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## Effect of Amitriptyline on Adrenergic Receptor Number and Second Messenger Function in Rat Brain

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**Abstract:** Radioligand binding studies were done to investigate the effect of chronic administration of Amitriptyline on  $\alpha_1$ -adrenoceptor ( $\alpha_1$ -AR) receptor mediated response to inositol triphosphate (IP3) in rat brain. Our studies revealed a significant decrease in the densities of  $\alpha_1$ -ARs in cortex and cerebellum of rat brain after chronic administration of Amitriptyline (10 mg kg<sup>-1</sup> b.wt.). However, there was no significant change in the affinity of [<sup>3</sup>H]prazosin to  $\alpha_1$ -ARs. Displacement studies showed that Amitriptyline has higher affinity for  $\alpha_1$ -AR with a K<sub>i</sub> value of 182±16 nM. Significant change was observed in basal IP3 activity in cortex and cerebellum after Amitriptyline exposure. In cortex and cerebellum of experimental rats the NE (Norepinephrine) stimulated IP3 activity was significantly decreased (1460±102 DPM/g tissue; p<0.0001; 1188±112 DPM/g tissue; p<0.0001), when compared to NE stimulated IP3 activity (4152±286 and 3952±245 DPM/g tissue, respectively) in control rats. The decrease in NE stimulated IP3 activity in both regions may be due to the significant downregulation of  $\alpha_1$ -ARs in cortex after Amitriptyline exposure as these sites are positively coupled to IP3. The observed significant decrease in  $\alpha_1$ -ARs with concomitant decrease in NE stimulated IP3 activity, after Amitriptyline treatment, suggests that Amitriptyline which has high affinity for these sites, acts by modulating the  $\alpha_1$ -AR receptor mediated response in brain.

**Key words:** Amitriptyline, *in vivo*, chronic,  $\alpha_1$ -AR, [<sup>3</sup>H]prazosine, IP3, rat brain

### INTRODUCTION

Recent studies have shown the possibility that some points along a G-protein signaling cascade represents a molecular target for AD (Anti Depressant) therapy that might lead toward a unifying hypothesis for depression. Almost all AD drug development over the past 50 years have concentrated on the design of drugs that selectively inhibit the reuptake or metabolism of 5-HT and/or NE. However, the notional ability of ADs to increase synaptic levels of 5-HT and/or NE acutely is at odds with the delay in their onset of therapeutic action. In recognition of this paradox, attention is turned towards slower, adaptive changes in postsynaptic 5-HT and NE receptors, resulting in alteration of gene expression of certain neurotrophic factors as being the final common mechanism by which ADs exert their effects.

The  $\alpha_1$ -ARs are located in the central and Peripheral Nervous System (PNS). In the CNS, they are predominantly located postsynaptically where they mediate an excitatory role. The  $\alpha_1$ -AR subtypes have

been classified as the  $\alpha_1A$ ,  $\alpha_1B$  and  $\alpha_1D$ -ARs (Han *et al.*, 1987). All the subtypes produce changes in cellular activity by increasing intracellular levels of free Ca<sup>2+</sup>. They do this by coupling with phospholipase C (PLC) through Gq/Gp which initiates the hydrolysis of a membrane phospholipid, phosphatidylinositol bisphosphate (PIP2), to produce two second messengers, diacylglycerol (DAG) which activates Protein Kinase C (PKC) and inositol triphosphate (IP3) which acts on specific intracellular receptors to release sequestered Ca<sup>2+</sup> (Berridge and Irvine, 1989). This pathway also stimulates phospholipase A2 and phospholipase D and arachidonic acid release (Harrison *et al.*, 1991). The  $\alpha_1$ -ARs are also coupled directly to Ca<sup>2+</sup> influx through the receptor operated Ca<sup>2+</sup> channel (Han *et al.*, 1987). Autoradiographic studies show high concentrations of  $\alpha_1$ -ARs in olfactory bulb, thalamus, medial geniculate nucleus and neocortex and lower levels in hippocampus (Palacios *et al.*, 1987).

It has now become clear that  $\alpha_1$ -ARs belong to the GTP-binding protein (G-protein) coupled family. It is also

established that these receptors are positively coupled to PLC through G-protein. The therapeutic mechanism of action of NE reuptake blockers is considered to be related to enhancement of NE transmission with concomitant activation of  $\alpha_1$ -ARs receptors.  $\alpha_1$ -AR sites have been considered to be functionally relevant to the effects of certain antidepressants (ADs) (Ressler and Nemeroff, 1999). Chronic treatment with Imipramine and other ADs has been shown to decrease (Mizuta and Segawa, 1989) or do not change (Newman *et al.*, 1990; Odagaki *et al.*, 1991)  $\alpha_1$ -ARs receptors in various regions of rat brain.

