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Increased 5-HT_{2C} receptor binding in the brain stem and cerebral cortex during liver regeneration and hepatic neoplasia in rats

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Abstract

In the present study, serotonin 2C (5-HT_{2C}) receptor binding parameters in the brainstem and cerebral cortex were investigated during liver generation after partial hepatectomy (PH) and *N*-nitrosodiethylamine (NDEA) induced hepatic neoplasia in male Wistar rats. The serotonin content increased significantly ($p<0.01$) in the cerebral cortex after PH and in NDEA induced hepatic neoplasia. Brain stem serotonin content increased significantly ($p<0.05$) after PH and ($p<0.001$) in NDEA induced hepatic neoplasia. The number and affinity of the 5-HT_{2C} receptors in the crude synaptic membrane preparations of the brain stem showed a significant ($p<0.001$) increase after PH and in NDEA induced hepatic neoplasia. The number and affinity of 5-HT_{2C} receptors increased significantly ($p<0.001$) in NDEA induced hepatic neoplasia in the crude synaptic membrane preparations of the cerebral cortex. There was a significant ($p<0.01$) increase in plasma norepinephrine in PH and ($p<0.001$) in NDEA induced hepatic neoplasia, indicating sympathetic stimulation. Thus, our results suggest that during active hepatocyte proliferation 5-HT_{2C} receptor in the brain stem and cerebral cortex are up-regulated which in turn induce hepatocyte proliferation mediated through sympathetic stimulation.

Keywords: 5-HT_{2C}; Brain stem; Cerebral cortex; Liver regeneration; Hepatic neoplasia; Partial hepatectomy

1. Introduction

The brain plays an important regulatory role in hepatic functions [1]. Early reports suggest that the central serotonergic neurons participate in the regulation of sympathetic nerve discharge. 5-HT neurons have an inhibitory influence on central sympathetic pathways [2] while recent work indicates that 5-HT facilitates central sympathetic nerve activity. A selective 5-HT2 receptor agonist, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) [3] produced a tremendous increase in sympathetic nerve discharge [4]. 5-HT2 antagonists ketanserin and LY-53587 reversed the massive increase in sympathetic discharge produced by DOI. Thus 5-HT2 receptors can increase sympathetic nervous discharge [5]. Transection of the spinal cord above the area innervating the liver resulted in decreased DNA synthesis [6]. Altered brain neurotransmission was reported in different liver diseases [7–9] and a role for brain serotonin has been suggested in hepatic encephalopathy [10].

5-HT_{2C} receptors [formerly termed 5-HT_{1C}] are widely expressed in the brain and appear to mediate many important effects of 5-HT [11,12]. 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 [13]. 5-HT has a regulatory role in neuronal proliferation, migration and differentiation, and in preventing apoptotic cell death [14]. Brain stem and cerebral cortex 5-HT_{2C} receptor binding was decreased during pancreatic regeneration [15]. The involvement of brain stem GABAergic neurotransmission in the regulation of the hepatic proliferation through the sympathetic stimulation was reported from our laboratory [16]. Oral carcinoma suppressed the expression of 5-HT transporter in the dorsal raphe nucleus [17] elucidating the CNS serotonergic alteration during neoplasia.

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The liver regeneration after partial hepatectomy (PH), and *N*-nitrosodiethylamine (NDEA) induced hepatocellular carcinoma in rats are established models to study the normal and neoplastic cell proliferation [18–20]. Although there are several studies regarding the brain control of hepatocyte proliferation, how brain serotonergic system responds to the hepatocyte proliferation and neoplasia remains undocumented. So in the present study we analysed the brain stem and cerebral cortex 5-HT content and 5-HT binding parameters during hepatic regeneration and hepatic neoplasia in rats.

2. Materials and methods

2.1. Chemicals

5-Hydroxytryptamine (5-HT), Mesulergine, (±)Norepinephrine, Sodium octyl sulphonate, Ethylenediamine tetra acetic acid (EDTA) were purchased from SIGMA Chemical Co., St. Louis, USA [N^6-methyl-^{3}H]mesulergine (Sp. activity 79.0 Ci/mmol) and [^{3}H]Thymidine (Sp. activity 25 Ci/mmol), were purchased from Amersham Life Science, UK. All other reagents were of analytical grade purchased locally.

