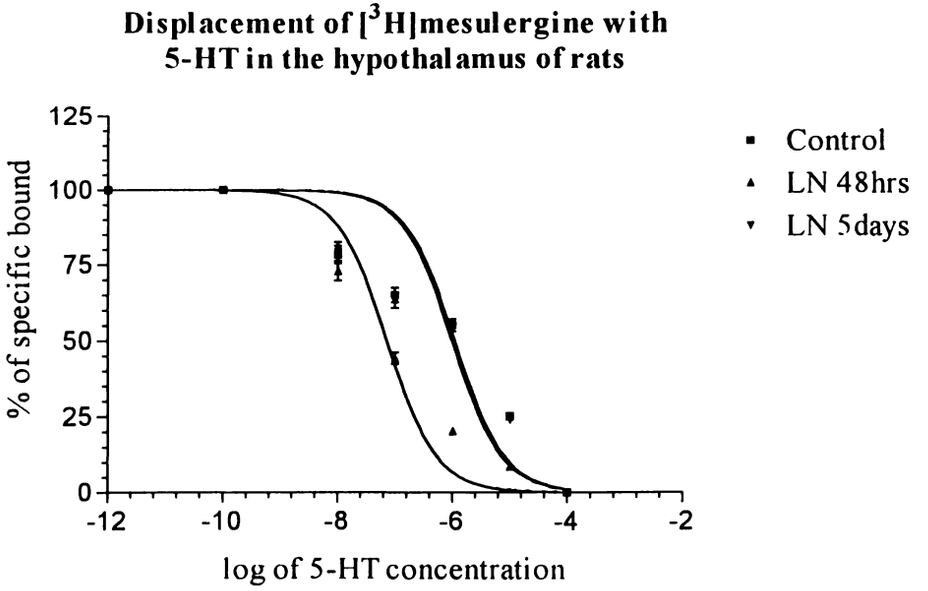
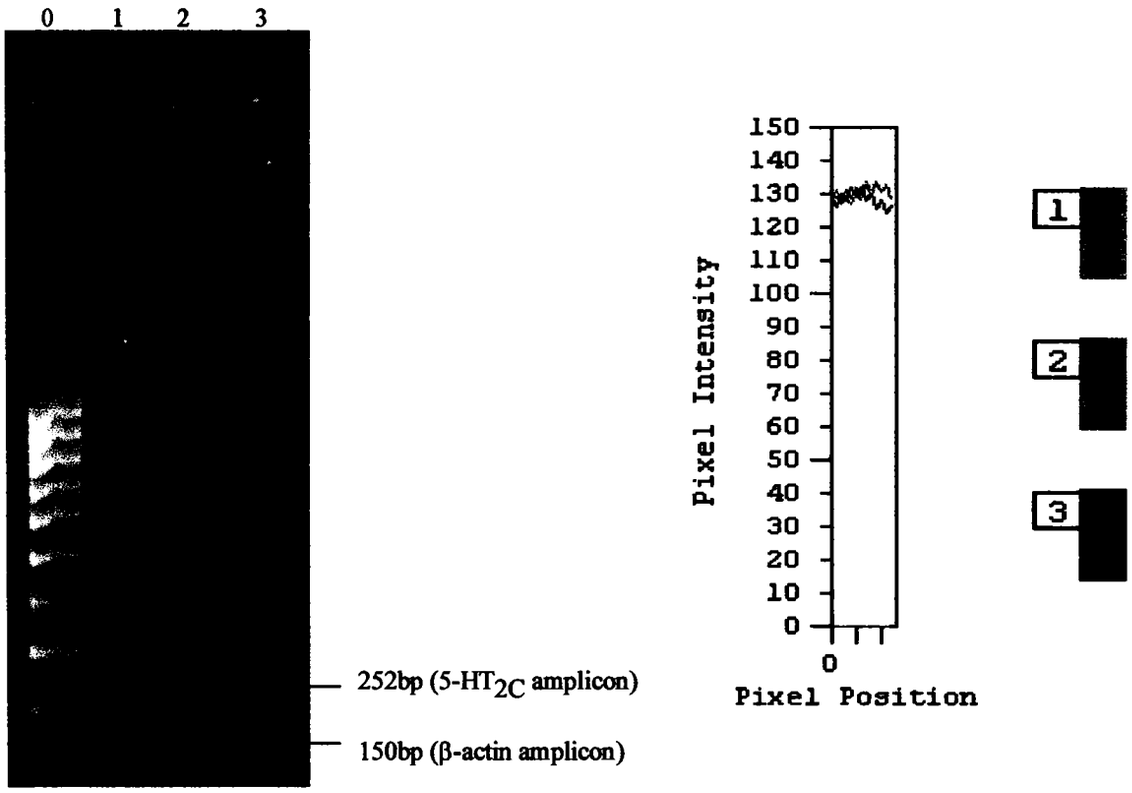


Figure - 47
RT-PCR amplification product of 5-HT_{2C} receptor mRNA from the hypothalamus of rats



Band properties of 5-HT_{2C} receptor mRNA RT-PCR amplicon

Lane No	Raw Volume	Area	Peak
1	13253	102	131.91
2	26432	207	130.97
3	30434	238	131.68

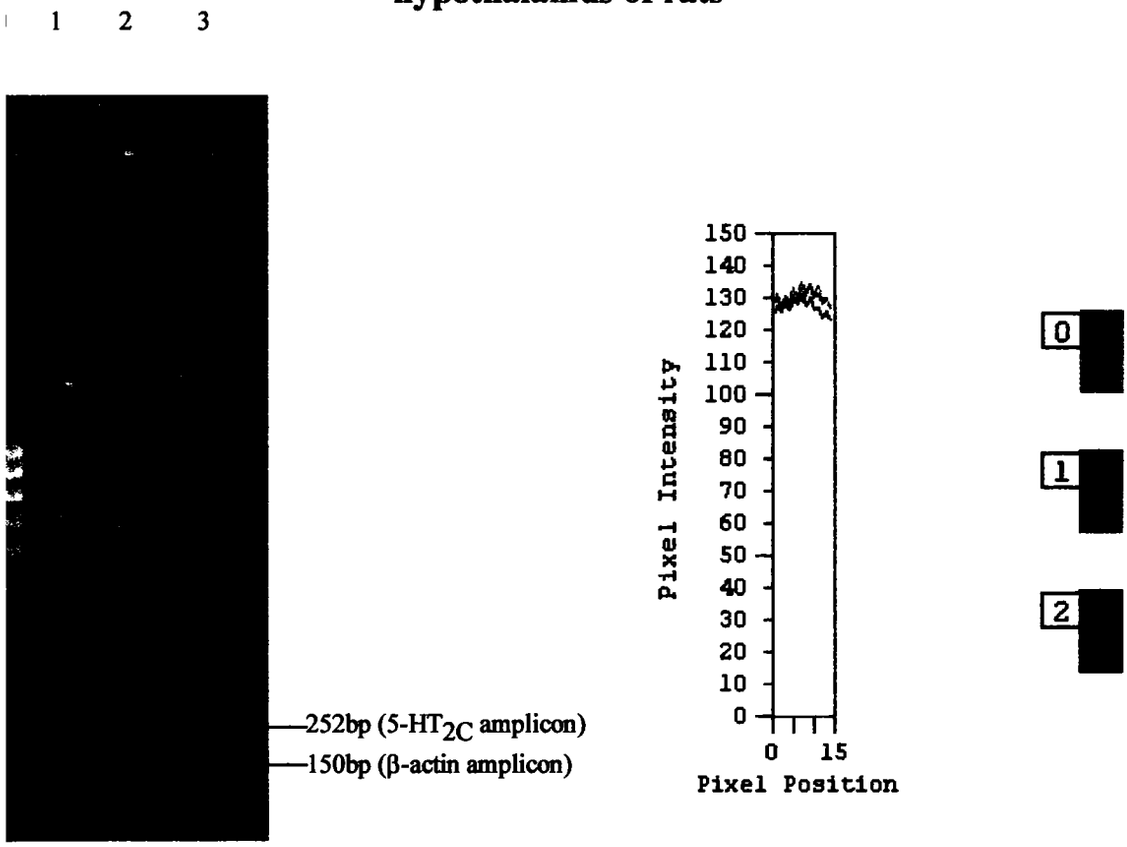
0 – 100bp ladder

1 – Control

2 –Partially Hepatectomised

3 – *N*-nitrosodiethylamine Treated

Figure - 48
RT-PCR amplification product of 5-HT_{2C} receptor mRNA from the hypothalamus of rats



Band properties of 5-HT_{2C} receptor mRNA RT-PCR amplicon

Lane No	Raw Volume	Area	Peak
1	13253	102	131.91
2	30060	102	134.91
3	12802	102	130.78

0 – 100bp ladder

1 – Control

2 –Lead Nitrate 48 hours

3 – Lead Nitrate 5 days

Figure - 49

Scatchard analysis of high affinity [^{3}H]8-OH DPAT receptor binding against 5-HT in the liver of rats