Majority of studies on Amitriptyline have been done by studying the effect on  $\alpha_1$ -ARs alone, in isolated regions, or without studying the changes in linked second messenger system. In the present study the effect of chronic administration of Amitriptyline on  $\alpha_1$ -ARs linked PLC linked IP3 system is studied in rat brain along with the *in vitro* effect on [ $^3$ H]prazosin binding to cortical and cerebellar membranes.

## MATERIALS AND METHODS

[ $^3$ H]prazosin (s.a. 27 Ci/mmol) and [ $^3$ H]myoinositol (Ci/mmol) were obtained from Amersham Int. (UK) and New England Nuclear (USA), respectively. Phentolamine, AMI were obtained from Sigma Chemicals (USA); 96-well microplates with glass fibre (GF/B) filters were obtained from M/s. Millipore, India. Other chemicals, of analytical grade, were obtained from local chemical suppliers.

**Animals and administration of drugs:** Adult male Sprague-Dawley rats weighing 200-250 g, were used for all the experiments. Animals (10 rats for each drug-a total of 30 rats), procured from Central Animal Research Facility (CARF), NIMHANS, were housed in cages (four rats per cage) and exposed to regular day/night period with food and water *ad libitum*. AMI (10 mg kg<sup>-1</sup> b.wt.), injected intraperitoneally, once daily, for a period of 30 days. As the clinical efficacy of ADs has been observed over 3-6 weeks of administration, their effects on  $\alpha_1$ -AR were studied after chronic administration for 30 days. Control rats received 0.5 mL of saline, by same route for the same period. All the animals were sacrificed by decapitation 24 h after the last injection. Brains were removed on ice-cold petri dish, cerebral cortex and cerebellum dissected out and used for membrane preparation. Tissues obtained from three rats were pooled for receptor binding assay.

**Membrane preparation:** Cortical and cerebellar tissues from three rats were pooled and membranes were obtained for radioligand binding and displacement studies. Crude

membrane pellet was obtained from brain tissue, homogenized in 20 volumes of Tris-HCl buffer (50 mM, pH 7.4) containing 0.32 M sucrose, following the procedure described by Creese and Snyder (Creese and Snyder, 1978) and as described earlier (Ramakrishna and Subhash, 2010). The pellet was resuspended in 50 mM sodium-potassium phosphate buffer (pH 7.4) for [ $^3$ H]prazosin binding studies. Protein concentration was estimated by Lowry's method (Lowry *et al.*, 1951) and made to 1 mg mL<sup>-1</sup> using respective buffers.

**Drug displacement studies:** The displacement of [ $^3$ H]prazosin binding to  $\alpha_1$ -ARs sites by Amitriptyline was done in cortical and cerebellar membranes of control rat brain. An aliquot of membrane suspension was incubated with 2.0 nM of [ $^3$ H]prazosin for  $\alpha_1$ -ARs sites, with different concentrations of Amitriptyline (10<sup>-11</sup> to 10<sup>-4</sup> M) for 2 h, at 37°C. Reaction was stopped by the addition of ice-cold Na<sup>+</sup>-K<sup>+</sup> buffer (pH 7.4) and rapidly filtered through Millipore Multiscreen filtration unit under vacuum. The filters were dried and the filters were punched into the scintillation vials containing 3 mL of scintillation fluid. Vials were allowed to equilibrate overnight and radioactivity was counted in a beta counter. The data from binding and displacement experiments was analysed using "LIGAND" programme (McPherson, 1983) and the maximal binding (B<sub>max</sub>), dissociation constant (K<sub>d</sub>), inhibition constant (K<sub>i</sub>) and concentration of drug required to displace 50% of radioligand bound (IC<sub>50</sub>) were obtained.

