ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Differential responses of amygdala and hippocampus consequent to $A\beta_{40}$ and $A\beta_{42}$ induced toxicity in the rat brain: A comparative study.

Neha Mishra, Rameshwar Singh, Deepak Sharma*

School of Life Sciences, Jawaharlal Nehru University, New Delhi, India.

Manuscript Info

Manuscript History:

Received: 14 November 2015
Final Accepted: 22 December 2015
Published Online: January 2016

Key words:
Amygdala; Hippocampus; Oxidative stress; Amyloid beta-protein (1-40); Amyloid beta-protein (1-42); Cognition; Social behavior.

Corresponding Author

Deepak Sharma.

Abstract

Amyloid beta (Aβ) peptides are the principal constituents of senile plaques of Alzheimer's disease (AD) brain, and are thought to play an important role in the etiology and pathogenesis of AD. The present study assesses the responses of brain regions (hippocampus and amygdala) to amyloid toxicity in the light of behavioral and oxidative parameters. Aggregated $A\beta_{40}$ and $A\beta_{42}$ were stereotaxically injected into the hippocampus or amygdala, and their effects on cognitive (Morris water maze test) and non-cognitive (fear, anxiety, general emotional state: open field and light and dark chamber tests) behaviors were studied. Since human-specific social behaviors (empathy, sympathy etc.) also exist in rodents, the effect on these behaviors was also determined by three-chamber social behavior test. The oxidative stress generated by amyloid-β peptides is thought to contribute to the disease-associated behavioral deficits. Therefore, the present study also investigated the oxidative stress produced in the rat brain following amyloid injections. The oxidative stress produced by Aβ peptides was higher in the hippocampus compared with that in the amygdala. Similarly greater behavioral anomalies were caused in animals with intrahippocampal administration than in those with intraamygdalar administration. Thus, hippocampus showed a higher vulnerability to amyloid toxicity than amygdala. Furthermore, the results demonstrated that the oxidative stress spread from the injected site to distant brain regions like cortex, midbrain, cerebellum, and medulla. The results also showed that compared with $A\beta_{40}$, $A\beta_{42}$ generated higher levels of oxidative stress and produced more severe behavioral deficits.

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Introduction:-

The deposition and accumulation of amyloid β-peptide (Aβ) in neuritic plaques and neurotoxicity of Aβ are considered central to the pathogenesis of Alzheimer's disease (AD) (Hardy et al., 1991; Hardy et al., 1992; Masters et al., (1985); Wisniewski et al., 1994). Exogenously injected Aβ rodent model is a common model used in the Alzheimer's disease related research. The amyloid plaques are said to be the loci of oxidative stress as Aβ can produce reactive oxygen species (Behl et al., 1992; Behl et al., 1994; Pike et al., 1991). AD brain cells exhibit abnormally high amounts of oxidatively modified proteins (Hensley et al., 1995; Lyras et al., 1997; Smith et al., 1991), lipids (Hajimohammadreza et al., 1990; Hensley et al., 1995; Lovell et al., 1995; Lovell et al., 1997; Lyras et al., 1997; Marcus et al., 1998; McIntosh et al., 1997; Subbarao et al., 1990.), and DNA (Gabbita et al., 1998; Markesbery et al., 1999; Mecocci et al., 1994.). Aβ peptide-induced oxidative stress is also thought to contribute to cognitive deficits (Jhoo et al., 2004).

Characteristic behavioral symptoms associated with AD are of great clinical interest and numerous researchers have administered different amyloid peptides into the brain to study various behavioral anomalies and biochemical effects, yielding many contradicting reports of amyloid toxicity (Borbély et al., 2014; Cioanca et al., 2014; Cleary et

al., 1995; De Ferrari et al., 2003; Dornam et al., 1993; Flood et al., 1991; Flood et al., 1994; Ginsberg et al., 2013; Giovannelli et al., 1995; Hritcu et al., 2014; Maurice et al., 1996; McDonald et al., 1994; Richardson et al., 2002; Sigurdsson et al., 1995; Stepanichev et al., 2003.).

A plausible explanation for such contradictory reports could be that not all brain regions are comparable and are vulnerable to amyloid toxicity to varied degrees. No study has been performed till date to simultaneously assess the effect of injecting an amyloid peptide into more than one brain region simultaneously. There is a great behavioral impact of AD and therefore, experimentally it is of interest to study the behavioral consequences following intra-brain injections of Aβ in different brain sites.

The present study was therefore aimed at determining the behavioral anomalies and severity of oxidative stress produced in the different rat brain regions following injections of aggregated $A\beta_{40}$, the most abundant amyloid form (Irvine et al., 2008), into the amygdala or hippocampus with a view to assess the differential susceptibility of the two brain regions i.e. effect of same peptide on different brain regions to assess the vulnerability of the brain region to amyloid induced toxicity. The results were further confirmed with aggregated $A\beta_{42}$, considered to be the most toxic and may be critically important in the oxidative stress of the AD brain (Jarrett et al., 1993; Xiao et al., 2015). Furthermore, we also examined whether effects spread and appear in the distant sites. Finally, the effects of $A\beta_{40}$ and $A\beta_{42}$ pathology were also compared i.e. the effect of different peptides on the same brain region to assess the inherent toxicity of the peptide.

The hippocampus and amygdala are among the brain regions that are severely affected in AD. Hippocampus is intricately involved in memory and cognition and is one of the primary centers for accumulation of amyloid plaques. Amygdala also shows severe pathology in AD and is reported to be one of the sites where the density of senile plaques is very high, in the brain of AD patients (Shoghi-Jadid et al., 2002). It together with the hippocampus is a part of the limbic system, and is involved in emotional conditioning. Hippocampus (Du, 2001; Jack et al., 1997; West et al., 1994), and amygdala (Cuenod, 1993) also shows physiological modifications in Alzheimer's disease. Thus, these regions were chosen for the study.

Since Aβ-induced cognitive behavioral neurotoxicity occurs in AD, studies of cognitive dysfunction and behavioral consequences in Aβ-induced pathology in experimental animals are considered to be of great interest (Richardson et al., 2002; Yamada et al., 1999). Therefore, the present study also focuses on the behavioral consequences of the two Aβ peptides by studying cognitive (spatial cognition) as well as non-cognitive (fear, anxiety, general emotional state) behaviors using Morris water maze test, open field test, light and dark chamber test. As human-specific behaviors such as empathy, sympathy and pro-social behaviors also exist in rodents (Mogil, 2012), and have been shown to be altered in AD patients, it should be of interest to investigate whether these behaviors are also impaired in experimental animals with Aβ-induced pathology. In the present study, therefore, social behavior was also studied in Aβ-injected animals by using three-chamber sociability test (Kerr et al., 2013). In previous studies, Aβ effects on non-cognitive behaviors (i.e. sociability) have not been studied.

The oxidative stress was assessed by determining the levels of antioxidant enzymes: superoxide dismutase, glutathione peroxidase, glutathione reductase, and oxidative damage indicators: lipid peroxidation, protein oxidation, and the total thiol content in brain regions. Most studies so far have focused on the oxidative stress manifested by lipid peroxidation and protein oxidation. In the present study hence, additional oxidative stress parameters i.e., glutathione peroxidase and reductase, superoxide dismutase and total thiol content were also studied. Since secondary oxidant toxic products such as hydroxynonenal can diffuse from their site of origin (i.e. amyloid plaques) and cause damage at more distant sites (Varadarajan et al., 2000), it was also considered to be of interest to determine the extent of the oxidative stress in the sites distant from the areas where Aβ was injected.

Materials and methods:-

Reagents:-

$A\beta_{42}$ and $A\beta_{40}$ were procured from Sigma Aldrich Chemical Company USA in lyophilized powder form. Di-nitrophenylhydrazine (DNPH), guanidine HCl and BSA (bovine serum albumin) were purchased from the Sigma Chemical Co., USA. All other chemicals were obtained from Merck and Hi-media. All chemicals used were of analytical grade.

Amyloid beta aggregation:-

$A\beta_{42}$ aggregation was performed as per the protocol of Soto et al., (1998). The lyophilized powder was dissolved in 16.7% dimethylsulfoxide (DMSO) to prepare a 2nmolar $A\beta_{42}$ solution. The $A\beta_{42}$ was incubated for 48 hours at 37°C (without shaking) to induce aggregation. $A\beta_{40}$ was aggregated into beta sheets following the protocol of Zheng et al., (2008). The lyophilized powder was dissolved in 0.9% saline to a concentration of 5mg/ml and incubated at 37°C (without shaking) for one week to induce aggregation.

Animals:-
Forty-eight male Wistar rats of 6 months age at the beginning of the experiment were taken for this study. Rats were housed in pairs, in standard laboratory cages 8 × 12 × 5-in. made of polypropylene with stainless-steel covers, and maintained at 23 ± 4 °C, under a 12-hour- light/12-hour-dark cycle. All experimental protocols were approved by the Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA) and the Institutional Animal Ethical Committee (IAEC) of Jawaharlal Nehru University, New Delhi, India. Each animal was provided *ad libitum* access to food and water. The health status of each rat was checked by observing various criteria such as: tail sores, posture hunch, grooming, nose red rim, red eye rims, tumors, and teeth (Sharma et al., 1993). After surgery rats were housed individually and continuously monitored for their health status.

The animals were divided into eight groups containing 6 animals in each group (n = 6).

Group 1: consisted of animals in which saline was injected into the hippocampus ($A\beta_{40}$ Hippocampus Control). This is the control group for group 2 animals. Group 2: consisted of animals in which $A\beta_{40}$ was injected into the hippocampus ($A\beta_{40}$ Hippocampus Test). Group 3: consisted of animals in which saline was injected into the amygdala ($A\beta_{40}$ Amygdala Control). This is the control group for group 4 animals. Group 4: consisted of animals in which $A\beta_{40}$ was injected into the amygdala ($A\beta_{40}$ Amygdala Test). Group 5: consisted of animals in which 16.7% DMSO in distilled water was injected into the hippocampus ($A\beta_{42}$ Hippocampus Control). This is the control group for group 6 animals. Group 6: consisted of animals in which $A\beta_{42}$ was injected into the hippocampus ($A\beta_{42}$ Hippocampus Test). Group 7: consisted of animals in which 16.7% DMSO in distilled water was injected into the amygdala ($A\beta_{42}$ Amygdala Control). This is the control group for group 8 animals. Group 8: consisted of animals in which $A\beta_{42}$ was administered into the amygdala ($A\beta_{40}$ Amygdala Test).

Surgery procedures:-
Animals were anesthetized with isoflurane (5%) and were placed in a stereotaxic apparatus (Surgivet, ISOTEC 4) for administration of Aβ peptides via stereotaxic surgery under 2.5% anesthesia. The head was shaved, a surgical incision was made in the scalp and the underlying muscles and tissues were cleared to expose the skull surface. Burr holes, 0.5mm in diameter, were drilled into the skull bone at the stereotaxically marked sites. Stereotaxic co-ordinates for the hippocampus were: -3.0mm posterior to bregma, -2.0mm lateral and -3.3mm ventral to skull surface; and for amygdala were: -3.0mm posterior to bregma, -4.6mm lateral and -8.8mm ventral to skull surface. The stereotaxic co-ordinates were calculated using the Paxinos and Watson's rat brain atlas (2007). 3 µl $A\beta_{42}$, and 2µl $A\beta_{40}$ and of the before mentioned concentration, were delivered bilaterally for the test subjects and corresponding volumes of their respective vehicles were delivered in the control subjects using a cannula and Hamilton syringe. After the injections, the burr holes were sealed with bone wax, the incision was stitched up and the animal was kept under observation till recovery.

Behavioral tasks:-
Animals were given a recovery period of one week after the surgery, before the commencement of behavioral experiments. The details of the housing of animals and the timeline of surgery and behavioral tests performed are given in Table1.