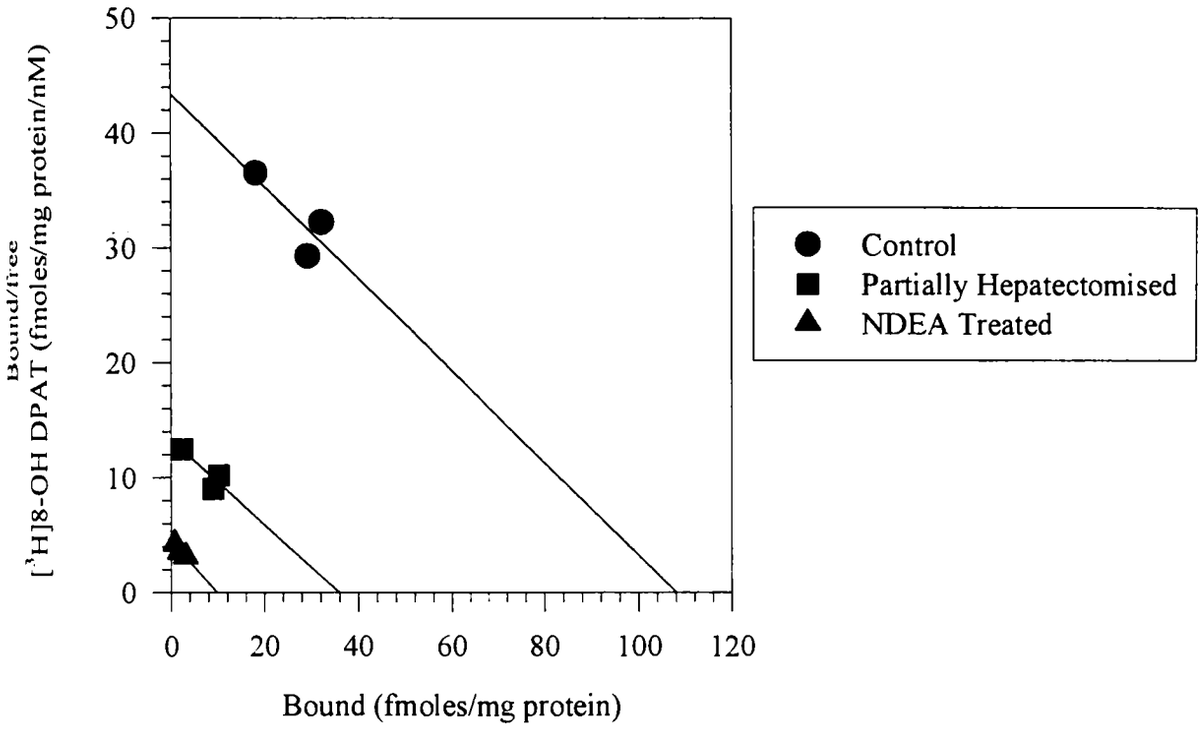


Table - 34

[^{3}H]8-OH DPAT high affinity receptor binding parameters in the liver of rats

Experimental Group	B_{max} (fmoles/mg protein)	K_d (nM)
Control[¶]	109.00 ± 4.67	2.49 ± 0.16
Partially Hepatectomised	38.30 ± 1.45***	2.92 ± 0.38
NDEA Treated	9.86 ± 0.37***	2.15 ± 0.01

***p<0.001, 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 - 50

Scatchard analysis of high affinity [^{3}H]8-OH DPAT receptor binding against 5-HT in the liver of rats

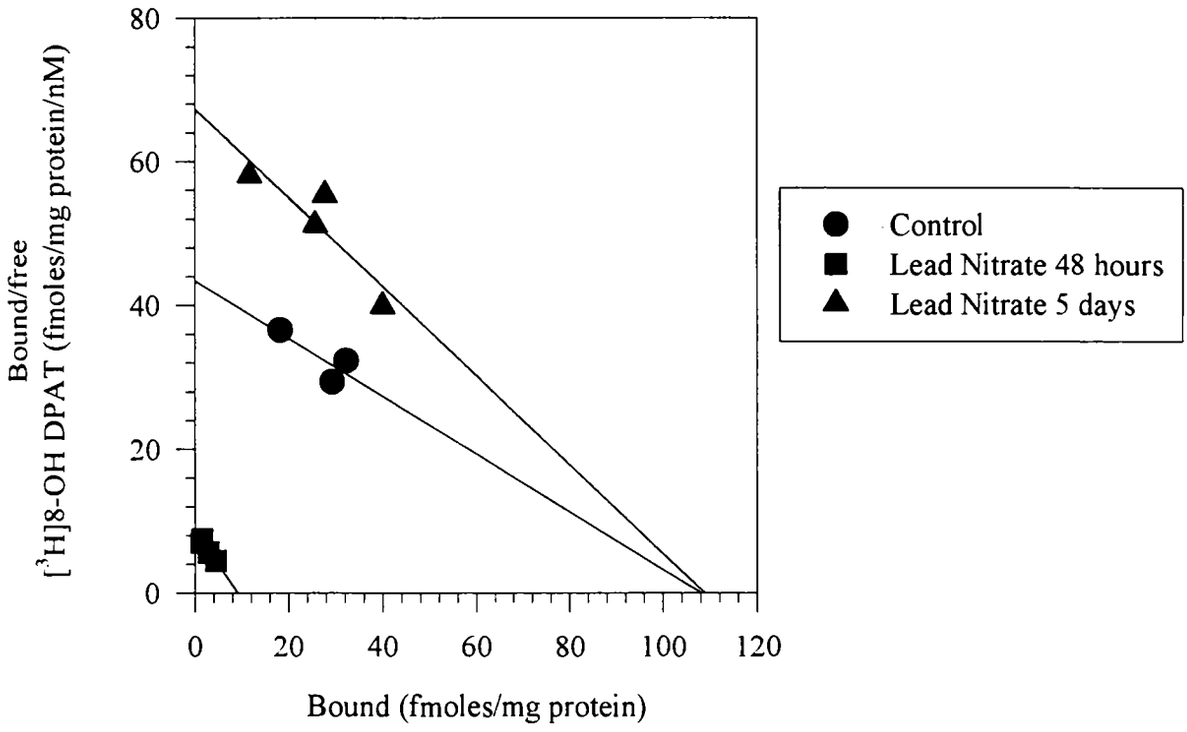


Table-35

[^{3}H]8-OH DPAT high affinity receptor binding parameters in the liver of rats

Experimental Group	B_{max} (fmoles/mg protein)	K_d (nM)
Control[¶]	109.00 ± 4.67	2.49 ± 0.16
Lead Nitrate 48 hours	9.13 ± 0.18***	1.08 ± 0.38*
Lead Nitrate 5 days	108.3 ± 8.30	1.62 ± 0.14*

***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.

Figure - 51

Scatchard analysis of low affinity [^{3}H]8-OH DPAT receptor binding against 5-HT in the liver of rats

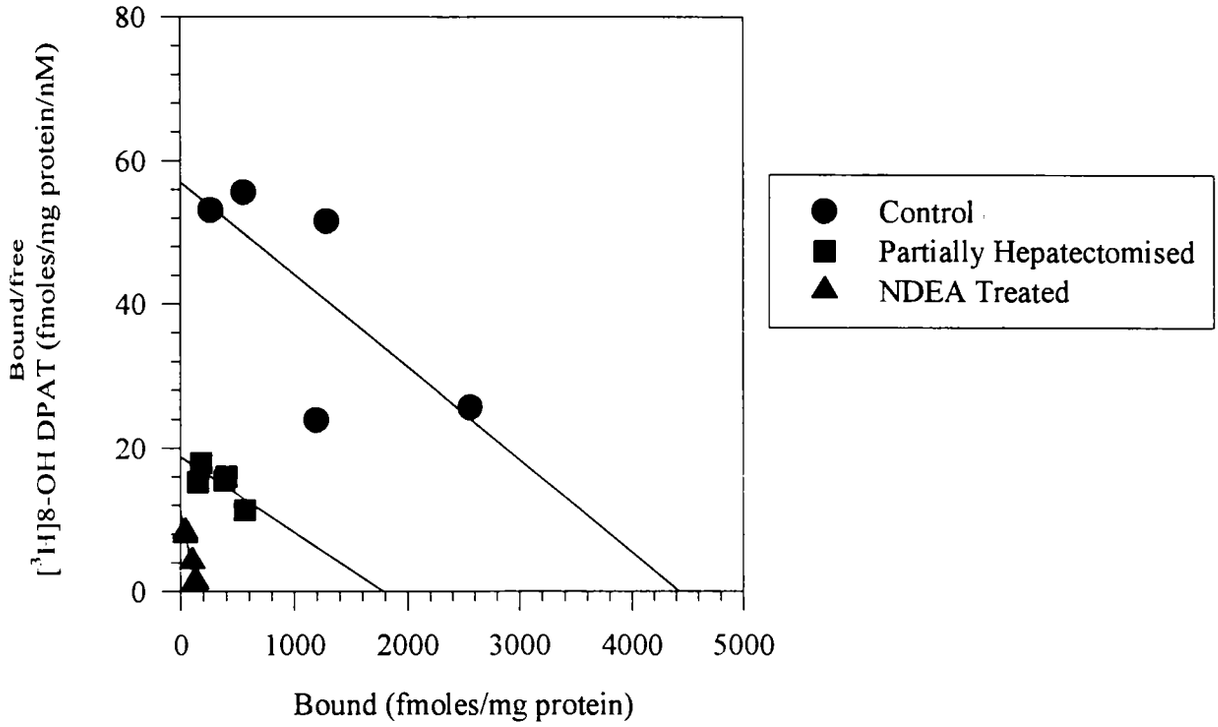


Table - 36

[^{3}H]8-OH DPAT low affinity receptor binding parameters in the liver of rats

Experimental Group	B_{max} (fmoles/mg protein)	K_d (nM)
Control[¶]	4366.67 ± 166.67	74.72 ± 3.02
Partially Hepatectomised	1810.00 ± 101.32***	94.72 ± 0.97*
NDEA Treated	152.00 ± 7.29***	13.54 ± 0.63***

***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.

Figure - 52

Scatchard analysis of low affinity [^{3}H]8-OH DPAT receptor binding against 5-HT in the liver of rats