**$\alpha_1$ -adrenoceptor binding:** Membranes obtained from cortex and cerebellum of both experimental and control rat brain were used for binding studies. The density of  $\alpha_1$ -ARs was estimated by using [ $^3$ H]prazosin following essentially the procedure described by Hyttel *et al.* (1992). In brief: an aliquot of membrane (100  $\mu$ g protein) was incubated with 6-8 different concentrations of [ $^3$ H]prazosin (0.05-0.60 nM) in 50 mM sodium-potassium phosphate buffer (pH 7.4) in a 96 well microplate with GF/B filters, for 30 min at 37°C. Non-specific binding was defined by using phentolamine (10  $\mu$ M), the reaction mixture was rapidly filtered under vacuum. The filters were punched by punching manifold, directly into scintillation vials containing 5 mL of scintillation fluid and allowed to equilibrate overnight. Radioactivity was measured using  $\beta$ -counter (Packard, USA) with 67% efficiency.

**IP3 estimation:** The measurement of [ $^3$ H]IP3 levels was done according to the method of Chuang, D.M

(Chuang, 1989) and as reported earlier (Devaki *et al.*, 2006; Subhash and Jagadeesh, 1997). Briefly; cross-chopped slices (300  $\mu$ M) from rat brain samples (0.5 g) were prepared and incubated in oxygenated KRB buffer (pH 7.4) with 0.5  $\mu$ Ci of [ $^3$ H]myo-inositol (s.a. 17.1 Ci/mmol) for 1 h at 37°C. Tissues were washed with the same buffer and incubated with AMI (10 mM). Assay was terminated by the addition of 10% TCA. Samples were kept on ice for 20 min and homogenized. Then the tissues were sedimented by centrifugation (3000 g). Supernatant was washed 4-5 times with water-saturated diethylether. Diethylether washed TCA extract, containing the inositol phosphates, was then neutralized with NaHCO<sub>3</sub> (5 mM). [ $^3$ H]IP<sub>3</sub> formed was separated by anion-exchange chromatography (Dowex-formate form). Inositol phosphates were separated by gradient elution with ammonium formate and formic acid (Subhash and Jagadeesh, 1997). The bound [ $^3$ H]IP<sub>3</sub> was eluted from the column using 0.8 M formate and 0.1 M formic acid. The eluent was mixed with 10 mL of scintillation cocktail and radioactivity was measured in liquid scintillation counter after overnight equilibration. The amount of [ $^3$ H]IP<sub>3</sub> formed was expressed as DPM/g tissue.

**Data analysis:** The data from the binding experiments was analyzed using 'LIGAND' software programme (McPherson, 1983) to obtain the equilibrium dissociation constant (K<sub>d</sub>), the density of receptors (B<sub>max</sub>) and the Hill co-efficients. The B<sub>max</sub> and K<sub>d</sub> values were expressed in fmol/mg protein and nM, respectively. All the data is expressed as Mean $\pm$ SD. The statistical analysis was done by the Student's t-test. Differences were considered to be significant at  $p < 0.05$ .

## RESULTS

Total  $\alpha$ 1-ARs sites were labeled by [ $^3$ H]prazosin in cortex and cerebellum of both control and experimental rat brain. The density of  $\alpha$ 1-ARs sites was 73.2 $\pm$ 3.3 fmol/mg protein in cortex and 67.2 $\pm$ 1.6 fmol/mg protein in cerebellum of control rat brain. After exposure to amitriptyline there was a significant downregulation of total  $\alpha$ 1-ARs sites in all regions (Table 1). In cortex 54% of total  $\alpha$ 1-ARs sites were decreased (33.7 $\pm$ 1.9 fmoles/mg protein;  $p < 0.0001$ ), where as in cerebellum there was a 37% decrease in total  $\alpha$ 1-ARs sites (42.5 $\pm$ 4.2 fmoles/mg protein;  $p < 0.0001$ , Fig. 1). The affinity of [ $^3$ H]prazosin to  $\alpha$ 1-ARs was also similar in both the regions (Table 1). The Hill coefficient values were near to unity in all regions of