Morris water maze test:-
Morris water maze task was performed to investigate visuo-spacial memory and hippocampal integrity, in accordance with the method described by Morris, (1984) with minor modifications, following the protocol of Jyoti et al (2009). Briefly, the maze consisted of a black painted circular tank of 168 cm diameter and 50 cm depth; divided into 4 equal quadrants, each represented by maze cues of different sizes, shapes and colors. A black circular platform (camouflaged) with an escape of 15 cm diameter and 2.0 cm under the surface of the water was positioned at the center or in one of the quadrants. Animals were habituated to the experimental conditions prior to experimentation by placing them on the water tank for 60sec without platform and were monitored to establish their swim speeds were similar. Thereafter, initial training for one day was given to animals to find the platform within 60

sec, failing which they were guided to reach the platform and allowed to remain on the platform for 15 sec. Each rat was placed inside the water tank facing the tank wall, at one of the four randomly selected entry points.

The time taken by the animal to reach the platform was termed latency time and was recorded. Each rat was exposed to the task for six consecutive days with ten probe trials of 60s each per day per animal. Recording was performed from 11:00 AM to 2:00 PM to exclude the performance variations resulted due to circadian rhythmicity.

Open field test:-

Open field test is a test for anxiety-like behavior in animals (Prut et al., 2003) and was performed by modifying the method as described by Sethi et al., (2008). The test is based on the premise that when an animal is subjected to an unknown environment from which escape is prevented (by surrounding walls), rodents spontaneously prefer the periphery of the apparatus to activity in the central parts of the open field. In our study, open field test was performed in a square arena (70cmX70cmX106cm) with a floor divided into 49 identical squares of 10 cm length. The field was divided into peripheral squares (24) and the central squares (25). At the beginning of the test, the rat was placed in the center of the open field. Before each trial, the field was cleaned thoroughly with 0.1% acetic acid solution.

The parameters evaluated were horizontal locomotor activity or ambulatory activity (total ambulatory activity; and percentage ambulatory activity in the center vs. periphery) defined as number of squares crossed; vertical locomotor activity or rearing frequency defined as number of times the animals stood on their hind legs (Colomina et al., 1999; Suarez-Fernandez et al., 1999); and defecation index calculated by counting the number of fecal boles. The test was performed for 5 consecutive days with six trials of 3min each per day per animal.

Light-dark chamber test:-

The light and dark chamber test was conducted to assess the anxiety levels of the subjects and corroborate them with open field test. As has been entrenched, this test is principled on the conflicting paradigms of exploratory behavior of novel environment and nocturnal habitat and aversion of open fields in rats. It was conducted as per the method described by Miller et al., (2011). The light-dark box was made of transparent ($38X35X38cm^3$) and black opaque ($38X26X38cm^3$) plexiglas, connected by a $10\times10cm^2$ door. The light chamber was well illuminated (640lux) and was the aversion chamber as compared to the opaque small dark chamber, which was the safe chamber. The rats were naïve to the apparatus. Animals were placed in the middle of the light chamber facing the side away from the dark chamber and then released. Parameters monitored were: percentage of time spent in the light chamber vs. dark chamber, full body transitions between light and dark chambers or crossings, and rearing activity in the light chamber. The test was conducted for 2 consecutive days with three trails of 5min each per day per animal.

Three-chamber social behavior test:-

The three-chamber social behavior test was conducted to assess the social and individual recognition/avoidance of the subjects. The test is based on the principle established by J. N. Crawley, (2004) of sociability in rodents. The apparatus was made of three identical chambers ($31.5X31.5X31.5cm^3$) made of transparent plexiglas, connected by a $10\times10cm^2$ doors. Test subject was housed with a same sex, age matched conspecific animal 4 days prior to the beginning of experiment and throughout the duration of the experiment. In the left and right chambers, familiar rat (animal the subject is housed with) and unfamiliar/novel rat (animal the subject has had no encounter with previous to the experiment day) are kept in bar-wired cages. The bars in the left and right cages are such that the animals can interact without fighting or harming each other. Test subject was placed in the middle chamber and allowed to explore the apparatus. Parameters monitored were duration of time spent in the each chamber, the chamber of first entry i.e. primary entry, and close contacts with the familiar or unfamiliar/novel animal. The chamber of primary entry was explored because Nadler, et al., (2004), had shown that the first minute of the trial was more important and usually the chamber of primary entry is the chamber where the rat spends more time later in the trial. Close contacts were defined as the tactile contacts made by the test animal with the wire-caged conspecifics, either with the nose (sniffs) or fore paws or any other body part actively touching the wired cage. Close contacts reflect actual physical acts of exploration, and curiosity towards, the conspecific versus nonsocial exploration of other areas of the chamber. If the time spent in the middle chamber is greater than the time spent in the left or right chambers (combined), the animal is said to be non-sociable as opposed to sociable if it prefers to stay in the left or right chamber. A greater affinity towards the familiar animal is marked as social familiarity and towards the unfamiliar/novel (used interchangeably in the text) is marked as social novelty. Prior to the commencement of the experiment, the subject is placed in the middle chamber for 10min habituation period, during which the central area

becomes a familiar "home base". The familiar and novel rats are also previously habituated to the wired cage, so that they are generally inactive and sit quietly in the wire cage during the test sessions. The position of the familiar and unfamiliar animals is changed periodically to eliminate position effects. The test was conducted for 2 consecutive days with three trials of 5min each per day per animal.

Preparation of tissue homogenates:-

Animals of each group were sacrificed by cervical dislocation after the behavioral experiments. Brains were quickly taken out and micro-dissected into amygdala, hippocampus, cortex, cerebellum, medulla, and midbrain, according to the stereotaxic atlas of Paxinos and Watson (Paxinos et al., 2007). The tissue was thoroughly washed with saline to remove blood and stored at -80◦C. Biochemical assays were performed separately in six animals of each group. Tissue samples were homogenized in 50 mMTris (pH 7.4) with a Potter-Elevehijam type homogenizer fitted with Teflon plunger. The homogenate was diluted 1:10 (with Tris, pH 7.4, buffer) and centrifuged at 6000 rpm for 5 min in a refrigerated centrifuge (Sorvall RCS or RC5C). The resulting pellet (P1), consisting of nuclear and cellular material, was discarded. The supernatant (S1), containing mitochondria, synaptosomes, microsomes and cytosol, was further ultracentrifuged at 25,000 rpm for 25 min to form mitochondrial pellet (P2). The resulting supernatant (S2) was used as such as cytosolic fraction. All biochemical tests were performed using the cytosolic fraction.

Biochemical assays:-

Superoxide dismutase (SOD) activity was measured by the method of Marklund et al., (1974). The SOD activity was assayed by following the auto-oxidation of pyrogallol at 420 nm using a Shimadzu UV-260A spectrophotometer. The activity was expressed as units/milligram protein, where a unit is equivalent to the amount of SOD required to inhibit the 50% of pyrogallol auto-oxidation. The enzyme glutathione peroxidase (GPx) was assayed according to the method of Flohe et al., (1984). The assay takes advantage of the concomitant oxidation of NADPH (nicotinamide di-phosphate reduced) by glutathione reductase (GR), which is measured at 340 nm. Enzyme activity is expressed as units/mg protein. The enzyme glutathione reductase (GR) was assayed according to the method of Mohandas et al., (1984), briefly the disappearance of NADPH at 340 nm and was calculated as nmol NADPH oxidized/min/mg protein using molar extinction coefficient of $6.22 \times 10^{-3} M^{-3} cm^{-3}$. Lipid peroxidation (LP) was estimated by measuring the TBA-RS levels spectrophotometrically at 532 nm according to the method of Ohkawa et al., (1979). LP levels were expressed as micromoles of TBA-RS formed per milligram protein. Protein oxidation (PO) was measured by estimating the protein carbonyl levels by the method of Reznick et al., (1994) and Lui et al., (2010). Protein carbonyl content was determined in the samples by measuring the DNPH adducts at 375 nm by using a Shimadzu UV-160A spectrophotometer. Carbonyl contents were calculated by using a molar extinction coefficient (e) of 22,000 $M^{-1}cm^{-1}$. Data were expressed as nanomoles carbonyl per milligram of soluble extracted protein. Total thiol (TT) was assayed by the method of Aksenov et al., (2001) on the basis of the reaction of 5,5-dithiobis- (2-nitrobenzoic acid) that is readily reduced by sulfhydryls forming a yellow substance, which is measured at 412 nm. Protein estimation was performed by method of Bradford et al., (1976) using bovine serum albumin as standard.

Statistical analysis:-

Data were expressed as mean±standard deviation (S.D.). Statistical comparison was performed by Repeated measures two-way ANOVA followed by Holm-Sidak pairwise analysis for observing the effect of each day of trail in Morris water maze. Two-way ANOVA followed by Holm-Sidak pairwise analysis was used for all the behavioral experiments and for the biochemical parameters. Calculated probabilities of < 0.05 were considered to be of significance and < 0.001 highly significant, respectively. Pearson's correlation analysis was performed for behavioral parameters: total ambulatory activity (OFT) and time spent with unfamiliar rat (three chambered social behavior test); and between all the parameters of three-chamber social behavior test. Co-efficient (r) values >0.5 were considered indicators of strong correlation, $0.4<r<0.5$ of medium correlation, and <4 of week correlation. Positive values indicated the factors tend to increase together and negative values indicated inverse relationship between the factors. All tests were performed using Sigma Plot software version 11.0.

Results:-

Behavioral effects following intrahippocampal/intraamygdalar $A\beta_{40}/A\beta_{42}$ injection

Morris water maze test:-
Administration of $A\beta_{40}$ or $A\beta_{42}$ intrahippocampally or intraamygdalarly increased the latency to reach the platform as compared to their respective controls (Fig.1). The effect of amyloid administration and latency to find the platform on consecutive days of trials [$F_{(5, 15)} = 17.7$; $p<0.001$] was found to be significant. The interaction between brain region and days (brain region X days) was not found to be significant [$F_{(5, 15)} = 0.08$; $p = 0.9$].

The interaction between the treatment and the brain region was found to be significant for day2 [$F_{(3,40)} = 77.4$; $p<0.001$], day3 [$F_{(3,40)} = 132.2$; $p<0.001$], day4 [$F_{(3,40)} = 52.9$; $p<0.001$] and day5 [$F_{(3,40)} = 45.3$; $p<0.001$]; and was not found to be significant for day1 [$F_{(3,40)} = 0.3$; $p=0.9$] and day6 [$F_{(3,40)} = 0.8$; $p=0.5$] of the trail. On day1 the effect of treatment was found to be significant [$F_{(3, 40)} = 50.8$; $p<0.001$] but the effect of brain region was not found to be significant [$F_{(1, 40)} = 0.01$; $p = 0.8$]. On day6 the effect of treatment [$F_{(3, 40)} = 43.8$; $p<0.001$] and brain region [$F_{(3, 40)} = 4.7$; $p<0.05$] were both found to be significant.

This confirms that amyloid beta has differential toxicity for different brain regions; hence some regions are more susceptible to amyloid induced damage compared with other regions. Upon comparing overall results the amygdalar region was more susceptible to toxicity by amyloid injections. Greater memory impairment was observed with $A\beta_{42}$ peptide and hence was found to be more toxic.

Open field test:-
Total horizontal locomotor activity or total ambulatory activity (total) (Fig.2A): Administration of $A\beta_{40}$ or $A\beta_{42}$ intrahippocampally or intraamygdalarly increased the total ambulatory activity as compared to their respective controls. The interaction of brain region of amyloid administration and the amyloid peptide (Brain region X Treatment) was significant [$F_{(3, 40)} = 111.4$; $p<0.001$].

Percentage horizontal locomotor activity or percentage ambulatory activity (percentage ambulatory activity in the center vs. the periphery) (Fig.2B): Administration of $A\beta_{40}$ or $A\beta_{42}$ intrahippocampally or intraamygdalarly increased the percentage ambulatory activity in the center vs. the periphery as compared to their respective controls. The interaction of brain region of amyloid administration and the amyloid peptide (Brain region X Treatment) was significant [$F_{(3, 40)} = 3.7$; $p<0.05$].

Vertical ambulatory activity or rearing frequency (Fig.2C): Administration of $A\beta_{40}$ or $A\beta_{42}$ intrahippocampally or intraamygdalarly significantly decreased the rearing frequency as compared to their respective controls. The interaction of brain region of amyloid administration and the amyloid peptide (Brain region X Treatment) was significant [$F_{(3, 40)} = 4.6$; $p<0.05$].