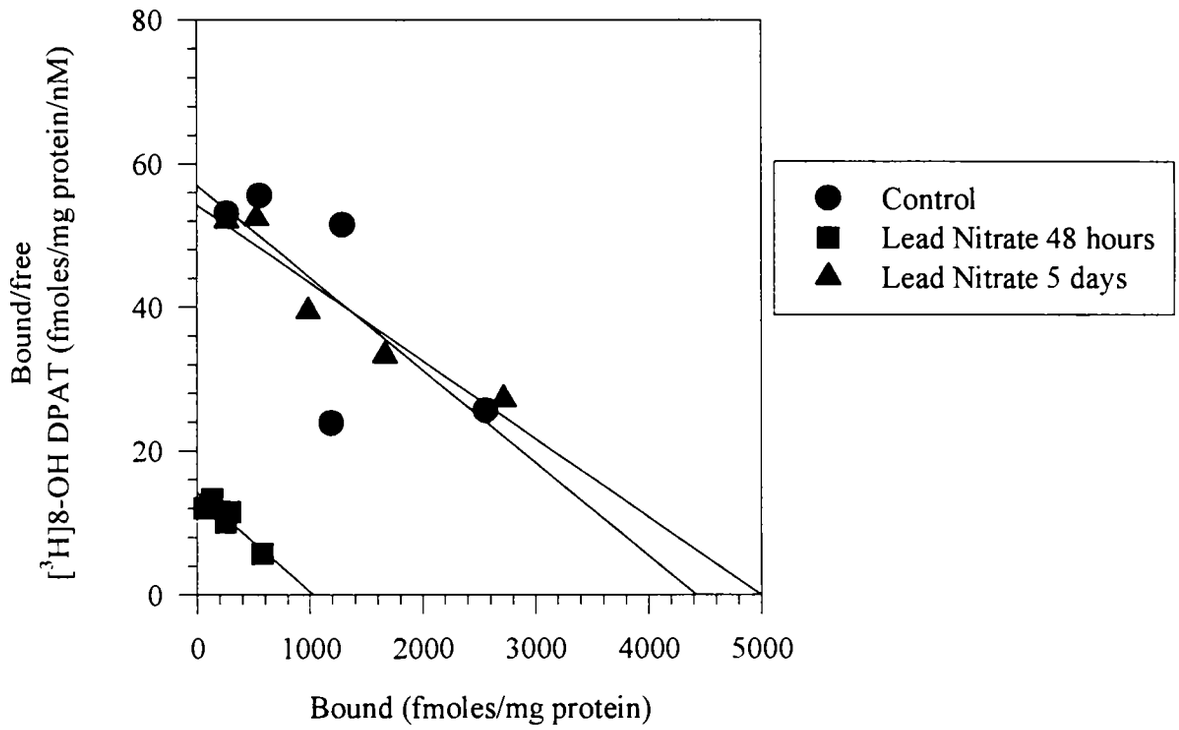


Table - 37

[^{3}H]8-OH DPAT low affinity receptor binding parameters in the liver of rats

Experimental Group	B_{max} (fmoles/mg protein)	K_d (nM)
Control[¶]	4366.67 ± 166.67	74.72 ± 3.02
Lead Nitrate 48 hours	1091.67 ± 46.39***	76.26 ± 3.14
Lead Nitrate 5 days	5000.00 ± 176.78**	91.25 ± 4.03*

***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]8-OH DPAT against 5-HT in the liver of rats

Experimental Group	Best-fit model	log (EC_{50})-1	log (EC_{50})-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Control[¶]	Two-site	-12.18	-5.64	4.70×10^{-13}	1.65×10^{-6}	-0.19
Partially Hepatectomised	Two-site	-12.15	-5.47	5.00×10^{-13}	2.44×10^{-6}	-0.19
N-Nitrosodiethylamine Treated	Two-site	-12.37	-6.46	3.06×10^{-13}	2.48×10^{-7}	-0.20

Values are mean of 4-6 separate experiments

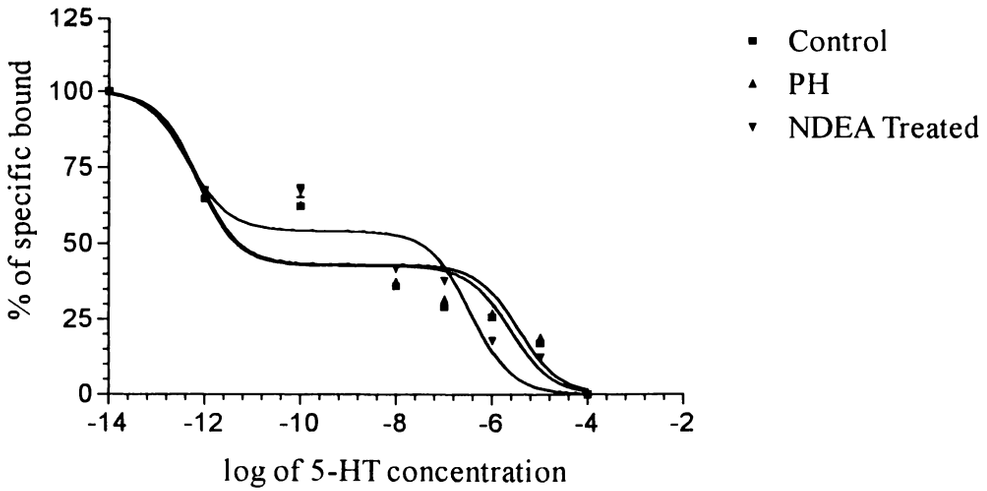
Data were fitted with an iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki - The affinity of the receptor for the competing drug. The affinity for the first and second site of the competing drug are designated as $Ki_{(H)}$ (for high affinity) and $Ki_{(L)}$ (for low affinity).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

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

PH – Partially Hepatectomised
NDEA Treated – *N*-Nitrosodiethylamine Treated

Figure - 53

Displacement of [^{3}H]8-OH DPAT with 5-HT in the liver of rats



Binding parameters of [^{3}H]8-OH DPAT against 5-HT in the liver of rats

Experimental Group	Best-fit model	log (EC_{50})-1	log (EC_{50})-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Control[¶]	Two-site	-12.18	-5.64	4.70×10^{-13}	1.65×10^{-6}	-0.19
Lead Nitrate 48hours	Two-site	-12.79	-5.82	1.17×10^{-13}	1.08×10^{-6}	-0.18
Lead Nitrate 5days	Two-site	-12.66	-4.97	1.57×10^{-13}	7.67×10^{-6}	-0.20

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.

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

LN 48hrs – Lead Nitrate 48 hours
LN 5days – Lead Nitrate 5 days

Figure - 54

Displacement of [^{3}H]8-OH DPAT with 5-HT in the liver of rats

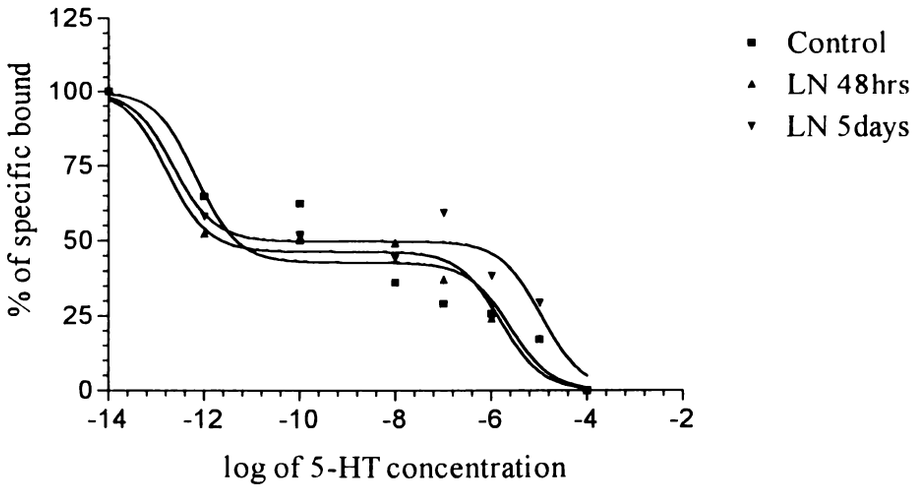
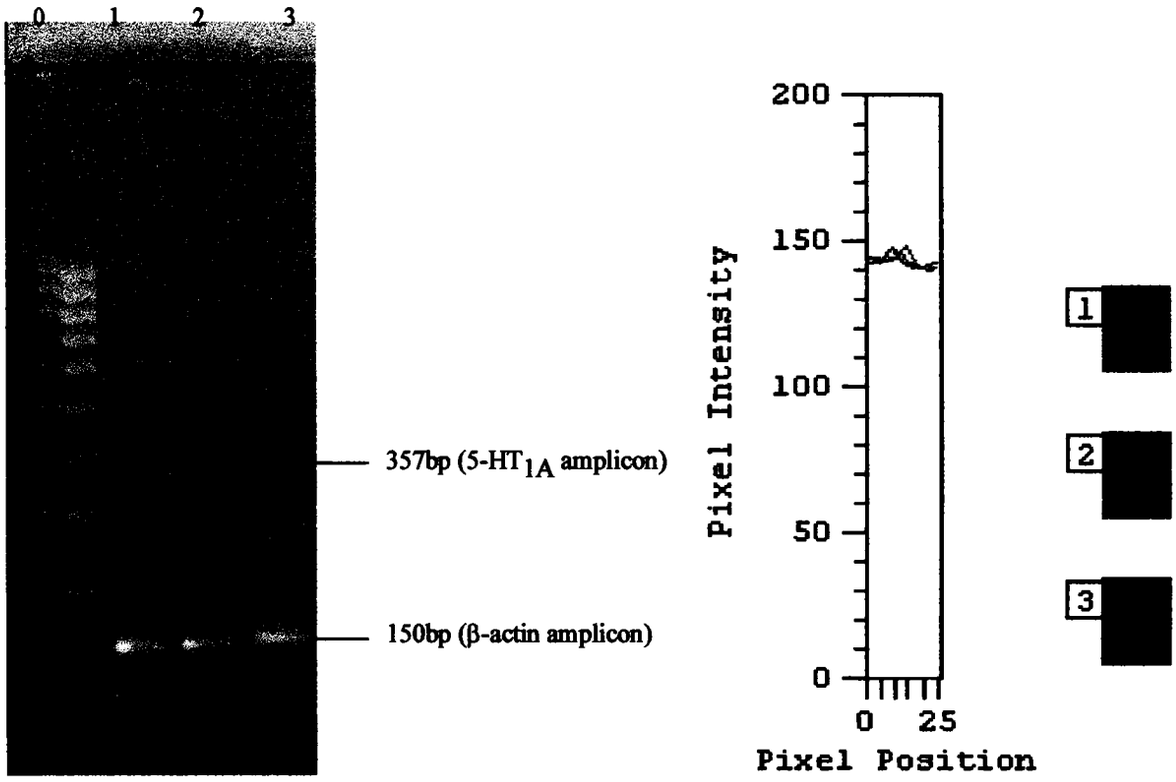