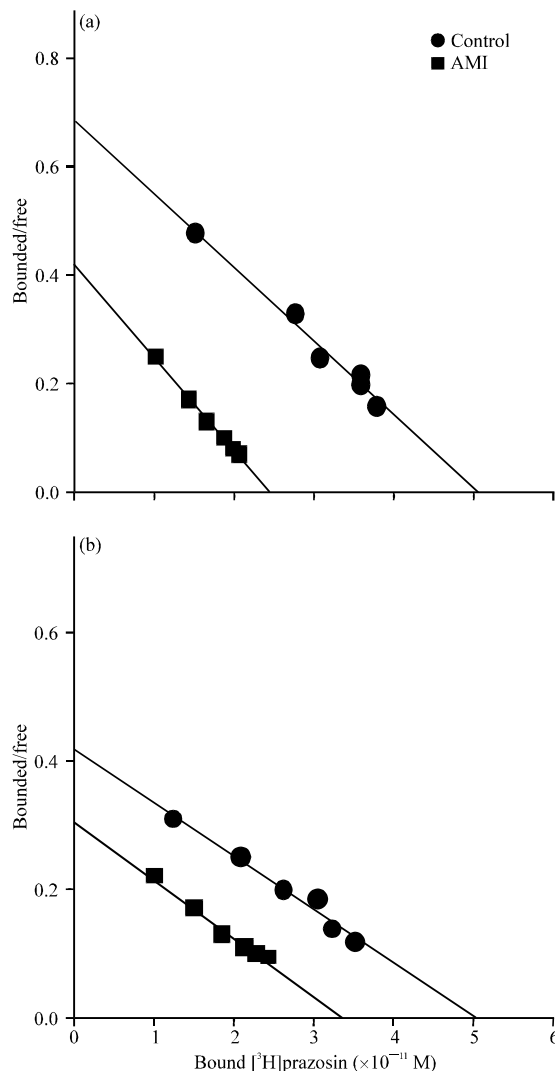


Fig. 1(a-b): Scatchard analysis of [ $^3$ H] prazosin binding to total  $\alpha$ 1-ARs sites in (a) Cortical and (b) Cerebellum membranes of rats control and exposed rats to amitriptyline (10 mg kg<sup>-1</sup> b.wt.) for 30 days

both control and experimental rat brain, suggesting that at this concentration [ $^3$ H]prazosin binds to single class of  $\alpha$ 1-ARs receptor sites.

The displacement of [ $^3$ H]prazosine binding to  $\alpha$ 1-ARs sites by Amitriptyline was studied in control rat cortex and cerebellum. It is observed that Amitriptyline displaces [ $^3$ H]prazosine binding to  $\alpha$ 1-ARs sites in a monophasic manner with K<sub>i</sub> of 182 $\pm$ 16 nM and an IC<sub>50</sub> of 558 $\pm$ 68, 290 $\pm$ 27 nM in both regions. The slope value of the displacement curve was 0.5, suggesting positive cooperativity of the drug towards  $\alpha$ 1-ARs sites (Fig. 2, 3).

Table 1: *In vivo* effect of Amitriptyline on  $\alpha_1$ -ARs sites in regions of rat brain

Regions	Bmax <sup>®</sup> Control	Bmax <sup>®</sup> Experimental	Kd (nM) Control	Kd (nM) Experimental
$\alpha_1$ -ARs in Cortex	73.2±3.3	33.7±1.9*	0.10±0.02	0.10±0.01*
$\alpha_1$ -ARs in Cerebellum	67.2±1.6	42.5±4.2*	0.11±0.01	0.10±0.02*

Binding experiments for  $\alpha_1$ -ARs sites were done using [<sup>3</sup>H]prazosin as described in text, in brain regions of rats exposed to Amitriptyline (10 mg kg<sup>-1</sup> b.wt.) for 30 days. Values are mean and SD of 3 experiments, each assayed in duplicate, <sup>®</sup>fmoles/mg protein, \*p<0.0001

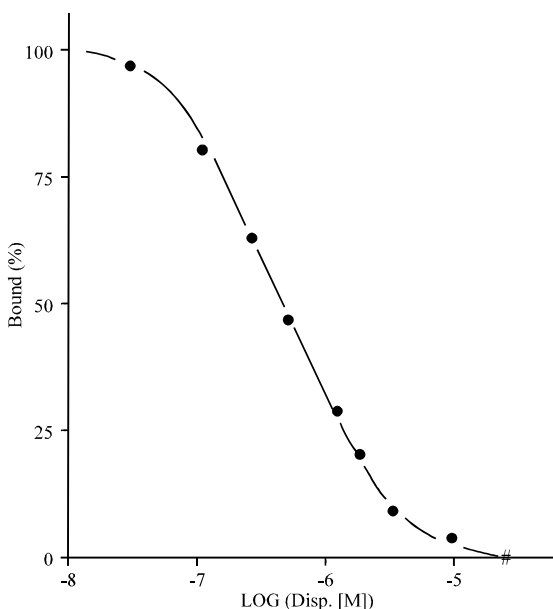


Fig. 2: Displacement of [<sup>3</sup>H]prazosin (0.2 nM) binding to  $\alpha_1$ -ARs sites in cortical membranes of control rats was done by incubating various concentrations ( $10^{-11}$ - $10^{-4}$  M) of amitriptyline at 37°C