Defecation index (Fig.2D): Administration of $A\beta_{40}$ or $A\beta_{42}$ intrahippocampally or intraamygdalarly significantly increased the defecation index as compared to their respective controls. The interaction of brain region of amyloid administration and the amyloid peptide (Brain region X Treatment) was significant [$F_{(3, 40)} = 3.0$; $p<0.05$].

Since all the parameters studied in open field test were found to show a significant interaction between the brain region of administration and amyloid peptide, hence, the same amyloid oligomer affects different brain regions differentially. Quantitatively greater anxiety, fear, and emotional alterations were observed with amygdalar administration of peptides; hence amygdala seemed to be more severely affected by amyloid toxicity.

Light and dark chambered test:-
Percentage of time spent in light vs. dark chamber (Fig.3A): Both $A\beta_{40}$- and $A\beta_{42}$-injected intrahippocampally and intraamygdalarly animals showed increased duration of time spent by them in the dark chamber as compared to their respective controls. The interaction of brain region of amyloid administration and the amyloid peptide (Brain region X Treatment) was significant [$F_{(3, 40)} = 89.4$; $p<0.001$].

Full body transitions between light and dark chamber or crossings (Fig.3B): Both $A\beta_{40}$- and $A\beta_{42}$-injected animals showed decreases in full body transitions from light chamber to dark chamber as compared to their respective controls. The interaction of brain region of amyloid administration and the amyloid peptide (Brain region X Treatment) was significant [$F_{(3, 40)} = 196.6$; $p<0.001$].

Rearing activity (Fig.3C): Both $A\beta_{40}$- and $A\beta_{42}$-injected animals showed decreases in rearing activity in the light chamber as compared to their respective controls. The interaction of brain region of amyloid administration and the amyloid peptide (Brain region X Treatment) was significant [$F_{(3, 47)}= 11.7$; $p<0.001$].

Upon comparing overall results, the hippocampus region was more susceptible to toxicity by amyloid injections. Greater anxiety was observed with $A\beta_{42}$ peptide.

Three-chamber social behavior test:-

Sociability (Fig.4A): Both $A\beta_{40}$- and $A\beta_{42}$-intrahippocampally and intraamygdalarly-injected animals showed increased duration of time spent by them in the middle chamber as compared to their respective controls. The interaction of brain region of amyloid administration and the amyloid peptide (Brain region X Treatment) was found to be significant [$F_{(3, 40)}= 4.6$; $p<0.05$].

Chamber of primary entry (Fig.4B): Both $A\beta_{40}$- and $A\beta_{42}$-intrahippocampaaly and intraamygdalarly injected animals showed decreased number of primary entries into the familiar chamber as compared to their respective controls. The interaction of brain region of amyloid administration and the amyloid peptide (Brain region X Treatment) was not significant [$F_{(3, 40)} = 1.6$; $p<0.21$]. The effect of brain region was found to be significant [$F_{(1, 40)} = 4.4$; $p<0.05$] and treatment was also found to be significant [$F_{(3, 40)} = 9.8$; $p<0.001$].

Time spent in the chamber of the familiar animal (Fig.4C) vs. novel (unfamiliar) animal (Fig.4D): Both $A\beta_{40}$- and $A\beta_{42}$-intrahippocampaaly and intraamygdalarly-injected animals showed decreases in the time spent with the familiar animal as compared to their respective controls. The amyloid injected animals also showed increases in the time spent with the unfamiliar animal as compared to their respective controls. The interaction of brain region of amyloid administration and the amyloid peptide (Brain region X Treatment) was found to be significant for time spent with familiar rat [$F_{(3,40)}= 65.6$; $p<0.001$]; and was also found to be significant for the time spent with unfamiliar rat [$F_{(3,40)}= 122.1$; $p<0.001$.

Close contacts with the familiar animal (Fig.4E) vs. novel (unfamiliar) animal (Fig.4F): Both $A\beta_{40}$- and $A\beta_{42}$-intrahippocampaaly and intraamygdalarly-injected animals showed decreases in the number of close contacts with the familiar animal as compared to their respective controls. The amyloid injected animals also showed increases in the number of close contacts with the unfamiliar animal as compared to their respective controls. The interaction of brain region of amyloid administration and the amyloid peptide (Brain region X Treatment) was significant for the close contacts with familiar rat [$F_{(3, 40)}= 5.0$; $p<0.001$] and close contacts with unfamiliar rat [$F_{(3, 40)}= 24.0$; $p<0.001$].

The overall results indicate that the hippocampus region was more susceptible to alterations in the sociability as compared to the amygdala, upon administration of the amyloid peptides. Quantitatively more deviation in social interaction was observed in the $A\beta_{42}$ peptide injected animals.

Correlation analysis:-

Pearson's correlation analysis was done to assess relationship between total ambulatory activity (OFT) and time spent with unfamiliar rat (three-chamber social behavior test): The increased ambulatory activity of the amyloid injected subjects in the open field test indicated hyperactivity or hyper reactivity of the animals to the test environment. These observations are similar to the increased locomotor activity of the young when circuits in the hippocampus are not mature. Thus, injection of amyloid into the brain could be resulting in disinhibition of hippocampal function in terms of controlling an ongoing behavior. These kinds of increases in activity at the behavioral level, in an unfamiliar open field were positively correlated with aggression towards a strange conspecific by Brain et al., (1969). Thus, correlation analysis was done between the total ambulatory activity and time spent with the unfamiliar rat in amyloid-injected rats and their respective control groups. In the amyloid-injected animals (Fig.5A.) a positive correlation ($r=0.583$; $p<0.001$) was observed between the two parameters; whereas, in the control animals no significant relationship was seen between the two variables ($r=0.263$, $p=0.215$) (Fig.5B).

Pearson's correlation analysis was also done to measure the degree of sociability by calculating the interaction between intra-social behavior parameters:

1. Chamber of primary entry and time with familiar animal.
2. Chamber of primary entry and time with unfamiliar animal.
3. Chamber of primary entry and close contacts with familiar animal.
4. Chamber of primary entry and close contacts with unfamiliar animal.
5. Time with familiar animal and time with unfamiliar animal.
6. Time with familiar animal and close contacts with familiar animal.
7. Time with familiar animal and close contacts with unfamiliar animal.
8. Time with unfamiliar animal and close contacts with familiar animal.
9. Time with unfamiliar animal and close contacts with unfamiliar animal.
10. Close contacts with familiar animal and close contacts with unfamiliar animal.

It was observed in the three-chamber social behavior test that the amyloid injected animals showed greater inclination towards the unfamiliar animals (spent more time with unfamiliar as compared to the familiar animal, larger number of close contacts towards the unfamiliar rat) as opposed to the control rats which had greater affinity for the familiar conspecifics (spent more time with familiar as compared to the unfamiliar animal, larger number of close contacts towards the familiar rat). From these it is apparent that time spent with the unfamiliar animal is strongly correlated to close contacts made with the unfamiliar animal ($r=0.851$; $p= 1.41e^{-7}$) and inversely correlated with the time spent with the familiar animal ($r= --0.983$; $p= 1.07e^{-17}$) as well as close contacts made with the familiar animal ($r= --0.654$; $p= 5.27e^{-4}$) for the amyloid injected animals (Fig.6A). The control animals on the other hand showed inverse relationship between the close contacts with familiar rat and the close contacts with unfamiliar rat ($r= --0.638$, $p= 7.92e^{-4}$) (Fig.6B).

Oxidative stress effects:-

Superoxide dismutase:-

Both $A\beta_{40}$- and $A\beta_{42}$-intrahippocampally and intraamygdalarly-injected animals showed decreases in the SOD levels as compared to their respective controls (Fig.7). The interaction of brain region of amyloid administration and the amyloid peptide (Brain region X Treatment) was found to be significant for hippocampus [$F_{(3, 40)}= 10.8$; $p<0.001$] and amygdala [$F_{(3, 40)}= 75.1$; $p<0.001$].

Comparing the changes in SOD levels in the local regions of administration of the peptides, the hippocampus was more susceptible to toxicity by the amyloid peptide.

Glutathione reductase:-

Both $A\beta_{40}$- and $A\beta_{42}$-intrahippocampally and intraamygdalarly-injected animals showed decreases in the GR levels as compared to their respective controls (Fig.8). The interaction of brain region of amyloid administration and the amyloid peptide (Brain region X Treatment) was significant for hippocampus [$F_{(3, 40)}= 164.0$; $p<0.001$] and amygdala [$F_{(3, 40)}= 57.8$; $p<0.001$].

Comparing the changes in GR levels in the local regions of administration of the peptides, the hippocampus was more susceptible to toxicity by the amyloid peptide.

Glutathione peroxidase:-

Both $A\beta_{40}$- and $A\beta_{42}$-intrahippocampally and intraamygdalarly-injected animals showed decreases in the GPx levels as compared to their respective controls (Fig.9). The interaction of brain region of amyloid administration and the amyloid peptide (Brain region X Treatment) was significant for hippocampus [$F_{(3, 40)}= 145.9$; $p<0.001$] and amygdala [$F_{(3, 40)}= 6.6$; $p<0.05$].

Comparing the changes in GPx levels in the local regions of administration of the peptides, the hippocampus was more susceptible to toxicity by the amyloid peptide.

Lipid peroxidation:-

Both $A\beta_{40}$- and $A\beta_{42}$-intrahippocampally and intraamygdalarly-injected animals showed increases in the LP levels as compared to their respective controls (Fig.10). The interaction of brain region of amyloid administration and the amyloid peptide (Brain region X Treatment) was significant for hippocampus [$F_{(3, 40)}= 195.2$; $p<0.21$] and amygdala [$F_{(3, 40)}= 144.9$; $p<0.001$].

Comparing the changes in LP levels in the local regions of administration of the peptides, the hippocampus was more susceptible to toxicity by the amyloid peptide.

Protein oxidation:-

Both $A\beta_{40}$- and $A\beta_{42}$-intrahippocampally and intraamygdalarly-injected animals showed increases in the PO levels as compared to their respective controls (Fig.11). The interaction of brain region of amyloid administration and the amyloid peptide (Brain region X Treatment) was significant for hippocampus [$F_{(3, 40)} = 161.8$; $p<0.001$] and amygdala [$F_{(3, 40)} = 35.0$; $p<0.001$].

Comparing the changes in PO levels in the local regions of administration of the peptides, the hippocampus was again more susceptible to toxicity by the amyloid peptide.

Total thiol:-

Both $A\beta_{40}$- and $A\beta_{42}$-intrahippocampally and intraamygdalarly-injected animals showed increases in the TT levels as compared to their respective controls (Fig.12). The interaction of brain region of amyloid administration and the amyloid peptide (Brain region X Treatment) was not found to be significant for hippocampus [$F_{(3, 40)} = 0.9$; $p<0.42$] and was found to be significant for amygdala [$F_{(3, 40)} = 4.0$; $p<0.05$].

Comparing the changes in TT levels in the local regions of administration of the peptides, the hippocampus was more susceptible to toxicity by the amyloid peptide.

Oxidative stress effects by $A\beta_{40}$ vs. $A\beta_{42}$:-

$A\beta_{42}$ seems to induce a greater degree of oxidative stress than $A\beta_{40}$ (Table-2). This can be established from the fact that the increases in PO levels and TT levels were quantitatively greater simultaneously with greater decreases in SOD and GPx levels in $A\beta_{42}$ intrahippocampally and intraamygdalarly-amyloid-injected animals as compared to $A\beta_{40}$ ones. Only LP was slightly more elevated by $A\beta_{40}$.

Discussion:-

The present study gives an account of the differential degree of vulnerability of different brain regions to amyloid induced toxicity. It also provides an exhaustive comparative account of behavioral changes and oxidative stress toxicity of $A\beta_{40}$ and $A\beta_{42}$ when injected into the hippocampus or amygdala regions of the rat brain, at the site of injection as well as distant regions of the rat brain. Both the cognitive and non-cognitive behaviors are significantly altered upon amyloid administration as compared to their respective controls. Also, both $A\beta_{40}$ and $A\beta_{42}$ are toxic at the site of administration (injection) and the toxic effects extend (spread) to the distant regions of the brain as well. Hippocampal region was found to be more susceptible to amyloid-induced toxicity than amygdala. Also, $A\beta_{42}$ seemed to produce quantitatively greater effects (both behavioral and oxidative stress) than $A\beta_{40}$.