Figure - 55
RT-PCR amplification product of 5-HT_{1A} receptor mRNA from the liver of rats



Band properties of 5-HT_{1A} receptor mRNA RT-PCR amplicon

Lane No	Raw Volume	Area	Peak
1	41873	288	148.25
2	41493	288	146.31
3	41619	288	146.81

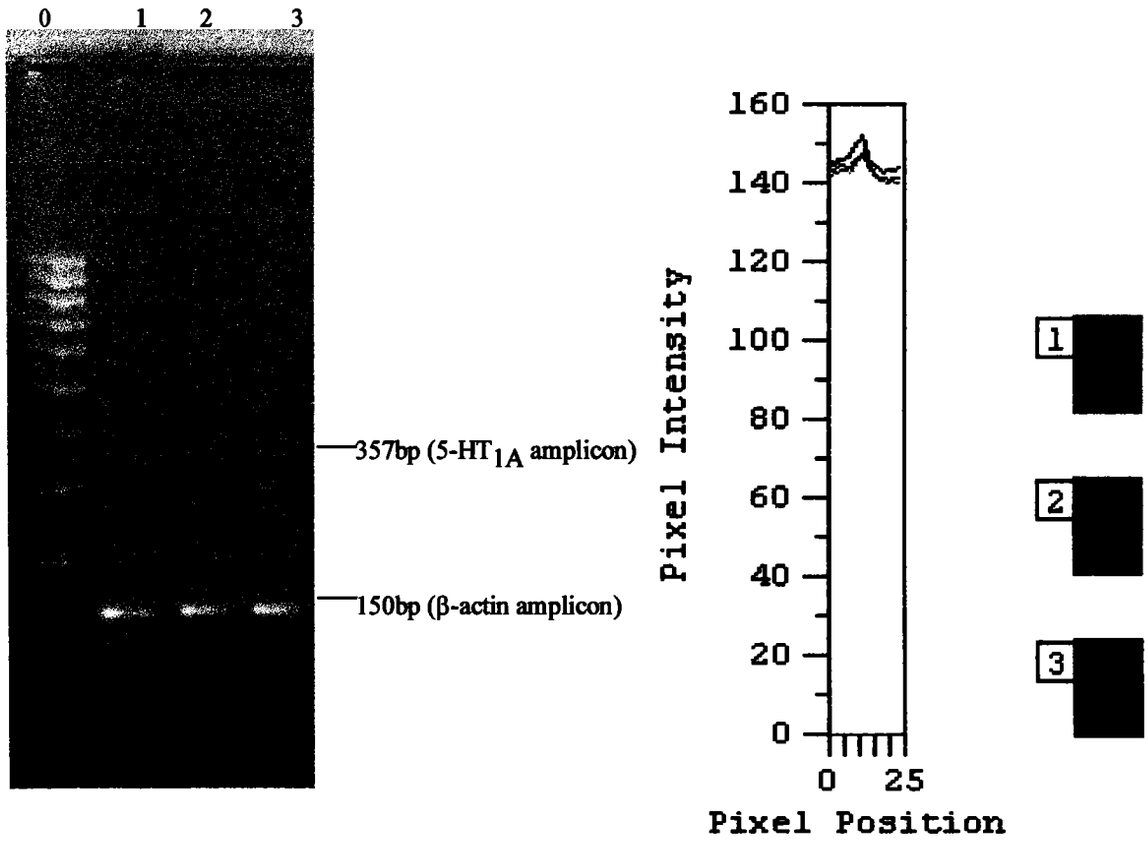
0 – 100bp ladder

1 – Control

2 – Partially Hepatectomised

3 – *N*-nitrosodiethylamine Treated

Figure - 56
RT-PCR amplification product of 5-HT_{1A} receptor mRNA from the liver of rats



Band properties of 5-HT_{1A} receptor mRNA RT-PCR amplicon

Lane No	Raw Volume	Area	Peak
1	41873	288	148.25
2	41921	306	146.79
3	52646	374	151.79

0 - 100bp ladder

1 - Control

2 - Lead Nitrate 48 hours

3 - Lead Nitrate 5 days

Figure - 57

Scatchard analysis of [^{3}H]Mesulergine binding against 5-HT in the liver of NDEA treated rats

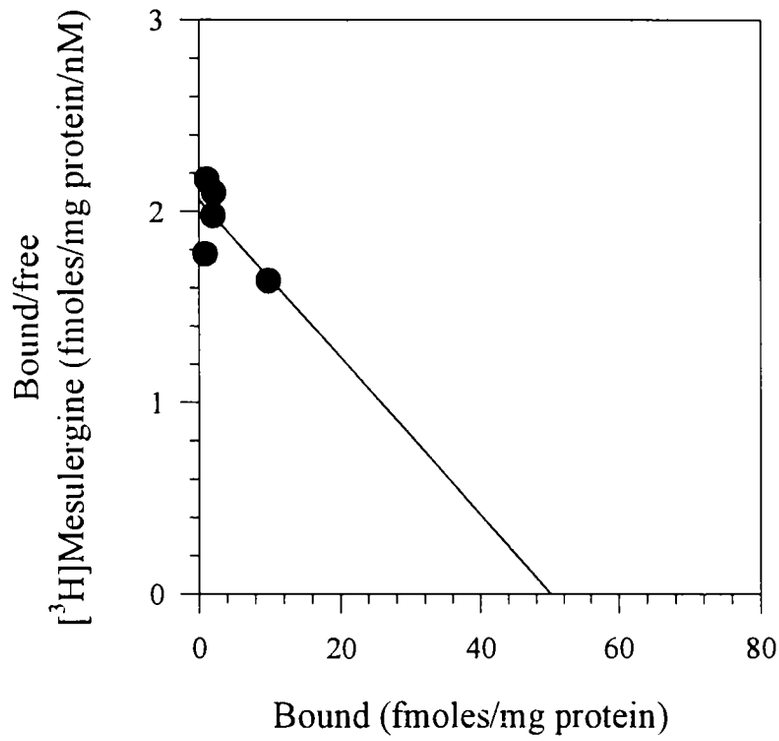


Table - 40

[^{3}H]Mesulergine binding parameters in the liver of NDEA treated rats*

B_{max} (fmoles/mg protein)	K_d (nM)
71.33 ± 0.88	35.24 ± 3.32

* 5-HT_{2C} receptor binding was observed only in *N*-nitrosodiethylamine treated (NDEA) not in other experimental groups.

Binding parameters of [^{3}H]mesulergine in the liver of NDEA treated rats

Experimental Group	Best-fit model	Log (EC50)	Ki	Hill slope
NDEA Treated	One-site	-7.19	3.91×10^{-8}	-1.00

* 5-HT_{2C} receptor binding was observed only in *N*-nitrosodiethylamine treated rats but not in other experimental groups

Values are mean of 4-6 experiments

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

NDEA – *N*-Nitrosodiethylamine

Figure - 58

Displacement of [^{3}H]mesulergine with 5-HT in the liver of NDEA treated rats

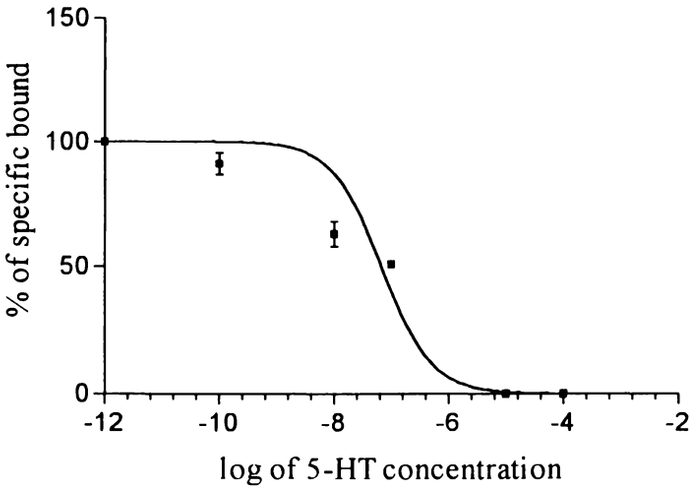
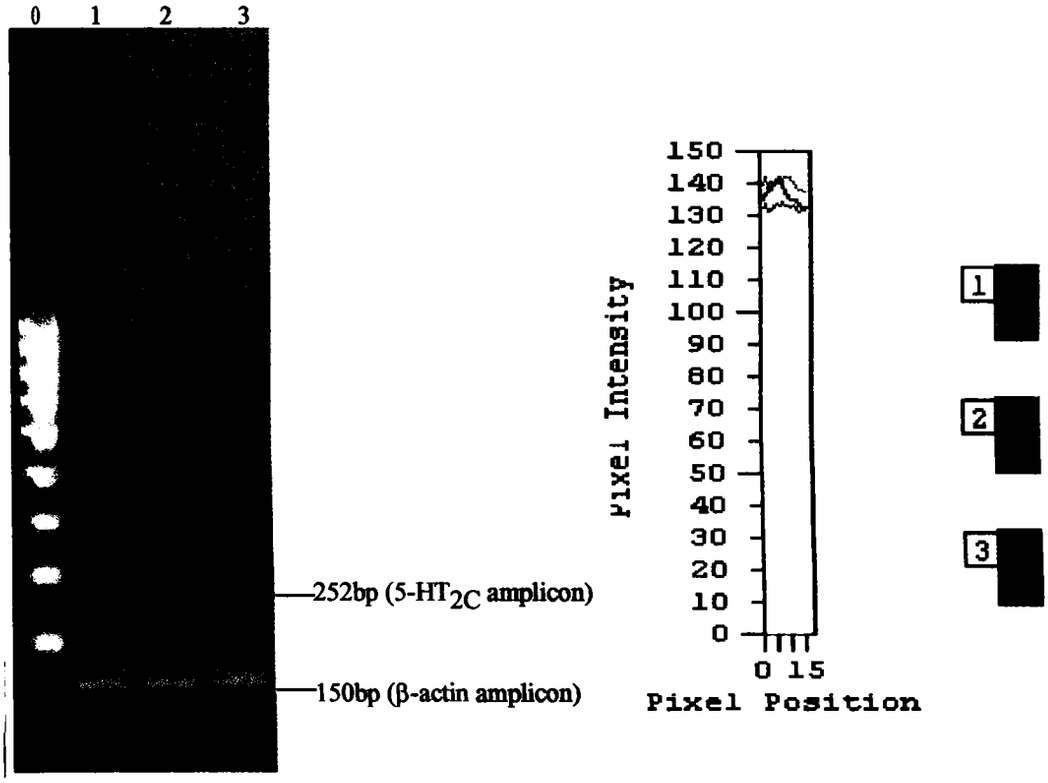


Figure - 59
RT-PCR amplification product of 5-HT_{2C} receptor mRNA from the liver of rats



Band properties of 5-HT_{2C} receptor mRNA RT-PCR amplicon*

Lane No	Raw Volume	Area	Peak
1	-	-	-
2	-	-	-
3	41302	300	141.8

5-HT_{2C} receptor mRNA expression was observed only in NDEA treated rats but not in other experimental groups.