2.2. Animals

Adult male Wistar rats weighing 200–300 g were obtained from Kerala Agricultural University, Mannuthy, Thrissur and used for all the experiments. All animals were housed under conditions of controlled temperature and light with free access to food and water. All animal care and procedures were in accordance with the Institutional and National Institute of Health guidelines.

2.3. Partial hepatectomy

Two-thirds of the liver constituting the median and left lateral lobes was surgically excised under light ether anaesthesia, following a 16-h fast [21]. 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.

2.4. N-nitrosodiethylamine treatment

Liver cancer was induced using NDEA [22]. The 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 22nd week. Neoplasia was confirmed by histological techniques.

Table 1
5-HT Content in the brain stem and cerebral cortex of rats (nanomoles/gm wet weight of the tissue)

Control	Brain stem	Cerebral cortex
	1.28±0.02	0.99±0.03
Partially hepatectomised	1.89±0.08*	2.15±0.28**
N-nitrosodiethylamine treated	3.22±0.03***	2.26+0.16**

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

Table 2
Levels of plasma norepinephrine in rats (nanomoles/ml)

Control	0.53±0.01
Partially hepatectomised	1.88±0.02**
N-nitrosodiethylamine treated	8.77±0.65***

***$p<0.001$, **$p<0.01$ compared with the control.
Values are mean ±SEM of 4–6 separate experiments.

2.5. Sacrifice of rats

The rats were sacrificed by decapitation. The brain stem (BS) and cerebral cortex (CC) were dissected out [23] and immediately stored at −70 °C for various experiments.

2.6. 5-HT quantification by HPLC

Brain 5-HT HPLC determinations were done by electrochemical detection [24]. The tissues from brain regions were homogenised in 0.4 N perchloric acid. The homogenate was centrifuged at 5000×*g* for 10 min at 4 °C (Kubota Refrigerated Centrifuge, Japan) and the clear supernatant was filtered through 0.22 μm 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 75 mM sodium dihydrogen orthophosphate buffer containing 1 mM sodium octyl sulphonate, 50 mM EDTA and 7% acetonitrile (pH 3.25), filtered through 0.22 μm filter delivered at a flow rate of 1.0 ml/min. 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.

2.7. Analysis of circulating norepinephrine

Plasma norepinephrine (NE) was extracted from 1 ml of plasma and diluted twice with distilled water. To it 50 μl of 5 mM sodium bisulphite was added, followed by 250 μl of 1 M Tris buffer, pH 8.6. Acid alumina (20 mg) was added, shaken in the cold for 20 min and was washed with 5 mM sodium bisulphite. Catecholamines were extracted from the final pellet of alumina with 0.1 N perchloric acid, mixed well and 20 μl of filtered sample was analysed [25].

2.8. 5-HT$_{2C}$ receptor binding studies

[^{3}H]mesulergine binding to 5-HT$_{2C}$ receptor in the synaptic membrane preparations were assayed as previously described [26]. Crude synaptic membrane preparation was suspended in

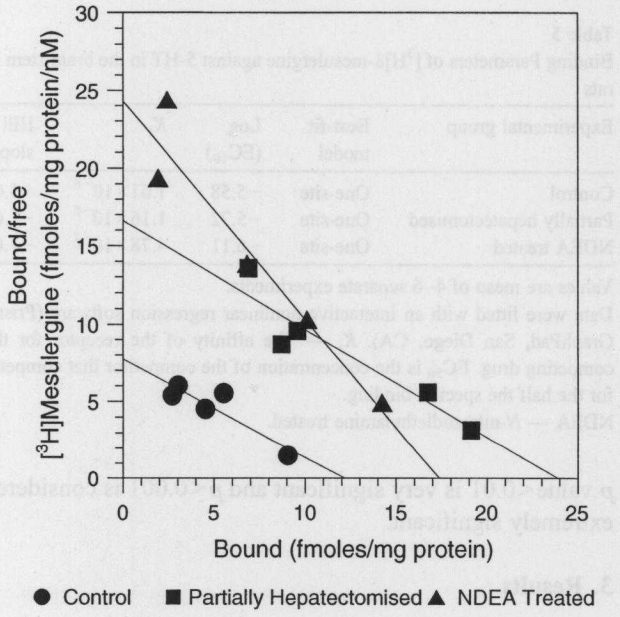

Fig. 1. Representative figure for Scatchard analysis of [^{3}H]mesulergine binding against 5-HT in the brain stem of rats (see Table 3). Crude synaptic membrane preparation was suspended in 50 mM Tris-HCl buffer pH 7.4 and used for assay. 0.1 nM–6 nM of [^{3}H]mesulergine was incubated with and without excess of unlabelled 5-HT (100 μM). Tubes were incubated at 25 °C for 60 min and filtered rapidly through GF/C filters (Whatman). 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.