Behavioral parameters:-

$A\beta_{40}$- and $A\beta_{42}$-injected rats exhibited longer latency period to reach the hidden platform in the Morris water maze test as compared to the controls. This indicates that both peptides caused reduction in the learning abilities of the animals. Previous studies have suggested that oxidative stress caused by Aβ peptide may contribute also to the learning-memory deficits (Jhoo et al., 2004). Intraamygdalarly-injected animals showed greater cognitive impairment than intrahippocampally-injected animals. This is consistent with the greater role the hippocampus plays in memory formation. This result is also in concordance with previous studies, which have demonstrated cognitive anomalies, and memory impairment in the AD brain models and Aβ-injected animals by Morris water maze test (De Ferrari et al., 2003; Sigurdsson et al., 1995; Sigurdsson et al., 1997), passive avoidance test (Harkany et al., 1998; McDonald et al., 1994; Sigurdsson et al., 1995; Sigurdsson et al., 1997), and radial arm maze test (McDonald et al., 1994). Ours is, however, the first study to demonstrate that Aβ-injected into the amygdala also causes cognitive decline. Previous studies have, however, shown histopathological damage in amygdala by Aβ-injections (Sigurdsson et al., 1997). Further $A\beta_{42}$ seemed to cause quantitatively greater impairment of spatial cognition than $A\beta_{40}$. Thus, greater cognitive impairment produced by $A\beta_{42}$ may be due to the higher oxidative stress caused by the peptide. This will be consistent with the conclusion that $A\beta_{42}$ is more toxic than $A\beta_{40}$. Our results also indicate that besides $A\beta_{42}$, $A\beta_{40}$ also significantly contributes to Aβ toxicity.

As is apparent from the present results derived from the open field and light-dark chamber tests, both $A\beta_{40}$ and $A\beta_{42}$ significantly elevate the anxiety and fear levels in the test subjects as compared to the controls. This can be derived from the increased activity in the periphery as compared to the center, increased defecation and lowering of rearing activity of the amyloid injected animals as compared to the controls. Our study has provided novel data from the light and dark chambered test. The results indicate that $A\beta_{40}$- and $A\beta_{42}$-injected animals showed quantitatively comparable impairments in anxiety, fear and emotional response levels taking into account both open field test and light-dark chamber test.

The present results obtained from the three-chamber social behavior test demonstrate that the amyloid injected rats (both intrahippocampally- and intraamygdalar-injected ones) and their respective controls are social in behavior, however, their preferences for familiar animal or novel animal were significantly varied. Familiarity is known to be crucial to empathetic responses in rodents such as mice (Mogil, 2012). Many studies show that rodents become friendly with and enjoy the company of the other rodents they are caged with, they will even share their food peacefully with their cage mates, and have been demonstrated to learn tricks to free their cage mates when trapped and share their food with them (Ben-Ami Bartal et al., 2014). It was observed that the amyloid injected animals showed greater inclination towards the unfamiliar animals (spent more time with unfamiliar as compared to the familiar animal, larger number of close contacts towards the unfamiliar rat) as opposed to the control rats which had a greater affinity for the familiar conspecifics (spent more time with familiar as compared to the unfamiliar animal, larger number of close contacts towards the familiar rat). Also, the control animals showed greater curiosity than the amyloid-injected subjects, as was indicated by the greater number of total close contacts made by them. The control animals also exhibited greater number of rearings in open field test and light-dark chamber test. In $A\beta_{40}$- and $A\beta_{42}$-injected animals all the sociability parameters were significantly different as these animals preferred unfamiliar animals to associate with. To further strengthen the social preference by the amyloid-injected and control animals, Pearson's correlation coefficient was calculated for intra-social behavior parameters (chamber of primary entry, time with familiar, time with unfamiliar, close contacts with familiar, and close contacts with unfamiliar rat) (Fig.6A and 6B). It can be observed that time spent with the unfamiliar animal is strongly correlated to close contacts made with the unfamiliar animal and inversely correlated with the time spent with the familiar animal as well as close contacts made with the familiar animal (Fig.6A.) for the amyloid injected animals. The control animals on the other hand showed inverse relationship between close contacts with familiar rat and close contacts with unfamiliar rat (Fig.6B.).

This social behavior test has not been previously applied to Aβ peptide-administered experimental animal models. There are reports of problems with sociability in Alzheimer's disease patients (Gauthier et al., 1996; Rayner et al., 1997); the present study would thus suggest that Aβ-related neurotoxicity might also be responsible for sociability impairment in animal models. It is also of interest to point out here that impaired social behavior is exhibited in rats prenatally exposed to valproic acid, and the impairment is mediated by alterations in endocannabinoid system (Ben-Ami Bartal et al., 2014; Kerr et al., 2013).

Another observation made from the behavior experiments was that, increases in total ambulatory activity in the open field test were positively correlate time spent with the unfamiliar rat in the three-chamber social behavior test (Fig.5.) in the amyloid-injected animals. The increased ambulatory activity of the amyloid injected subjects in the open field test indicated hyperactivity or hyper reactivity of the animals to the test environment. These observations are similar to the increased locomotor activity of the young when circuits in the hippocampus are not mature. Thus, injection of amyloid into the brain could be resulting in disinhibition of hippocampal function in terms of controlling an ongoing behavior. These kinds of increases in activity at the behavioral level in an unfamiliar open field have also been positively correlated with aggression towards a strange conspecific by Brain et al., (1969). Thus, Pearson's correlation analysis was done between the total ambulatory activity and time spent with the unfamiliar rat in amyloid-injected rats and their respective control groups to form a statistical basis for the disinhibition. In the amyloid-injected animals (Fig.5A.) a positive correlation was observed between the two parameters; whereas, in the control animals no significant relationship was seen between the two variables (Fig.5B.). Thus, these results could indicate that upon amyloid administration there is disinhibition of hippocampal function, leading to increased locomotor activity as a function of emotional response to an unfamiliar environment (open field) and also socially inappropriate behavior upon the initial encounter with an adult stranger i.e. disinhibited attachment (Nakazawa and Tang, 2006).

Oxidative parameters:-

The present study shows that both $A\beta_{40}$ and $A\beta_{42}$ significantly increased the oxidative stress at the site of injection in the brain following intrahippocampal and intraamygdalar administration. Furthermore, the effects spread to distant regions of the brain. This is evident from the $A\beta_{40}$- and $A\beta_{42}$-induced increases in the levels of LP, PO, and increased TT levels, along with decreased activities of anti-oxidant enzymes: SOD, GR, and GPx; at the site of injection i.e. hippocampus or amygdala as well as the distant regions i.e. cortex, midbrain, cerebellum, and medulla.

The existence of AD-induced oxidative stress manifested by enhanced LP and PO in the brain is well known (Anantharaman et al., 2006; Butterfield and Lauderback, 2002; Varadarajan et al., 2000). Although the pathogenesis of the oxidative stress is not very clear, it appears to be associated with Aβ pathology. Both peptides i.e. $A\beta_{40}$ and $A\beta_{42}$ are thought to mediate neurotoxicity through oxidative mechanisms because they can generate oxygen free radicals directly or indirectly, cause mitochondrial dysfunction, stimulate nitric oxide production and alter Ca^{2+} homeostasis (Butterfield and Lauderback, 2002; Jhoo et al., 2004). In the present study therefore, in vivo oxidative stress consequences following intrahippocampal and intraamygdalar administration of Aβ peptides were studied. Besides these two brain regions, other regions of the brain were examined to determine the spread of the oxidative damage to distant areas.

The results obtained from our study showed that both Aβ peptides (40 and 42) produced a decrease in the activity of enzymes important in regulating oxidative stress i.e. SOD, GR and GPx in the hippocampus and amygdala. Simultaneously, there were increases in the levels of LP, and PO. TT levels also increased in peptide-injected regions, and this increase will contribute to diminution in GPx and GR enzymes (Cumming et al., 2004). Significant decreases in the protein expression of antioxidant enzymes have been observed in the rat brain after continuous intracerebroventricular infusion of $A\beta_{42}$ (Kim et al., 2003). Thus, deficiency of antioxidant enzyme activity in Aβ-peptide-injected brain should be responsible for the elevation of LP and PO following Aβ-injections. Free radical induced LP is widespread in the AD brain (Butterfield and Lauderback, 2002). Thus, deficient or insufficient antioxidant enzyme activity (antioxidant capacity) in the AD brain may contribute to Aβ-induced elevation in oxidant products in the AD brain (Varadarajan et al., 2000). Increases in antioxidant enzyme activities observed in the AD brain appear to be compensatory rises in response to free radical generation (Lovell et al., 1995; Markesberry, 1997). Compensatory increases have also been observed in experimental studies where $A\beta_{42}$ was intracerebroventricularly-injected (Jhoo et al., 2004). Similarly no changes in the activity of the antioxidant enzymes reported in several studies would appear to be the failure of the antioxidant system enzymes to increase their activity to counter the oxidative process. For example, it was reported that there was no elevation in the activity of GPx in the hippocampus, and temporal-frontal lobes of the AD patients (Kish et al., 1986). However, in some studies of the AD brain significant decline in the antioxidant enzymes have been found. The activity of catalase was found reduced in the basal ganglia, amygdala, and parietal-temporal cortex in the AD brain (Gsell et al., 1995). Reduction of SOD activity in the frontal cortex, hippocampus, and cerebellum in AD has also been reported (Richardson, 1993). There have also been reports of diminished SOD activity in the caudate nucleus (Marklund et al., 1985), and reduction in SOD activity in the frontal and temporal lobes; and reduction in catalase activity in the temporal cortex (Marcus et al., 1998). Recently, significant decline in glutathione, GPx, glutathione-S-transferase, and SOD activities in the frontal cortex of AD patients has also been reported. Of interest also are data from AD animal models (Ansari and Scheff, 2010). Decreases in the levels of Mn-SOD in the brain of double knock in mouse model of AD have also been seen (Anantharaman et al., 2006). Taken together these data from the AD brain indicate that the decline in antioxidant enzymes often occurs in the AD brain. In addition it is also obvious that in the AD brain the antioxidant enzyme system fails to up regulate the antioxidant defenses to counter the oxidative stress pathology. Therefore, declines in antioxidant enzymes observed in the present study on the Aβ-injected brain would appear to reflect the consequences of Aβ-pathology.

The present study also focused on the oxidative stress effects appearing in brain regions distant from the brain regions into which Aβ-peptides were injected. It is known that secondary oxidative stress products such as hydroxynonenal or acrolein can diffuse from their site of origin to cause damage at more distant sites (Kerr et al., 2013). In our study, distant effects possibly caused by diffusion of the secondary products were found to be similar to the local effects (i.e. oxidative stress effects produced in the injected regions). Thus, elevated oxidative stress generated (i.e. increased LP and PO; and diminished antioxidant enzymes) in the hippocampus (injected region) was similar to the oxidative stress observed at the distant sites i.e. amygdala, cortex, midbrain, cerebellum and medulla. In the same way, elevated oxidative stress in the amygdala (injected region) was similar to the oxidative stress observed at distant sites i.e. hippocampus, cortex, midbrain, cerebellum and medulla. Consistent with our findings is the study by Sigurdsson et al., (1996) who found distant histopathological effects following unilateral Aβ-peptide

injections into the rat amygdala. In this study in vivo injections of $A\beta_{25-35}$ into the amygdala induced trans-synaptic cytoskeletal immunoreactions (tau immunoreactions), and the reactions gradually spread to distant brain regions: hippocampus, cingulate cortex, parietal cortex, pyriform cortex, hypothalamus, thalamus, globus pallidus, claustrum, substantia nigra, ventral pallidum/substantia innominate and the entorhinal cortex.