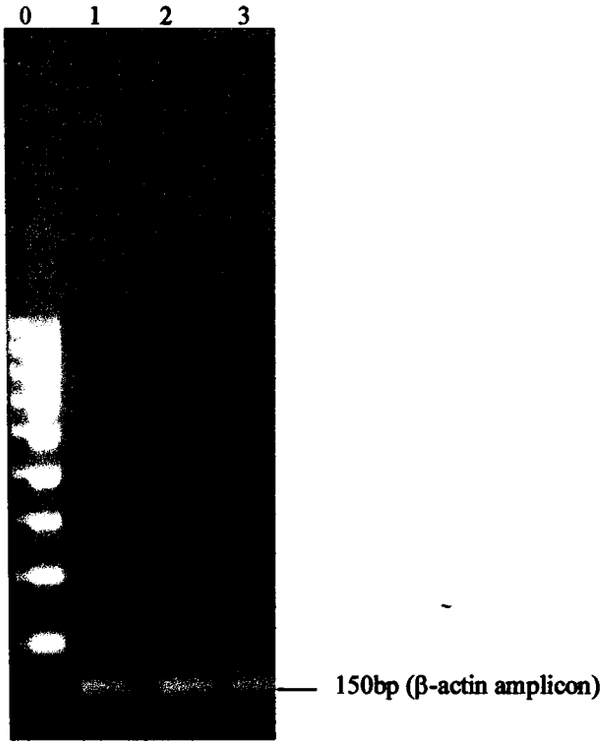
0 – 100bp ladder

1 – Control

2 – Partially Hepatectomised

3 – *N*-nitrosodiethylamine Treated

Figure - 60
RT-PCR amplification product of 5-HT_{2C} receptor mRNA from the liver of rats



Band properties of 5-HT_{2C} receptor mRNA RT-PCR amplicon*

Lane No	Raw Volume	Area	Peak
1	-	-	-
2	-	-	-
3	-	-	-

* 5-HT_{2C} receptor mRNA expression was observed only in NDEA treated rats but not in other experimental groups.
0 - 100bp ladder

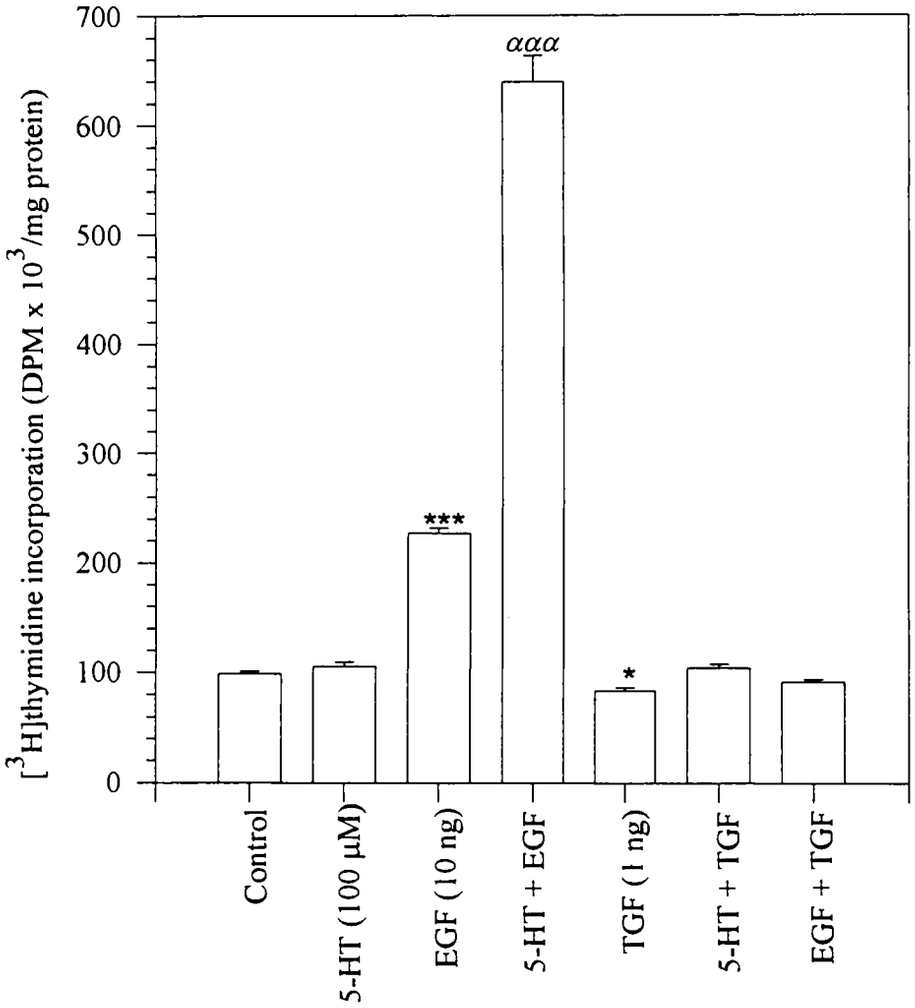
1 - Control

2 - Lead Nitrate 48 hours

3 - Lead Nitrate 5 days

Figure - 61

Effect of 5-HT on hepatocyte DNA synthesis



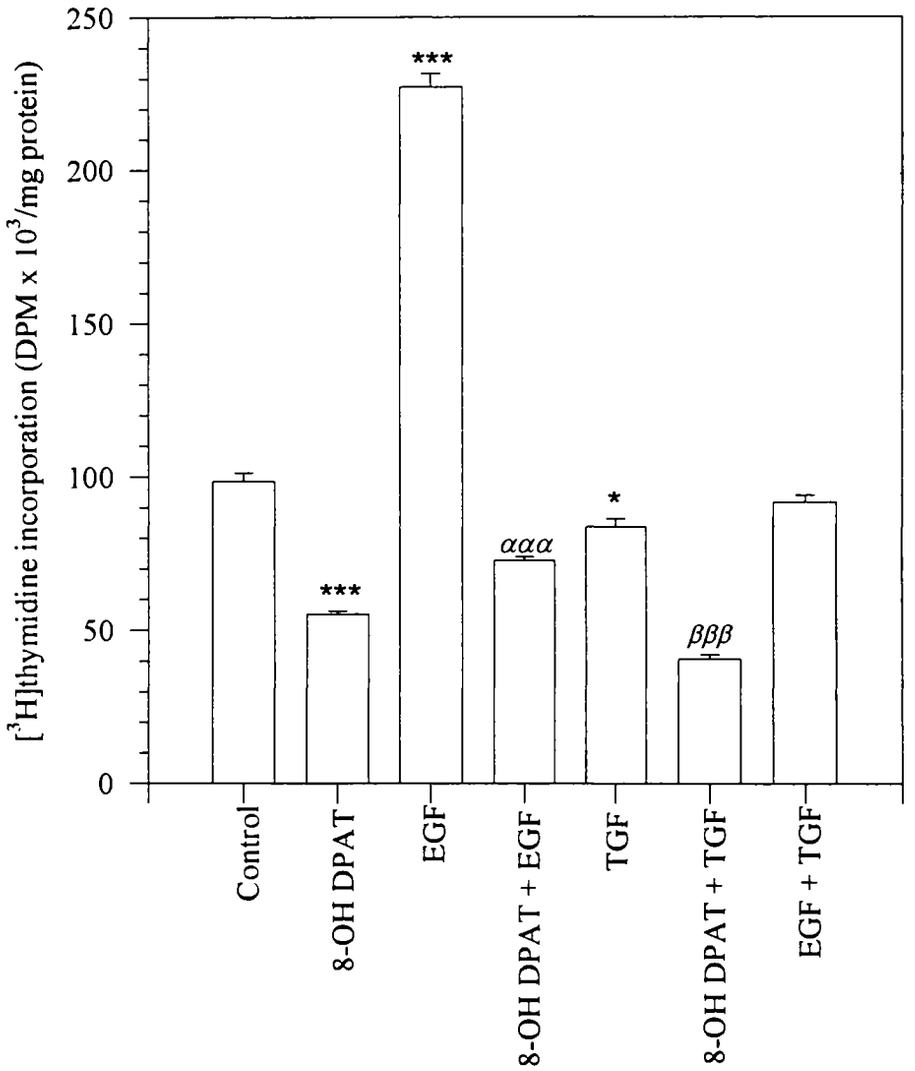
5-HT - 5-Hydroxy tryptamine
EGF - Epidermal growth factor
TGF - Transforming growth factor β1

*** $p<0.001$, * $p<0.05$ compared with the control
ααα $p<0.001$ compared with EGF

1×10^6 hepatocytes were incubated in serum-free William's E medium with 2.5mCi of [^{3}H]thymidine, 5-HT (100µM) and growth factors.