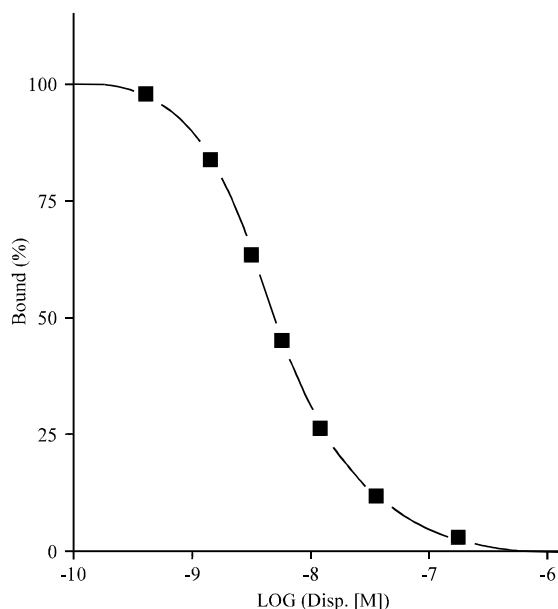


Fig. 3: Displacement of [<sup>3</sup>H]prazosin (0.2 nM) binding to  $\alpha_1$ -ARs sites in cerebellar membranes of control rats done by incubating various concentrations ( $10^{-11}$ - $10^{-4}$  M) of amitriptyline at 37°C

The displacement of [<sup>3</sup>H]prazosine binding to  $\alpha_1$ -ARs sites by Amitriptyline also showed a monophasic type of displacement. Amitriptyline displaces [<sup>3</sup>H]prazosine bound to  $\alpha_1$ -ARs sites with  $K_i$  of 290±27 nM and an  $IC_{50}$  of 730±77 nM. The slope was also near to one, suggesting that Amitriptyline binds to  $\alpha_1$ -ARs sites in a positive cooperativity manner (Fig. 2, 3).

Both basal and NE (Norepinephrine) stimulated  $IP_3$  activity was measured in cortex and cerebellum of both control and experimental rat brain. It is observed that there is a significant change in basal  $IP_3$  activity in both regions after Amitriptyline exposure. In cortex, NE stimulated  $IP_3$  activity was significantly decreased (47%, 810±76 DPM/g tissue,  $p<0.0001$ ), when compared to NE stimulated  $IP_3$  activity of 4152±286 pmoles/mg protein in control rats. In cerebellum, NE stimulated  $IP_3$  activity was significantly decreased (45%, 1188±112 DPM/g tissue,  $p<0.0001$ ), when compared to NE stimulated  $IP_3$  activity of 3952±245 pmoles/mg protein in control rats (Table 2). The decrease in NE stimulated  $IP_3$  activity in cortex and cerebellum may

be due to the significant downregulation of  $\alpha_1$ -ARs sites in both regions after Amitriptyline exposure, as these sites are positively coupled to PLC.

## DISCUSSION

Receptor binding characteristics of antidepressants have been studied in rat, pig and bovine brain, however, the effects of many antidepressants on receptor mediated second messenger system have not been extensively studied. Although reuptake inhibition alone may be sufficient to cause receptor regulation, direct interaction between ADs and receptors are possibility that have been suggested (Wong *et al.*, 1991). The profile of binding of TCA to a wide range of monoaminergic receptors are well documented. However the  $K_d$ ,  $K_i$ ,  $IC_{50}$ , type of cooperativity etc., are difficult to compare as different studies have been adopted different protocol for administration and reported changes are often inconsistent (Beasley *et al.*, 1992; Nojimoto *et al.*, 2010).

Table 2: Inositol Tri Phosphate (IP<sub>3</sub>) activity in brain regions of Amitriptyline treated rats

Regions	Basal	With NE	% of Stimulation
<b>Cortex</b>			
Control	1518±212	4152±286	175
Experimental	810±76*	1460±102*	80
<b>Cerebellum</b>			
Control	1488±112	3952±245	173
Experimental	798±81*	1188±112*	77

Values (pmoles/mg protein) are mean and SD of 3 experiments, each assayed in duplicate. IP<sub>3</sub> activity was assayed in presence and absence of NE (10 µM) in brain regions of rats exposed to Amitriptyline (10 mg Kg<sup>-1</sup> b.wt.) for 30 days. \*p<0.00001, compare to control