The sequence and length of an Aβ-peptide appear crucial to its neurotoxicity (Barrow and Zagorski, 1991). The neurotoxicity of the amyloid peptide seems to reside in the 25-35 sequence (Giovannelli et al., 1995) of the peptide. A number of studies have reported that the neurotoxicity of $A\beta_{40}$ and $A\beta_{42}$ is similar (May et al., 1992; Pike et al., 1993), and both peptides mediate toxicity through oxidative mechanism. However, $A\beta_{42}$ is often considered more neurotoxic (Richardson et al., 2002) because it may generate more free radicals than $A\beta_{40}$. For example, $A\beta_{42}$ injected in the mouse cerebral cortex produced larger glial fibrillary acidic protein immunoreactivity and greater reactive astrocytes than $A\beta_{40}$ in a study (Klien et al., 1999). Consistent with these studies, our data shows that the level of oxidative stress generated by $A\beta_{42}$ is greater than $A\beta_{40}$. This can be seen for Table-2, both in the hippocampus and amygdala, $A\beta_{42}$ elevated the protein oxidation by 528% and 356% respectively whereas $A\beta_{40}$ elevated it by 224% and 330%. Similarly, $A\beta_{42}$ caused greater impairment of the antioxidant enzymes GPx and SOD, and greater elevation in the thiol content. With respect to LP, however $A\beta_{40}$ appeared to be more toxic. In respect to the distant effects also, the oxidative stress effects were generally higher in the $A\beta_{42}$-injected subjects than in the $A\beta_{40}$-injected ones. Thus, the results derived from the present study lend support to the idea that $A\beta_{42}$ is more neurotoxic. Overall the oxidative stress alterations observed in the present study would seem to resemble that of the AD brain.

Numerous studies demonstrate that brain regions related to learning and memory are selectively affected by amyloid neurotoxicity. Amyloid administered centrally in the brain also induces distal morphological changes, damages cells and causes neuronal loss in neocortex, hippocampal subfields CA1 and CA3 and basal ganglia in mice (Maurice et al., 1998) and rats (Stepanichev et al., 2003) ranging from 6 weeks upto 6 months post-injection. Hippocampus and associated structures, dentate gyrus (DG) and entorhinal cortex (EC) are the primary regions affected and by amyloid induced neurotoxicity (Harris et al., 2010) and the progression of the neuronal disruption may be through anatomically and functionally connected brain regions (Braak et al., 2006; Braak and Braak, 1991; Buckner et al., 2005). Julie et al., (2010) showed that transgenic mice over expressing amyloid precursor protein (APP) in neurons of the superficial layers of the EC, lead to Aβ deposits in the hippocampus of older mice. They also showed that selective over expression of APP in EC neurons increased excitability and synaptic loss in DG and CA1 region of hippocampus. These results indicate that over expression of APP in other regions of the brain also leads to effects on hippocampus.

The reasons for greater degree of vulnerability of hippocampus could be multifold. It has been shown that Aβ peptides are cholinotoxic, and loss of cholinergic fibers has also been reported after injection of $A\beta_{25-35}$ (Harkany et al., 1999) and $A\beta_{42}$ (O'Mahony et al., 1998). Hippocampus receives a larger supply of cholinergic fibers from the medial septal nucleus and the vertical limb nucleus of the diagonal band (Mesulam et al., 1983). This rich supply could render hippocampus more susceptible to amyloid induced toxicity.

Amyloid also has a detrimental effect on calcium homeostasis, leading to reduction of calbindin in the brain. Studies have shown decreases in the levels of calbindin to be associated with hippocampal dysfunction and deficits in special learning (He et al., 2002; Molinari et al., 1996). Another facet of calcium homeostasis and increase in calcium load in the brain is its selective affinity for N-methyl-D-aspartate (NMDA) receptors. Activation of postsynaptic NMDA receptors in hippocampal pathways have been found to be essential for the induction of an activity-dependent synaptic modification called long-term potentiation (FTP) (Bliss and Collingridge, 1993; Collingridge et al., 1995), spatial learning and memory. Since Aβ peptides have adverse effects on calcium homeostasis and glutamate and glycine (Cowburn et al., 1997), this could lead to changes in NMDA receptor activity, further leading to poor long-term potentiation, memory formation and abnormal hippocampal activity, as these processes are intricately involved.

In the amygdala also, the NMDA receptors have been proposed to play a role in acquisition (but not expression) of conditioned fear-potential (Campeau et al., 1992; Miserendino et al., 1990), and second order fear conditioning (Gewirtz et al., 1997). But, NMDA receptors are not essential for all fear and emotional behaviors associated with amygdala, and thus, though we observe anomaly in amygdala associated behaviors upon amyloid injection, the aberrations are not as pronounced as in intrahippocampally-injected amyloid animals.

A number of studies propose that the local microenvironment of lipid membrane also contribute to the degree of damage to neuronal populations or disruption of essential pathways in different regions of the brain, for example, Mark et al., (1997) had also found 4-hydroxynonenal, an aldehydic product of lipid peroxidation, (HNE) to be neurotoxic to rat hippocampal neurons, possibly by disrupting Ca^{2+} homeostasis, reducing $Na^{+}K^{+}$ATPase activity; and, both $A\beta_{40}$ (Arispe et al., 1993) and $A\beta_{42}$ (Hirakura et al., 1999) have been shown to form ion channels by direct incorporation of Aβ peptide into lipid bilayer to form cation-selective ion channels (Arispe et al., 1993; Lin and Arispe, 2014). Further research needs to be done to assess the lipid bilayer membrane and its micro-dynamics to understand better how different amyloid peptides differ in mechanisms leading to the selective vulnerability of different brain region to amyloid toxicity.

The causes for disinhibition observed in our study could be many fold. Nicotinic acetylcholine receptors (nAChRs) are known to play a role in modulating cognitive functions, including learning and memory (Levin and Simon, 1998). The hippocampus receives extensive cholinergic innervation from the medial septum-diagonal band complex (Alonso and Amaral, 1995; Woolf et al., 1991; Yoshida and Oka, 1995), and there is strong expression of nAChRs in the hippocampus (Marin and Aceto, 1981). The high calcium permeability of the α7-containing nAChR (Castro and Albuquerque, 1995; Rathouz et al., 1996; Seguela et al., 1993) enables it to enhance the release of both glutamate and GABA via presynaptic mechanisms in the hippocampus (Alkondon et al., 1997; Gray et al., 1996; Radcliff and Dani, 1998; Radcliff et al., 1999). Activation of α7-containing and non-α7 receptors was shown to produce disinhibition of pyramidal neurons by suppressing tonic GABAA activity in the rat hippocampus (Ji and Dani, 2000). It was also proposed that activation of nAChRs on those interneurons that directly innervate pyramidal neurons causes the inhibition, and activating nAChRs on those interneurons that innervate other interneurons causes the disinhibition (Ji and Dani, 2000). Indirect excitation of pyramidal neurons of similar mechanism was also supported by another study, which demonstrated that nAChR activation could increase GABA activity in some interneurons (Alkondon et al., 1999). The nAChRs have the capacity to influence LTP/LTD by activating interneurons and, thereby, decreasing or increasing the postsynaptic depolarization of the pyramidal neurons (Ji and Dani, 2000).

In depth studies also need to be performed to quantify the cholinergic fibre loss and calcium homeostasis in different brain regions to delineate the region specific and neuronal specific mechanisms of amyloid toxicity. It has been well established that there is a selectively greater toxicity load on the regions concerned with memory and learning associated with AD. Thus, if the mechanisms underlying this phenomenon were known, therapeutic intervention customized for brain region-specific protection could be developed. Presently, development of most therapeutic interventions is based on reducing the plaque load or neurofibrilary tangles, considering that all the brain regions are similarly affected, and this could be one of the factors for non-development of any successful drugs or therapies for the disease till date.

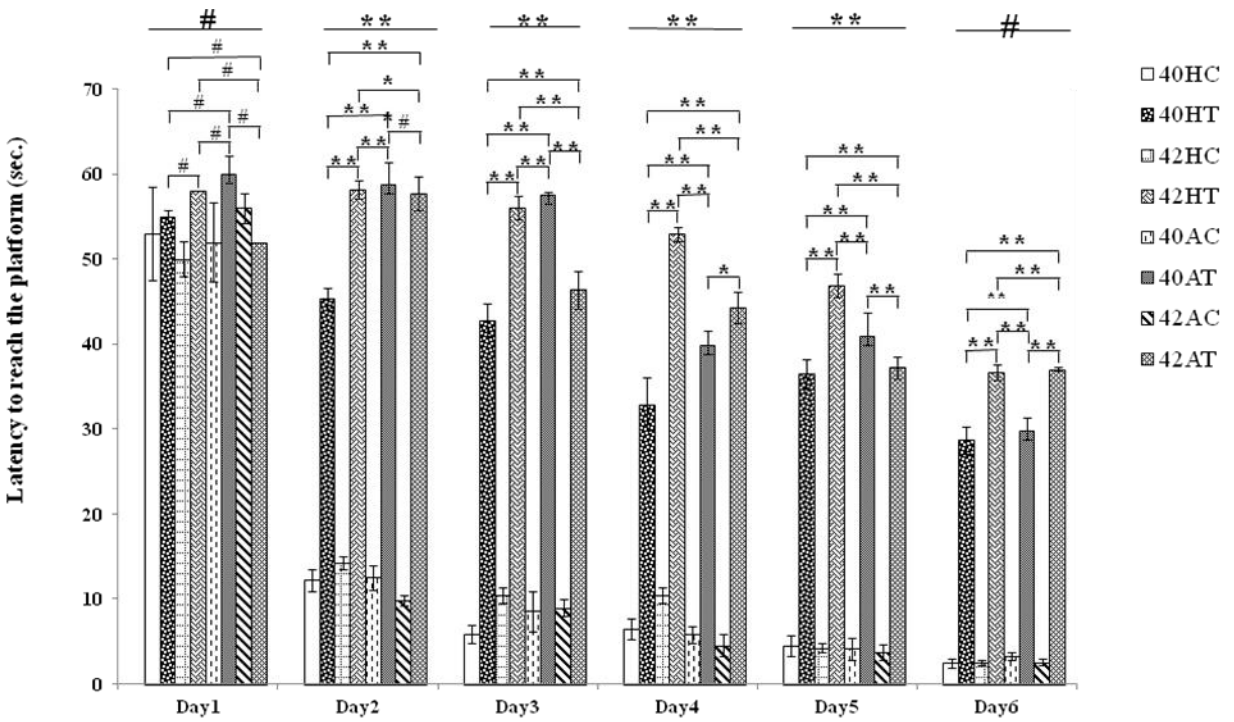


Fig1. Effect of amyloid beta toxicity on latency period in Morris water maze test. Latency to reach the platform is significantly increased upon $A\beta_{40}$ and $A\beta_{42}$ administration in both hippocampus and amygdala of the rat brain. Each data point represents the mean±SD of n=6 rats. The top-most bars in the figure represent the significance level of the interaction between treatment and trails per day of Morris water maze. All other bars represent the significance level between the various treatment groups.

All statistical analysis by Two-way ANOVA .$^{**}P<0.001$, $^{*}P<0.05$, $^{\#}$ Not significant.