Figure - 62

Effect of 8-OH DPAT on hepatocyte DNA synthesis

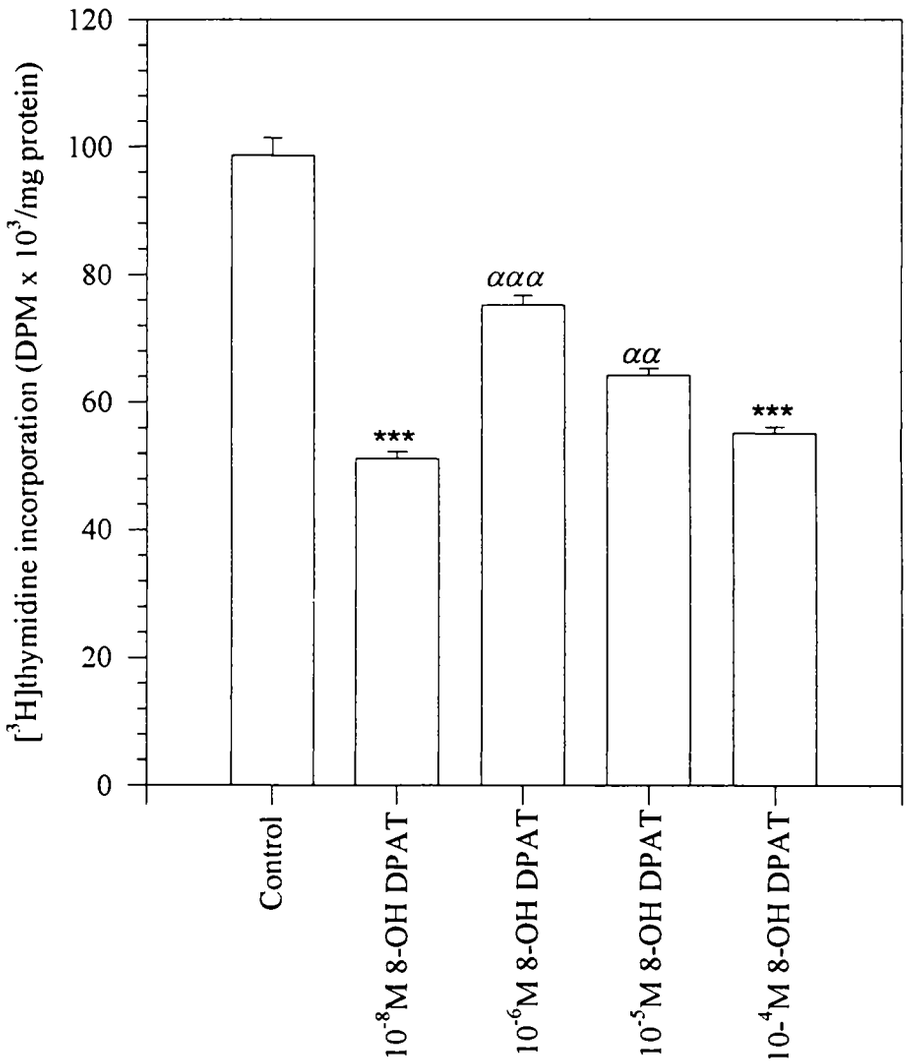


8-OH DPAT - 8-Hydroxy dipropylaminotetralin (100 μM)
EGF - Epidermal growth factor (10 ng)
TGF - Transforming growth factor β1 (1 ng)
*** $p<0.001$, * $p<0.05$ compared with the control
ααα $p<0.001$ compared with EGF
βββ $p<0.001$ compared with TGF β1

1×10^6 hepatocytes were incubated in serum-free William's E medium with 2.5μCi of [^{3}H]thymidine, 8-OH DPAT (5-HT_{1A} receptor agonist) and growth factors.

Figure - 63

Dose-dependent response of hepatocyte DNA synthesis to 8-OH DPAT

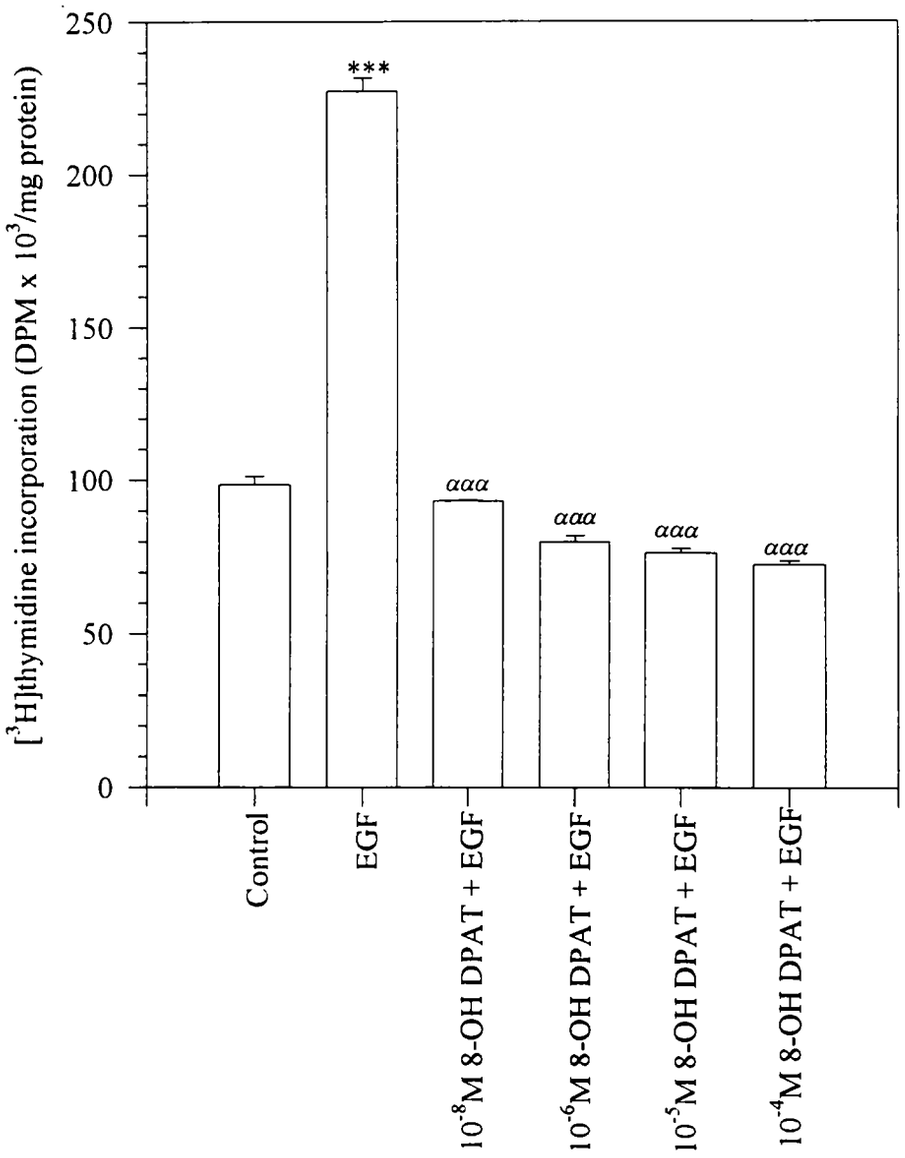


8-OH DPAT - 8-Hydroxy dipropylaminotetralin
*** $p<0.001$ compared with the control
$\alpha\alpha\alpha$ $p<0.001$, $\alpha\alpha$ $p<0.01$ compared with 10^{-4} M 8-OH DPAT

1×10^6 hepatocytes were incubated in serum-free William's E medium with 2.5mCi of [^{3}H]thymidine and different concentrations of 8-OH DPAT (5-HT_{1A} receptor agonist)

Figure - 64

Dose-dependent effect of 8-OH DPAT on EGF mediated hepatocyte DNA synthesis

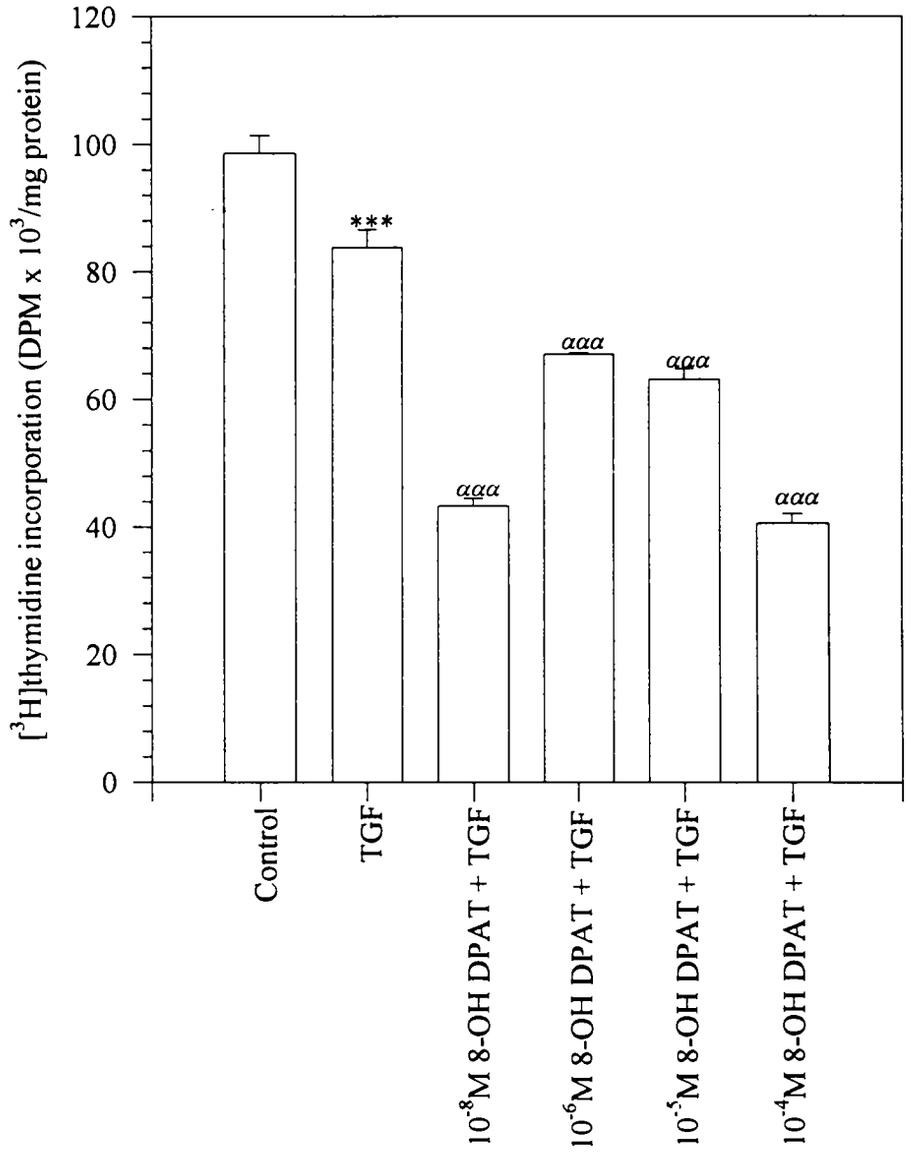


EGF - Epidermal growth factor
8-OH DPAT - 8-Hydroxy dipropylaminotetralin
*** $p<0.001$ compared with control
$\alpha\alpha\alpha$ $p<0.001$ compared with EGF