saturation binding experiments, 0.1 nM–6 nM of [^{3}H] mesulergine was incubated with and without excess of unlabelled 5-HT (100 μM) and in competition binding experiments the incubation mixture contained 1 nM 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 min and filtered rapidly through GF/C filters (Whatman). The filters were given three quick successive washings with 3 ml of ice-cold 50 mM 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.

2.9. Protein estimation

Protein concentrations were estimated [27] using bovine serum albumin as the standard.

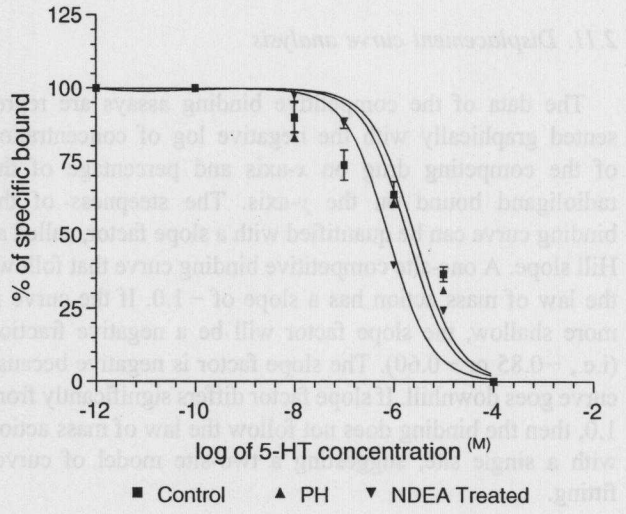

Fig. 2. Displacement of [^{3}H]mesulergine with 5-HT in the brain stem of Control, Partially hepatectomised and NDEA treated rats. Crude synaptic membrane preparation was suspended in 50 mM Tris-HCl buffer, pH 7.4 and used for assay. The incubation mixture contained 1 nM of [^{3}H]mesulergine with and without 5-HT at a concentration range of 10^{-12} M to 10^{-4} M. Tubes were incubated at 25 °C for 60 min and filtered rapidly through GF/C filters (Whatman). Bound radioactivity was determined with cocktail-T in a Wallac 1409 liquid scintillation counter.

2.10. Receptor binding parameters analysis

The receptor binding parameters were determined using Scatchard analysis [28]. 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. 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 nonlinear regression curve-fitting procedure (GraphPad PRISM™, San Diego, USA). The concentration of the 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 [29].

Table 3
[^{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	24.10±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.

Table 4
[^{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.

2.11. 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, called as 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.

2.12. Statistical analysis

The equality of all the groups is tested by the analysis of variance (ANOVA) technique for different values of p. Further the pair wise comparisons of all the experimental groups are studied using Students-Newman-Keuls test at different significance levels. The testing is performed using GraphPad Instat (version 2.04a, San Diego, USA). The significance level is expressed taking $p<0.05$ as the threshold value. p value<0.05 is considered as significant, p value<0.01 is very significant and $p<0.001$ is considered extremely significant.