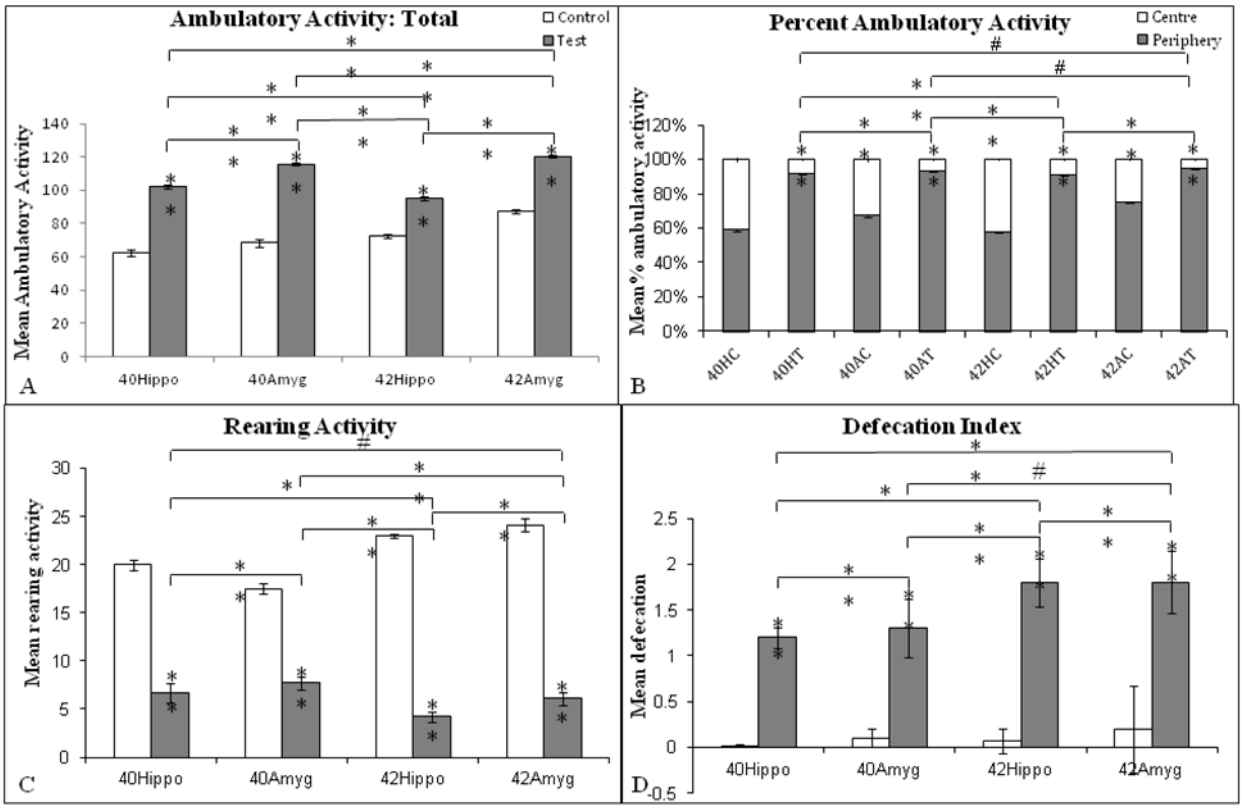


Fig2. Effect of amyloid beta toxicity on open field test parameters. (A) and (D) are increased; (B) and (C) are decreased, upon $A\beta_{40}$ and $A\beta_{42}$ administration in both amygdala and hippocampus of the rat brain. Each data point represents the mean±SD of n=6 rats. The symbols on the error bars represent significance of amyloid test values compared to their respective controls. The symbols on top horizontal bars represent the significance level between the various treatment groups. All statistical analysis by Two-way ANOVA. $^{**}P<0.001$, $^{*}P<0.05$, $^{\#}$Not significant.

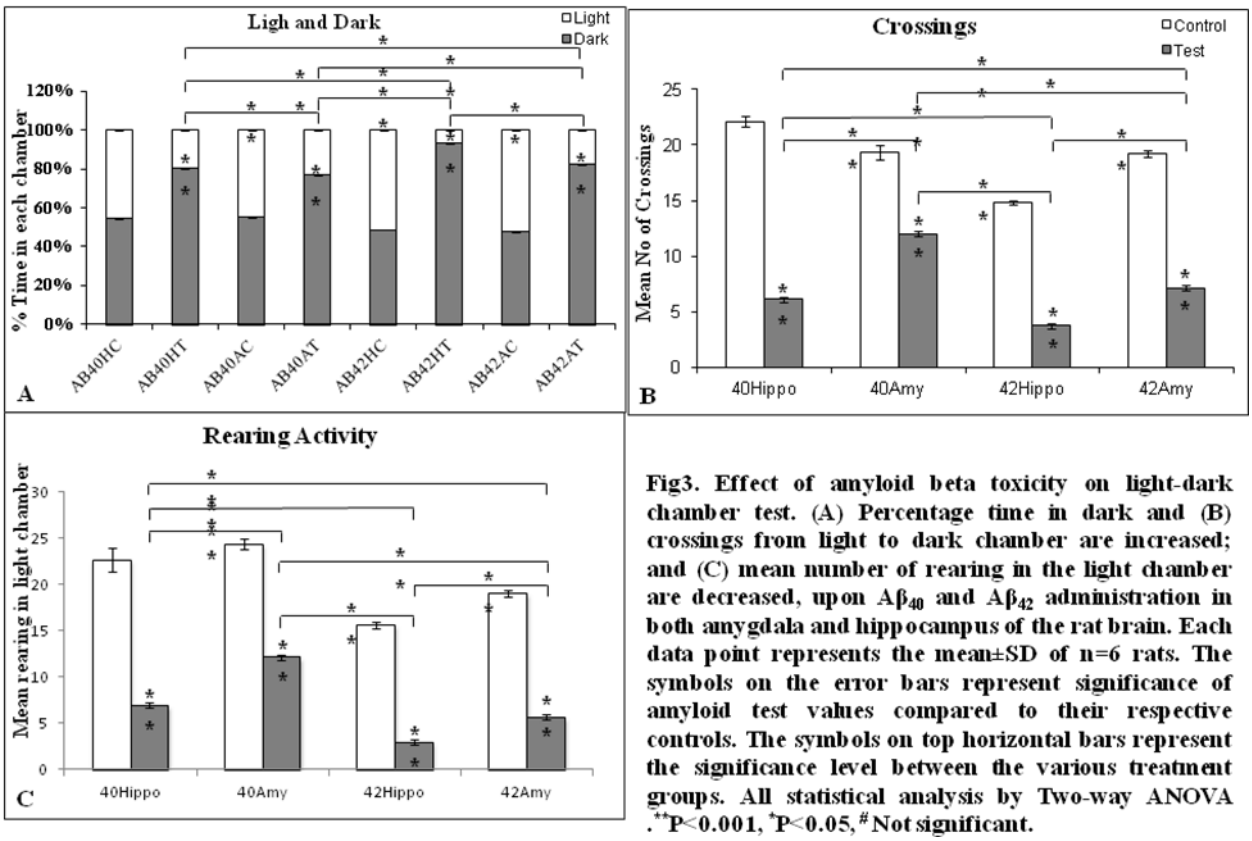


Fig3. Effect of amyloid beta toxicity on light-dark chamber test. (A) Percentage time in dark and (B) crossings from light to dark chamber are increased; and (C) mean number of rearing in the light chamber are decreased, upon $A\beta_{40}$ and $A\beta_{42}$ administration in both amygdala and hippocampus of the rat brain. Each data point represents the mean±SD of n=6 rats. The symbols on the error bars represent significance of amyloid test values compared to their respective controls. The symbols on top horizontal bars represent the significance level between the various treatment groups. All statistical analysis by Two-way ANOVA .$^{**}P<0.001$, $^{*}P<0.05$, $^{\#}$Not significant.

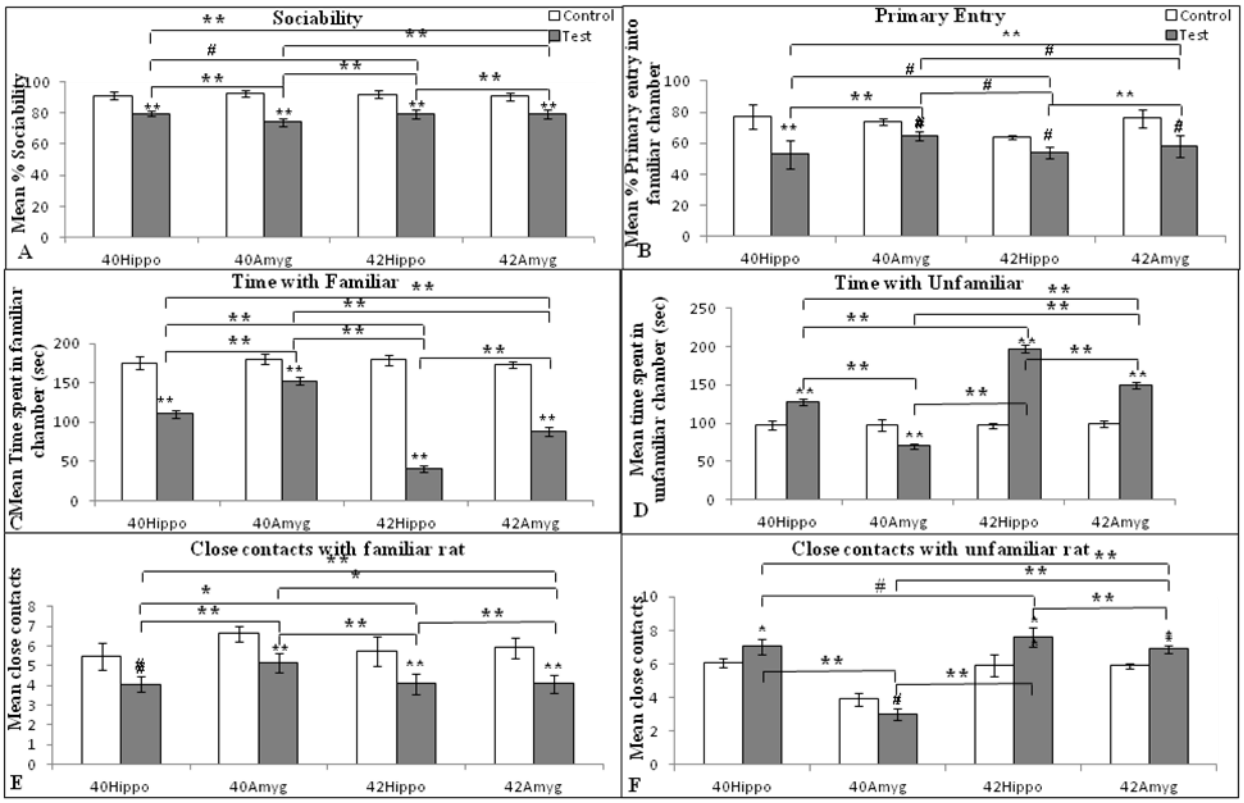


Fig4. Effect of amyloid beta toxicity on three-chamber social behavior. (C) Time with familiar rat and (E) Close contacts with the familiar rat are decreased; (D) Time with the unfamiliar rat and (F) Close contacts with the unfamiliar rat are significantly increased, upon $A\beta_{40}$ and $A\beta_{42}$ administration in both amygdala and hippocampus of the rat brain. Each data point represents the mean±SD of n=6 rats. The symbols on the error bars represent significance of amyloid test values compared to their respective controls. The symbols on top horizontal bars represent the significance level between the various treatment groups. All statistical analysis by Two-way ANOVA . $P<0.001$, * $P<0.05$, # Not significant.**

FIGURE.5.PEARSON'S CORRELATION PLOT BETWEEN TOTAL AMBULATORY ACTIVITY (OPEN FIELD TEST) AND TIME WITH UNFAMILIAR (THREE-CHAMBER SOCIAL BEHAVIOR TEST)

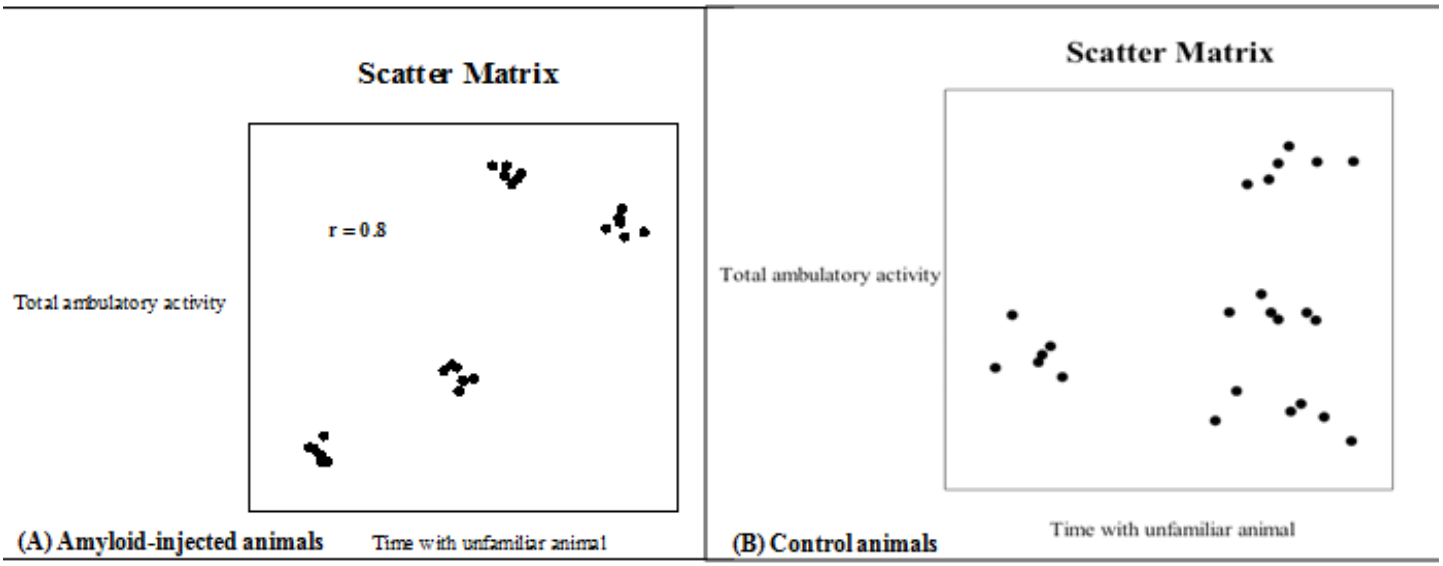


Fig5. Pearson's correlation scatter matrix (A) Amyloid injected animals (B) Control animals. Correlation was calculated between total ambulatory activity (open field test) and time spent with the unfamiliar animal (three chamber social behavior test). The amyloid injected animals show a strong positive correlation between the total ambulatory activity and the time spent with the novel animal ($r = 0.8$), whereas the control animals do not show any significant correlation between the two parameters. This could be indicative of a disinhibition of hippocampus, with relation to stopping an ongoing behavior. Co-efficient (r) values >0.5 were considered indicators of strong correlation, $0.4<r<0.5$ of medium correlation, and <4 of week correlation. Positive values indicated the factors tend to increase together and negative values indicated inverse relationship between the factors.