1×10^6 hepatocytes were incubated in serum-free William's E medium with 2.5mCi of [^{3}H]thymidine, different concentrations of 8-OH DPAT (5-HT_{1A} receptor agonist) and EGF

Figure - 65

Effect of 8-OH DPAT on TGFβ1 mediated hepatocyte DNA synthesis inhibition

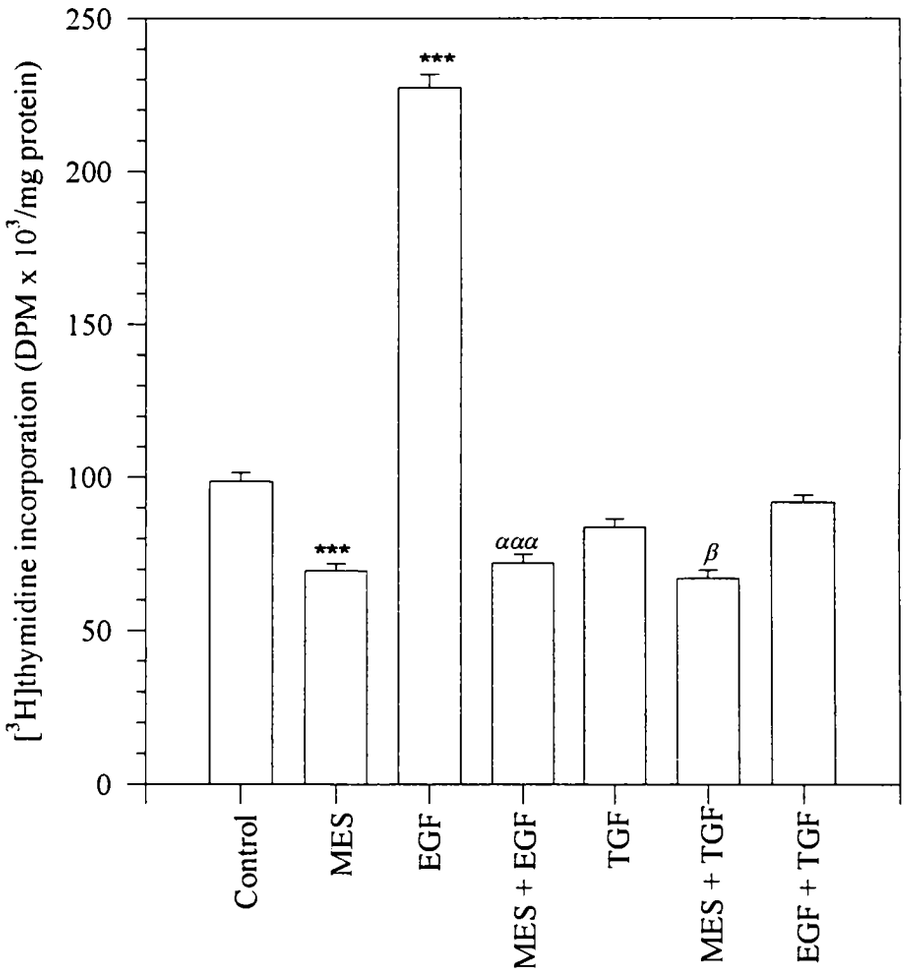


TGF - Transforming growth factor β1
8-OH DPAT - 8-Hydroxy dipropylaminotetralin
*** $p<0.001$ compared with control
$\alpha\alpha\alpha$ $p<0.001$ compared with TGFβ1

1×10^6 hepatocytes were incubated in serum-free William's E medium with 2.5mCi of [^{3}H]thymidine, different concentrations of 8-OH DPAT (5-HT_{1A} receptor agonist) and TGFβ1

Figure - 66

Effect of mesulergine on hepatocyte DNA synthesis

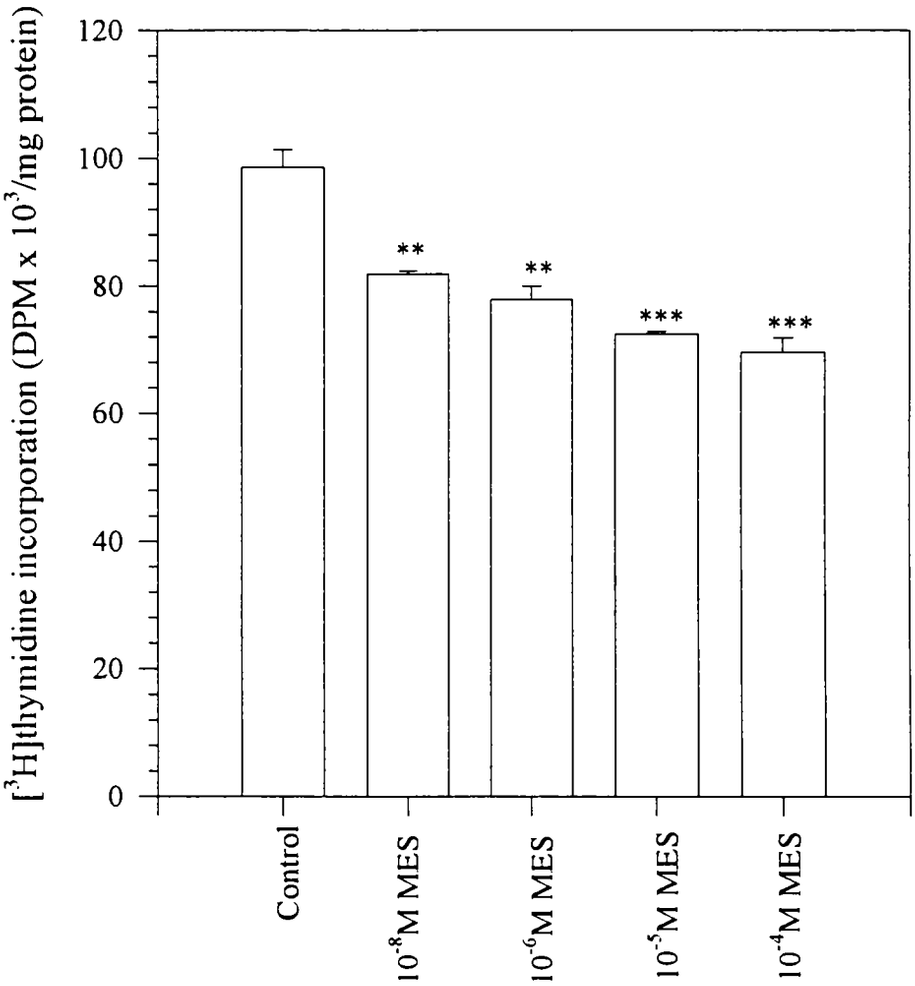


MES - Mesulergine (100μM)
EGF - Epidermal growth factor
TGF - Transforming growth factor β1
*** $p<0.001$ compared with the control
$\alpha\alpha\alpha$ $p<0.001$ compared with EGF
β $p<0.05$ compared with TGF

1×10^6 hepatocytes were incubated in serum-free William's E medium with 2.5mCi of [^{3}H]thymidine, mesulergine (5-HT_{2C} receptor antagonist), EGF and TGFβ1

Figure - 67

Dose-dependent effect of mesulergine on hepatocyte DNA synthesis



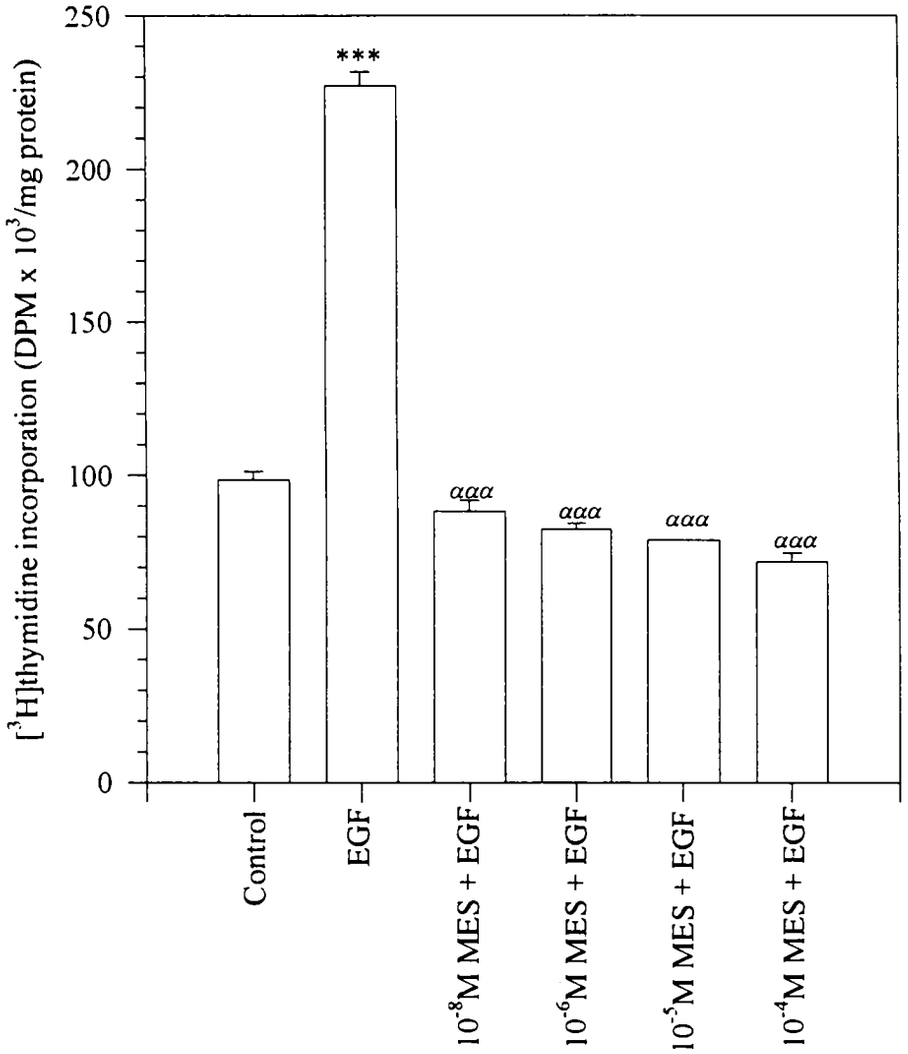
MES - Mesulergine

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

1×10^6 hepatocytes were incubated in serum-free William's E medium with 2.5mCi of [^{3}H]thymidine and different concentrations of mesulergine (5-HT_{2C} receptor antagonist)