Amitriptyline is a tricyclic antidepressant that was synthesised in 1960 and introduced as early as 1961 in the USA, but is still regularly used. It has also been frequently used as an active comparator in trials on newer antidepressants and can therefore be called a 'benchmark' antidepressant (Leucht *et al.*, 2012). Amitriptyline has been shown to have higher affinity than desipramine and Fluoxetine at  $\alpha_1$ -ARs (Ramakrishna and Subhash, 2010). Adaptive phenomena such as desensitization of autoreceptors are considered as an important factor in the achievement of therapeutic efficacy of AD drugs with chronic treatment. Long-term but not acute administration of ADs has been shown to significantly decrease the sensitivity of  $\alpha_1$ -ARs (Vila *et al.*, 1990). Earlier reports documented that the acute *in vivo* inhibition of NE neuronal uptake by ADs leads to the activation (through endogenous NE) of presynaptic inhibitory  $\alpha_1$ -ARs and prolonged *in vivo* inhibition of NE reuptake is followed by a slow desensitization process of the same receptors (Garcia-Sevilla and Zubieta, 1986). It has been reported that G-protein function may contribute to the complex neuro adaptive mechanisms involved in the clinical actions of ADs. Changes in receptor binding after chronic administration of ADs are perhaps of more relevance.

In this study, it has been observed that chronic administration of Amitriptyline results in significant down regulation of total  $\alpha_1$ -ARs sites in both regions studied. This is due to the fact that IP<sub>3</sub> is positively coupled to  $\alpha_1$ -ARs sites. Studies have also shown a region-specific alteration of G-protein induced activation of the phosphoinositide signal transduction system and G-protein  $\alpha$ -subunits that are involved in PLC formation, in the prefrontal cortex of suicide victims with major depression (Pacheco *et al.*, 1996). Therefore, the differential and region-specific effect of ADs on  $\alpha_1$ -ARs may be understood well by studying the G $\alpha$ -proteins and linked second messenger system.

In this study the basal IP<sub>3</sub> activity was affected by chronic exposure to Amitriptyline. However the response of IP<sub>3</sub> to NE was significantly decreased after exposure. However, it is argued that this effect of ADs on PLC

(Phospholipase-C) activity is not by a direct effect on catalytic unit of the enzyme but by suppression of the regulatory function of the N-protein (Yamaoka *et al.*, 1988). Amitriptyline displayed monophasic and sigmoidal competition against [<sup>3</sup>H]prazosin binding sites in rat cortex and cerebellum. The slope values for both sites were near to 1.0, with displacement occurring at nanomolar concentrations, suggesting high affinity of the drug to these sites. The observed down regulation of  $\alpha_1$ -ARs sites with concomitant decrease in NE sensitive IP<sub>3</sub> activity, with Amitriptyline treatment, suggests that Amitriptyline has antagonistic effect at these sites. Hypersensitivity of  $\alpha_1$ -ARs receptor mediated responses has been reported in certain neuropsychiatric disorders. The role of  $\alpha_1$ -ARs receptors in mechanism of action of certain TCA has been conveniently established by animal experiments. Amitriptyline, a more potent TCA, seems to act via  $\alpha_1$ -ARs with high affinity. The effect, though, is region specific. The results of this study suggest that the mechanism of action of Amitriptyline, apart from being TCA, is by downregulating  $\alpha_1$ -ARs which in turn stimulates IP<sub>3</sub>, resulting in increased PLC (Phospholipase-C) content of the brain. It has also been proposed that the desensitisation of  $\alpha_1$ -ARs receptors may underline the ability of chronic, but not acute, TCA administration to raise synaptic cleft NE. Evidences from various behavioural studies are consistent with the observation that  $\alpha_1$ -ARs act through  $\alpha_1$ -ARs receptor activation and clinical evidences suggest that  $\alpha_1$ -ARs antagonists have antidepressant efficacy. From this study it is observed that Amitriptyline has antagonist effect at  $\alpha_1$ -ARs sites, with high affinity, suggesting a possibility for its efficacy as therapeutic agent in conditions where  $\alpha_1$ -ARs sites are altered. The observed alterations in  $\alpha_1$ -Adrenergic receptor densities are likely to be a part of adaptive neuronal changes that occur after chronic administration of Amitriptyline and may be related to antidepressant effect of the drug. This study suggests that Amitriptyline which has high affinity at  $\alpha_1$ -AR sites, acts by modulating this receptor mediated IP<sub>3</sub>-PLC signal pathway.

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