FIGURE.6(A).PEARSON'S CORRELATION PLOT BETWEEN THE PARAMETRES OF THREE-CHAMBERED SOCIAL BEHAVIOR FOR AMYLOID-INJECTED TEST ANIMALS

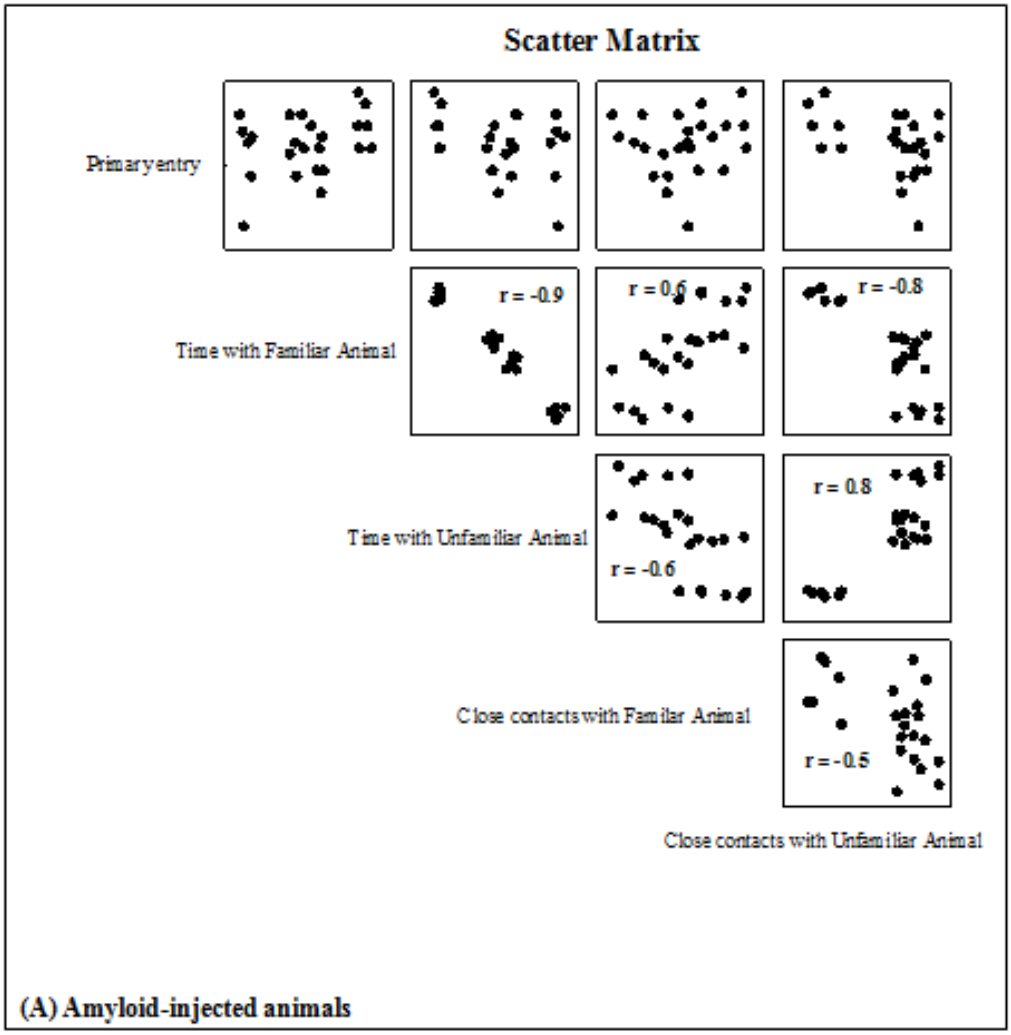
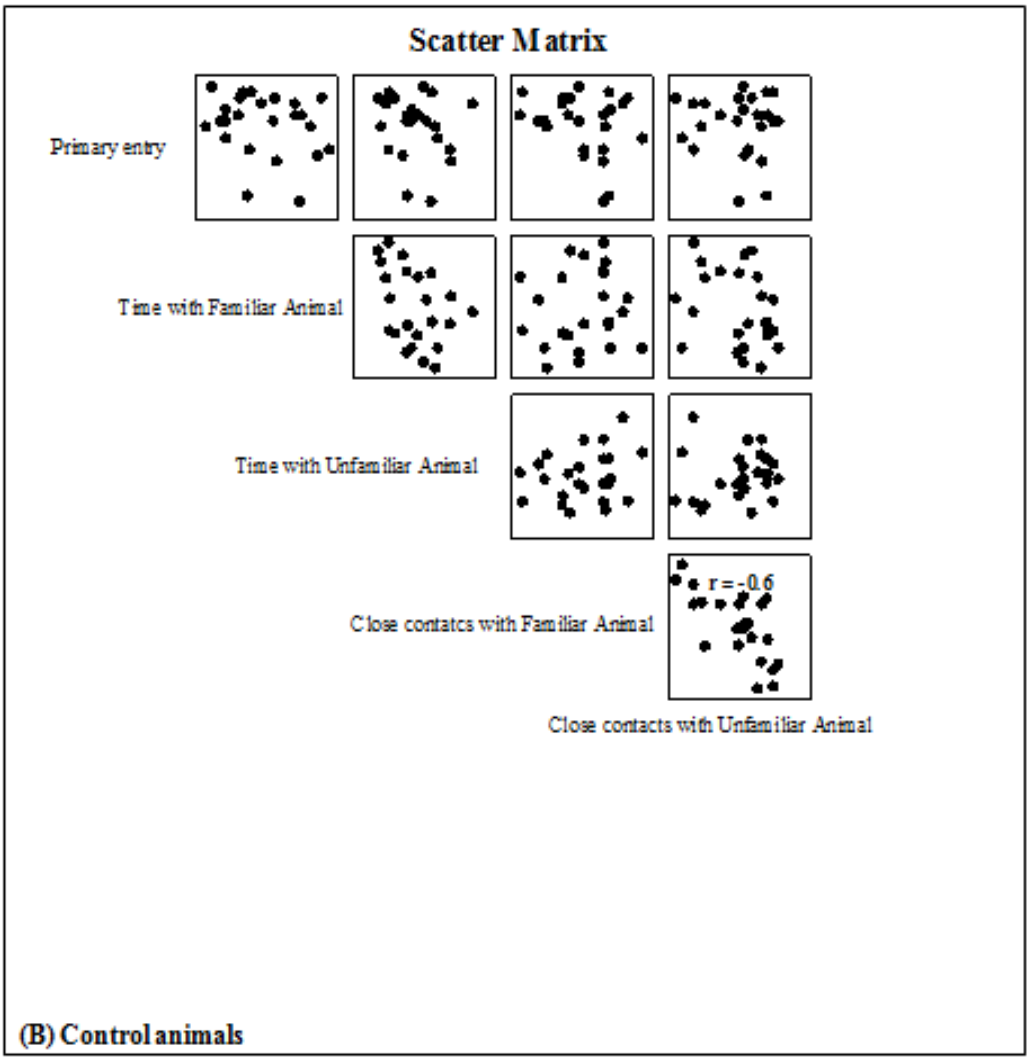


Fig6. Pearson's correlation scatter matrix chart depicting relationship between three-chamber social behavior test parameters in amyloid-injected animals (A) and control animals (B). These results indicate that the amyloid-injected animals have a preference towards the unfamiliar animals as apposed to the familiar ones. Also, the chamber of primary entry of the animal does not provide any indication towards the social inclination (social novelty or familiarity) or the animal. These results indicate that the control animals show a negative correlation between close contacts for the familiar and unfamiliar animals.

FIGURE.6(B).PEARSON'S CORRELATION PLOT BETWEEN THE PARAMETRES OF THREE-CHAMBERED SOCIAL BEHAVIOR FOR CONTROL ANIMALS



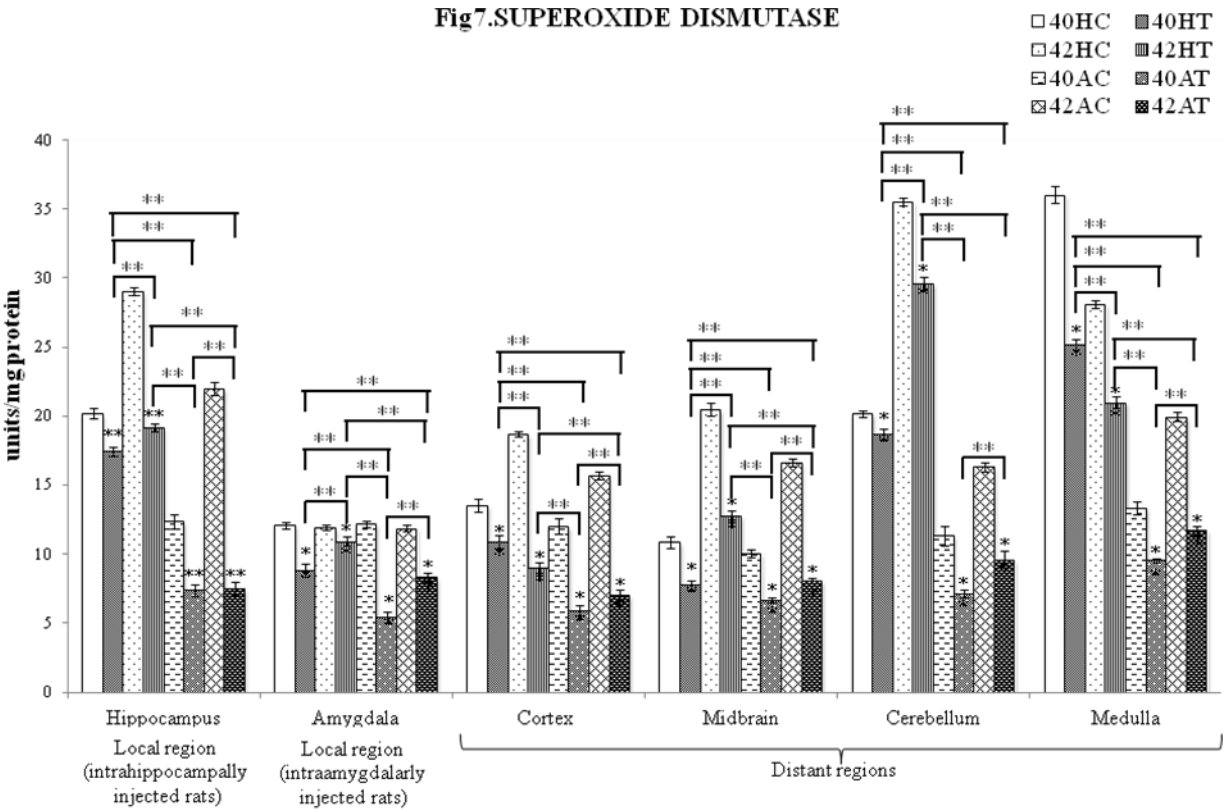


Fig7. Effect of amyloid beta toxicity on superoxide dismutase levels. SOD levels were decreased upon $A\beta_{40}$ and $A\beta_{42}$ administration in both hippocampus and amygdala of the rat brain, at the local site of injection i.e. hippocampus or amygdala, and also at distant regions. Each data point represents the mean±SD of n=6 rats. The symbols on the error bars represent significance of amyloid test values compared to their respective controls. The symbols on top horizontal bars represent the significance level between the various treatment groups. All statistical analysis by Two-way ANOVA. $^{**}P<0.001$, $^{*}P<0.05$, $^{\#}$Not significant.