Figure - 68

Dose-dependent effect of mesulergine on EGF mediated hepatocyte DNA synthesis

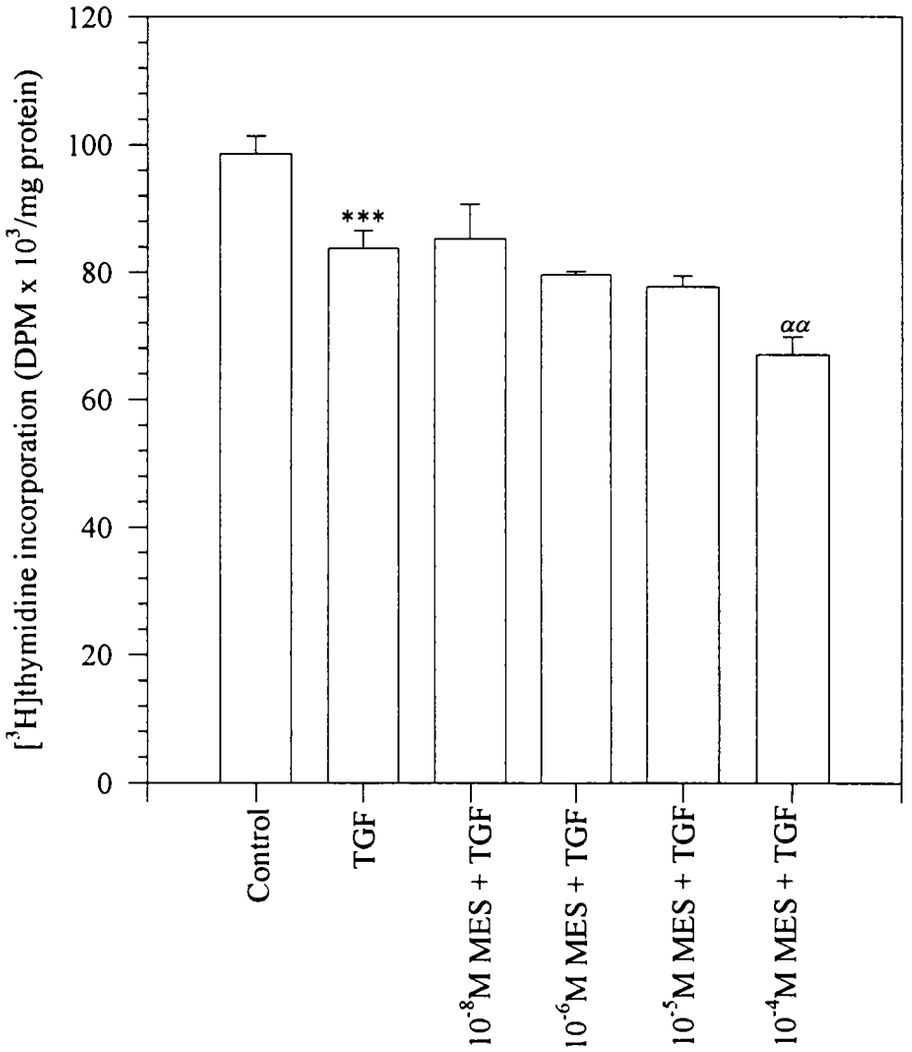


MES - Mesulergine
EGF - Epidermal growth factor
*** $p<0.001$ compared with the control
ααα $p<0.001$ compared with EGF

1×10^6 hepatocytes were incubated in serum-free William's E medium with 2.5mCi of [^{3}H]thymidine, different concentrations of mesulergine (5-HT_{2C} receptor antagonist) and EGF

Figure - 69

Dose-dependent effect of mesulergine on TGFβ1 mediated hepatocyte DNA synthesis suppression



MES - Mesulergine
TGF - Transforming growth factor β1
*** $p<0.001$ compared with the control
$\alpha\alpha$ $p<0.05$ compared with TGF
β $p<0.05$ compared with TGF

1×10^6 hepatocytes were incubated in serum-free William's E medium with 2.5mCi of [^{3}H]thymidine, different concentrations of mesulergine (5-HT_{2C} receptor antagonist) and TGFβ1

CONCLUSION

We conclude from our studies that the alterations of the 5-HT_{1A} and 5-HT_{2C} receptor function and gene expression in the brain stem, cerebral cortex and hypothalamus play an important role in the sympathetic regulation of cell proliferation, neoplastic transformation and apoptosis. Though many reports are there implicating the importance of sympathetic nervous system in cell proliferation, the involvement of specific receptor subtypes are not given emphasis. Receptor binding studies and RT-PCR analysis revealed that during hepatocyte proliferation brain and liver 5-HT_{1A} receptor was down regulated but during hepatocyte apoptosis the receptor was up regulated. Our results suggest an inhibitory role for 5-HT_{1A} receptor in hepatocyte proliferation i.e., the down regulation of this receptor facilitates hepatocyte proliferation. Brain 5-HT_{2C} receptor was up regulated during active hepatocyte proliferation without any significant change in hepatic apoptosis. During NDEA induced hepatocellular carcinoma 5-HT_{2C} receptor binding was observed in the liver which is not expressed in peripheral tissues in normal conditions. Therefore, 5-HT_{2C} receptor function mediates stimulation of hepatocyte proliferation but it has no significant role in hepatocyte apoptosis. These receptors may be regulating the hepatocyte cell proliferation through a sympathetic nervous system mediated mechanism. 5-HT_{1A} receptors mediate sympathetic inhibition and 5-HT_{2C} receptors mediate sympathetic stimulation. The circulating NE levels in the experimental groups are also consistent with our receptor data. *In vitro* studies revealed that 5-HT_{1A} receptor agonist, 8-OH DPAT and 5-HT_{2C} receptor antagonist, mesulergine inhibited the hepatocyte DNA synthesis. Also, 8-OH DPAT and mesulergine enhanced the mitoinhibitory effect of TGFβ1. Thus, the functional balance between 5-HT_{1A} and 5-HT_{2C} receptor plays an important role in regulating hepatocyte proliferation, neoplastic transformation and hepatic apoptosis. Loss of the ability to undergo apoptosis will ultimately result in carcinogenesis. The regulatory role of 5-HT_{1A} and 5-HT_{2C} receptor during neoplastic transformation and apoptosis could lead to possible therapeutic intervention in the treatment of cancers and have immense clinical importance.

SUMMARY

1. Liver regeneration after partial hepatectomy (PH) and 48 hours after lead nitrate treatment (LN) were used as model systems to study controlled cell proliferation in rats. 5 days after LN treatment was used as a model to study hepatic apoptosis. *N*-nitrosodiethylamine (NDEA) induced hepatocellular carcinoma was used as a model to study uncontrolled cell proliferation.
2. [^{3}H]thymidine incorporation was used as an index for hepatic DNA synthesis in PH and LN treated rats. DNA synthesis peaked at 24 hours after PH and 48 hours after LN treatment in rats. Thymidine kinase activity and histological studies showed tumourigenesis and increased DNA synthesis in NDEA treated rats. Hepatic apoptosis was confirmed by Giemsa staining and histological studies in rats 5 days after LN treatment.
3. 5-HT content was analysed using HPLC. It increased in the brain regions during active hepatocyte proliferation, i.e., in PH, 48 hours after LN treatment and NDEA treated rats. During hepatic apoptosis, i.e., in 5 days after LN treatment the brain 5-HT content decreased.
4. 5-HT receptor functional status was analysed by Scatchard and displacement analyses using [^{3}H] ligands. Receptor analysis was confirmed by studying the mRNA status of the corresponding receptor using RT-PCR. During active hepatocyte proliferation, 5-HT_{1A} receptors were down regulated and 5-HT_{2C} receptors were up regulated. During hepatic apoptosis, 5-HT_{1A} receptors were up regulated whereas there was no change in 5-HT_{2C} receptors.
5. Hepatic 5-HT content increased in PH, 48 hours after LN treatment and NDEA treated rats. Liver 5-HT content decreased in 5 days after LN treated rats. Hepatic 5-HT_{1A} receptor down regulation was observed during hepatocyte DNA synthesis but this receptor up regulation was found during hepatic apoptosis. During hepatic neoplasia induced by NDEA treatment 5-HT_{2C} receptor expression was observed which is absent in other groups of rats.
6. Plasma norepinephrine (NE) level increased during active hepatocyte proliferation, while it decreased during hepatic apoptosis.

In vitro studies showed that activation of 5-HT_{1A} receptor by adding 8-OH DPAT, a specific agonist, inhibited hepatocyte DNA synthesis. Also, the addition of mesulergine, a specific antagonist of 5-HT_{2C} receptor resulted in the inhibition of DNA synthesis.

Thus, it is evident from our results that brain and hepatic 5-HT_{1A} and 5-HT_{2C} receptor functional balance plays a major role in regulating the hepatocyte proliferation, neoplastic transformation and apoptosis.

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