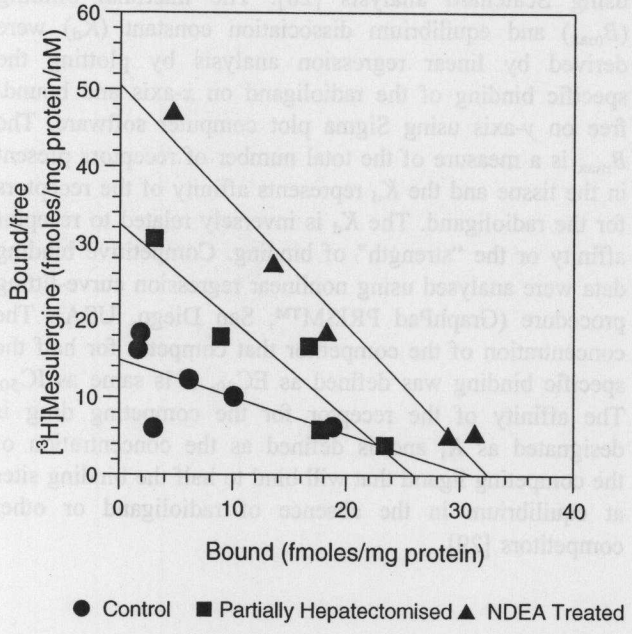

Fig. 3. Representative figure for Scatchard analysis of [^{3}H]mesulergine binding against 5-HT in the cerebral cortex of rats (see Table 4). Crude synaptic membrane preparation was suspended in 50 mM Tris-HCl buffer, pH 7.4 and used for assay. 0.1 nM–6 nM of [^{3}H]mesulergine was incubated with and without excess of unlabelled 5-HT (100 μM). Tubes were incubated at 25 °C for 60 min and filtered rapidly through GF/C filters (Whatman). Bound radioactivity was determined with cocktail-T in a Wallac 1409 liquid scintillation counter. Specific binding was determined by

Table 5
Binding Parameters of [^{3}H]8-mesulergine against 5-HT in the brain stem of rats

Experimental group	Best-fit model	Log (EC_{50})	K_i	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 separate experiments.
Data were fitted with an interactive nonlinear regression software (Prism, GraphPad, San Diego, CA). K_i — The affinity of 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 treated.

3. Results

There was a significant increase in brain stem 5-HT content in PH ($p<0.05$), and NDEA treated rats ($p<0.001$) compared with control. The Cerebral cortex 5-HT content also increased significantly in PH ($p<0.01$) and NDEA treated rats ($p<0.01$) compared with control (Table 1). The plasma NE levels increased significantly in PH ($p<0.01$) and NDEA treated ($p<0.001$) rats compared with control (Table 2).

Scatchard analysis of [^{3}H]mesulergine binding to brain stem synaptic membranes showed a significant increase ($p<0.001$) in the B_{max} of PH and NDEA treated rats. The K_d of the receptor binding showed a significant decrease ($p<0.001$) in NDEA treated rats (Fig. 1 and Table 3). The competition curve for 5-HT against [^{3}H]mesulergine fitted

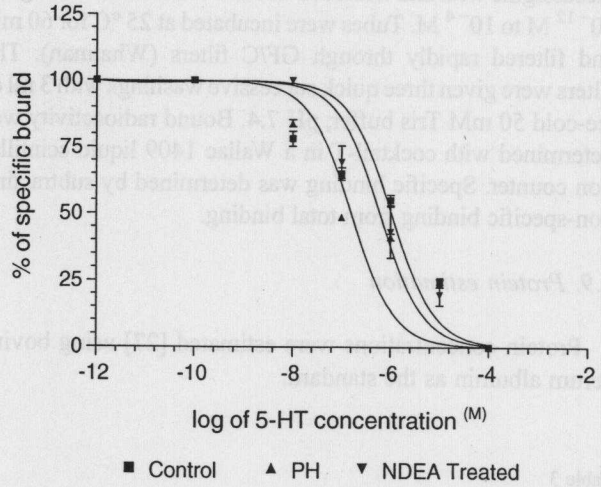

Fig. 4. Displacement of [^{3}H]mesulergine with 5-HT in the cerebral cortex of Control, partially hepatectomised and NDEA treated rats. Crude synaptic membrane preparation was suspended in 50 mM Tris-HCl buffer, pH 7.4 and used for assay. The incubation mixture contained 1 nM of [^{3}H]mesulergine with and without 5-HT at a concentration range of 10^{-12} M to 10^{-4} M. Tubes were incubated at 25 °C for 60 min and filtered rapidly through GF/C filters (Whatman). Bound radioactivity was determined with cocktail-T in a Wallac

Table 6
Binding parameters of [^{3}H]mesulergine against 5-HT in the cerebral cortex of rats

Experimental group	Best-fit model	Log (EC_{50})	K_i	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 separate experiments.
Data were fitted with an interactive nonlinear regression software (Prism, GraphPad, San Diego, CA). K_i — The affinity of 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 Treated.

for one-sited model in all the groups with Unity as Hill slope value. There was a decrease in K_i and log (EC_{50}) in NDEA treated rats compared with control indicating a shift towards higher affinity (Fig. 2, Table 5).

The B_{max} of the [^{3}H]mesulergine binding increased significantly ($p<0.001$) in the cerebral cortex of NDEA treated rats whereas the K_d of the receptor binding decreased significantly ($p<0.001$) in PH and NDEA treated rats compared with control (Fig. 3 and Table 4). 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 K_i and log (EC_{50}) in PH and NDEA treated rats indicating a shift in receptor towards higher affinity (Fig. 4 and Table 6) compared with control.

4. Discussion

The relationship between the functional status of the liver and that of the brain has been known for centuries [30]. In hepatic encephalopathy and other liver diseases, neurotransmission in the brain is reported to be altered [7–9]. The serotonin brain circuits are known to be involved in hepatic encephalopathy [10].

Lateral lesions of hypothalamus cause an increase in DNA synthesis during liver regeneration while sympathectomy and vagotomy block this effect [31]. Central thyrotropin-releasing hormone, one of the important peptide transmitter substance, regulates hepatic proliferation through autonomic nervous system [32].

Two models of cell proliferation were chosen for the present study: controlled hepatocyte proliferation after partial hepatectomy and uncontrolled hepatocyte proliferation during NDEA induced hepatic neoplasia. The animals were sacrificed at the peak of the DNA synthesis in the liver, based on previous reports and based on our [^{3}H]Thymidine incorporation studies (data not shown) [16,33]. 5-HT content was increased during active hepatocyte proliferation in brain stem and cerebral cortex and it was more pronounced in hepatic neoplasia model. 5-HT mediates mitogenic effects in many cell types [34,35]. 5-HT can act as a potent hepatocyte co-mitogen and induce DNA synthesis in primary cultures of rat hepatocytes [13]. Sympathetic innervation is important for hepatocyte proliferation [31]. 5-HT facilitates central sympathetic nerve activity [36]. Therefore the increase in the 5-HT content during active cell proliferation i.e., in PH and NDEA treated rats, may be triggering the sympathetic stimulation and thereby DNA synthesis. Increased plasma NE levels during active cell proliferation, i.e., in PH and NDEA treated rats support the involvement of sympathetic stimulation. Norepinephrine functions as a co-mitogen by enhancing the EGF mediated DNA synthesis in primary hepatocyte culture [37]. So, the increased brain 5-HT during liver regeneration after PH and NDEA treated hepatic neoplasia is suggested to facilitate the active cell proliferation possibly through sympathetic stimulation.

5-HT receptors can be classified into seven classes from 5-HT_1 to 5-HT_7 [38–40]. 5-HT_2 receptor is reported to be responsible for proliferation of rat vascular smooth muscle cells through an increase in intracellular Ca^{2+} [41–43]. 5-HT_{2C} receptors activate phospholipase C [44,45] and thereby influence cell proliferation. Phospholipase C is a well known second messenger which mediates cell proliferation. The 5-HT_{2C} receptor binding parameters determined by [^{3}H] mesulergine against 5-HT showed an increase in the brain stem receptor density in PH and NDEA treated rats, as indicated by the increased B_{max}. The affinity of the receptor increased in NDEA treated rats. The affinity shifted towards higher affinity site in PH and NDEA treated rats. When we analysed the 5-HT_{2C} receptor status in the cerebral cortex, we found that 5-HT_{2C} receptor number increased significantly in NDEA treated rats as indicated by an increased B_{max}. The affinity of the receptor increased in PH and NDEA treated rats as indicated by the decrease in the K_i and log (EC_{50}).

5-HT_2 receptors in glioma cells appear to regulate proliferation, migration, and invasion [46]. The 5-HT_2 receptor antagonists, ketanserin (10^{-6} M) and spiperone (10^{-6} M), blocked the stimulation of DNA synthesis by 5-HT indicating that the mitogenic activity of 5-HT is mediated through 5-HT_2 receptors. Displacement studies on [^{3}H]5-HT binding to crude membranes from control and regenerating liver tissue showed an increased involvement of 5-HT_2 receptors in the regenerating liver during the DNA synthetic phase [13].

We conclude from our studies that the brain 5-HT_{2C} receptor was up-regulated during liver regeneration after PH and in NDEA induced hepatic neoplasia. The increased 5-HT content and 5-HT_{2C} receptor in the brain stem and cerebral cortex is suggested to facilitate the active hepatocyte proliferation possibly through the sympathetic stimulation.

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