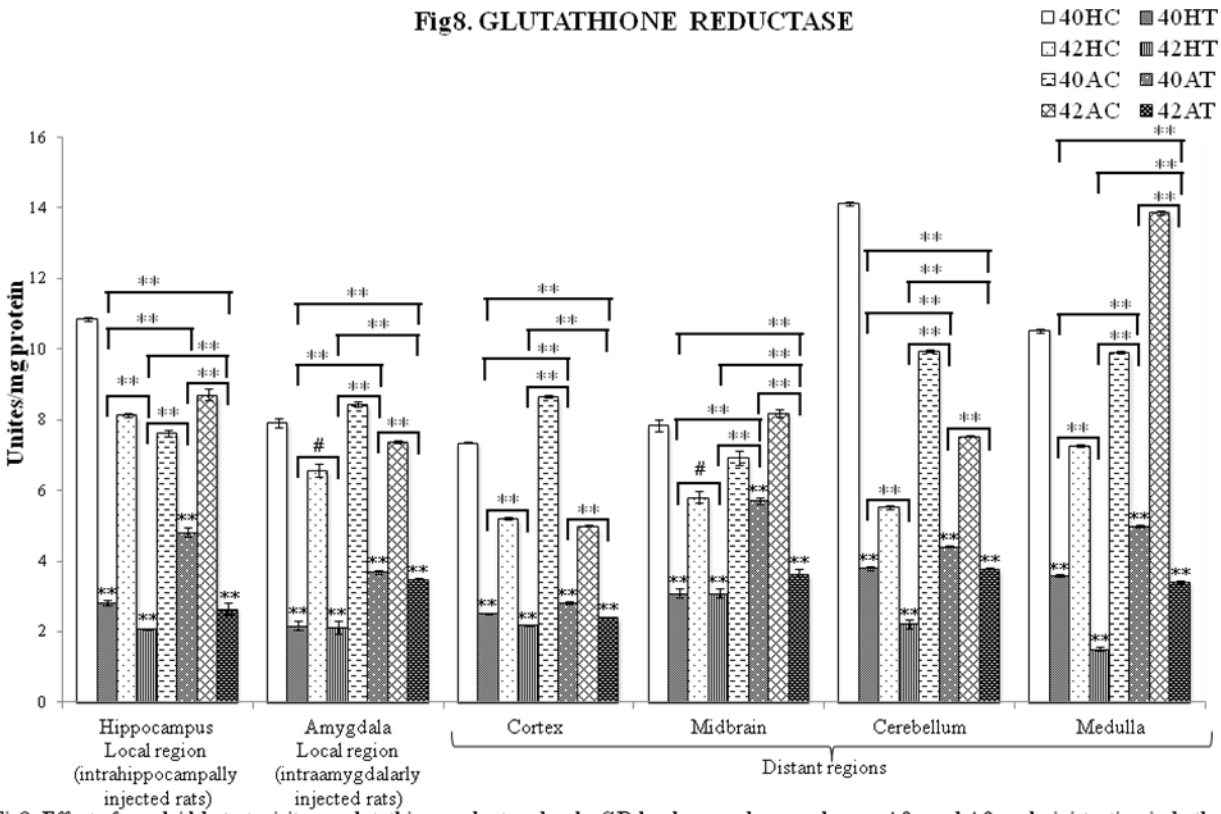


Fig8. Effect of amyloid beta toxicity on glutathione reductase levels. GR levels were decreased upon $A\beta_{40}$ and $A\beta_{42}$ administration in both hippocampus and amygdala of the rat brain, at the local site of injection i.e. hippocampus or amygdala, and also at distant regions. Each data point represents the mean±SD of n=6 rats. The symbols on the error bars represent significance of amyloid test values compared to their respective controls. The symbols on top horizontal bars represent the significance level between the various treatment groups. All statistical analysis by Two-way ANOVA. $^{**}P<0.001$, $^{*}P<0.05$, $^{\#}$ Not significant.

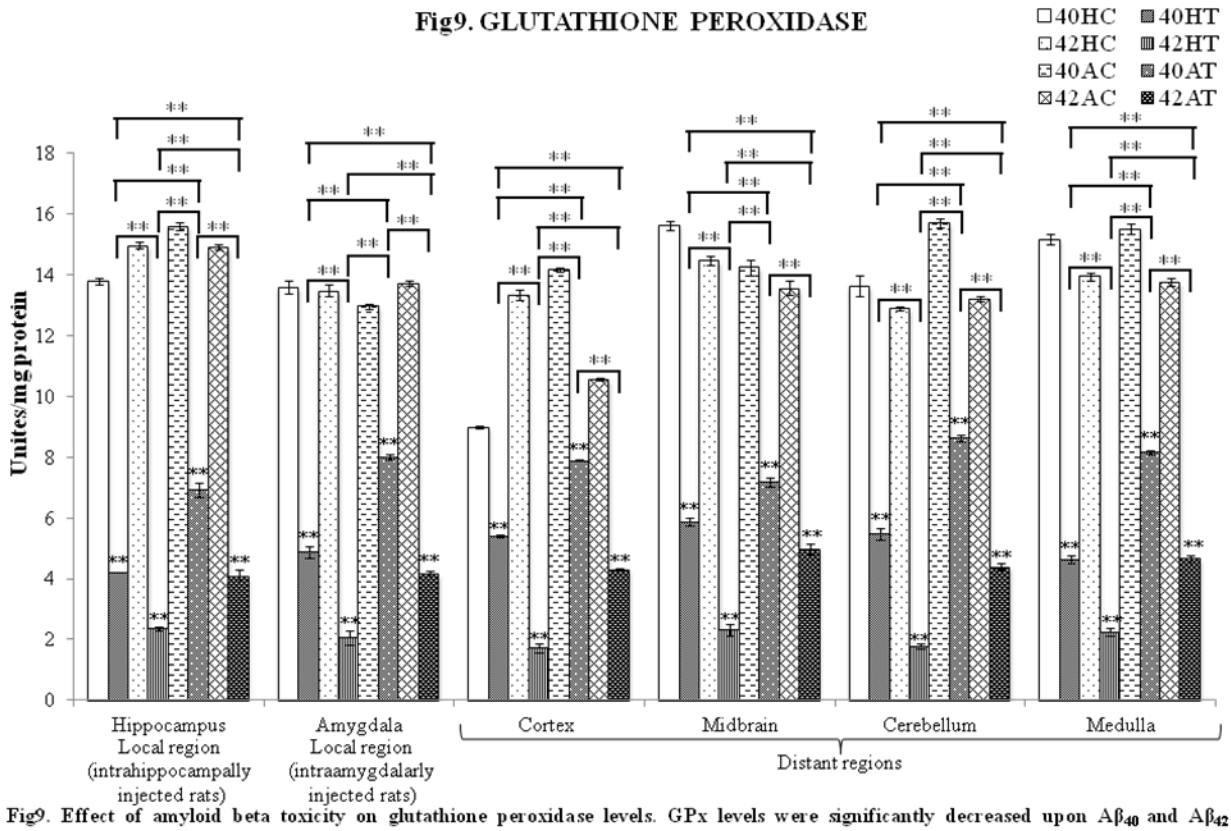


Fig9. Effect of amyloid beta toxicity on glutathione peroxidase levels. GPx levels were significantly decreased upon $A\beta_{40}$ and $A\beta_{42}$ administration in both hippocampus and amygdala of the rat brain, at the local site of injection i.e. hippocampus or amygdala, and also at distant regions. Each data point represents the mean±SD of n=6 rats. The symbols on the error bars represent significance of amyloid test values compared to their respective controls. The symbols on top horizontal bars represent the significance level between the various treatment groups. All statistical analysis by Two-way ANOVA. $^{**}P<0.001$, $^{*}P<0.05$, $^{\#}$ Not significant.

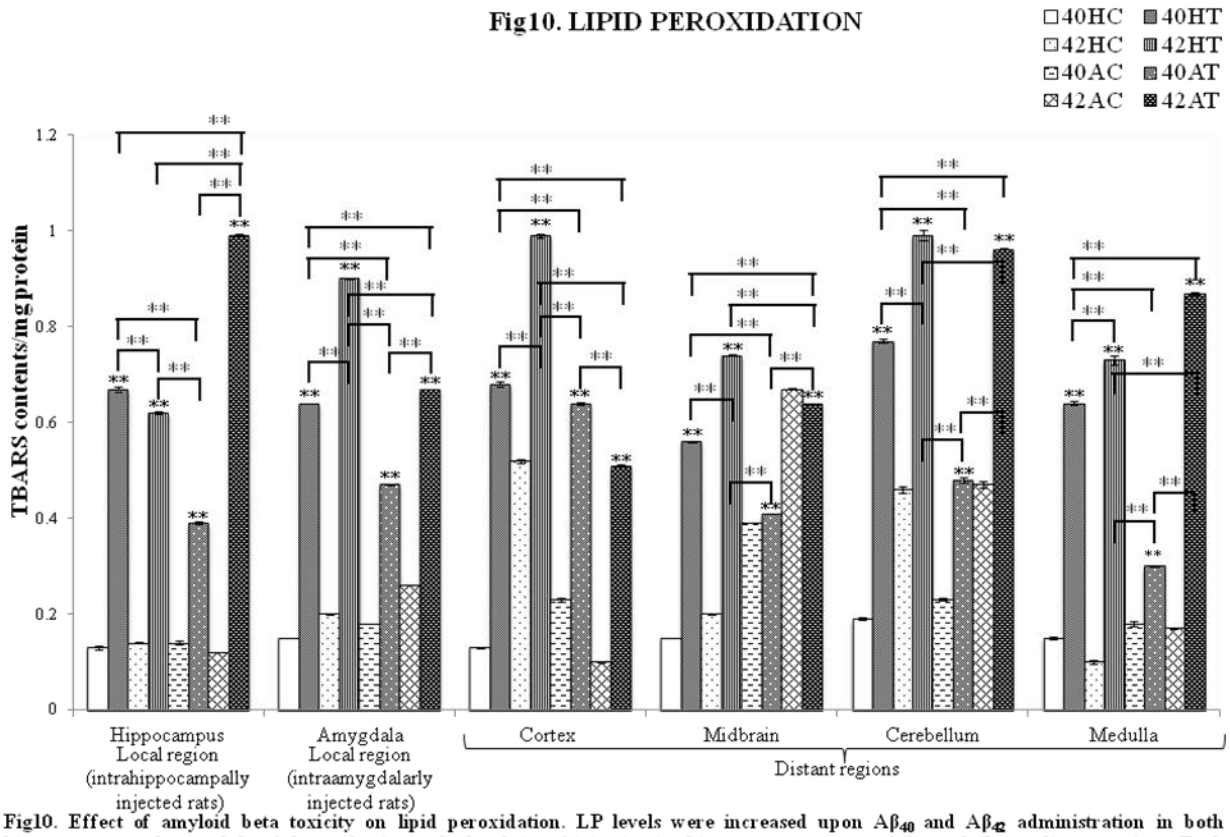


Fig10. Effect of amyloid beta toxicity on lipid peroxidation. LP levels were increased upon $A\beta_{40}$ and $A\beta_{42}$ administration in both hippocampus and amygdala of the rat brain, at the local site of injection i.e. hippocampus or amygdala, and also at distant regions. Each data point represents the mean ±SD of n=6 rats. The symbols on the error bars represent significance of amyloid test values compared to their respective controls. The symbols on top horizontal bars represent the significance level between the various treatment groups. All statistical analysis by Two-way ANOVA. $^{**}P<0.001$, $^{*}P<0.05$, $^{\#}$ Not significant.

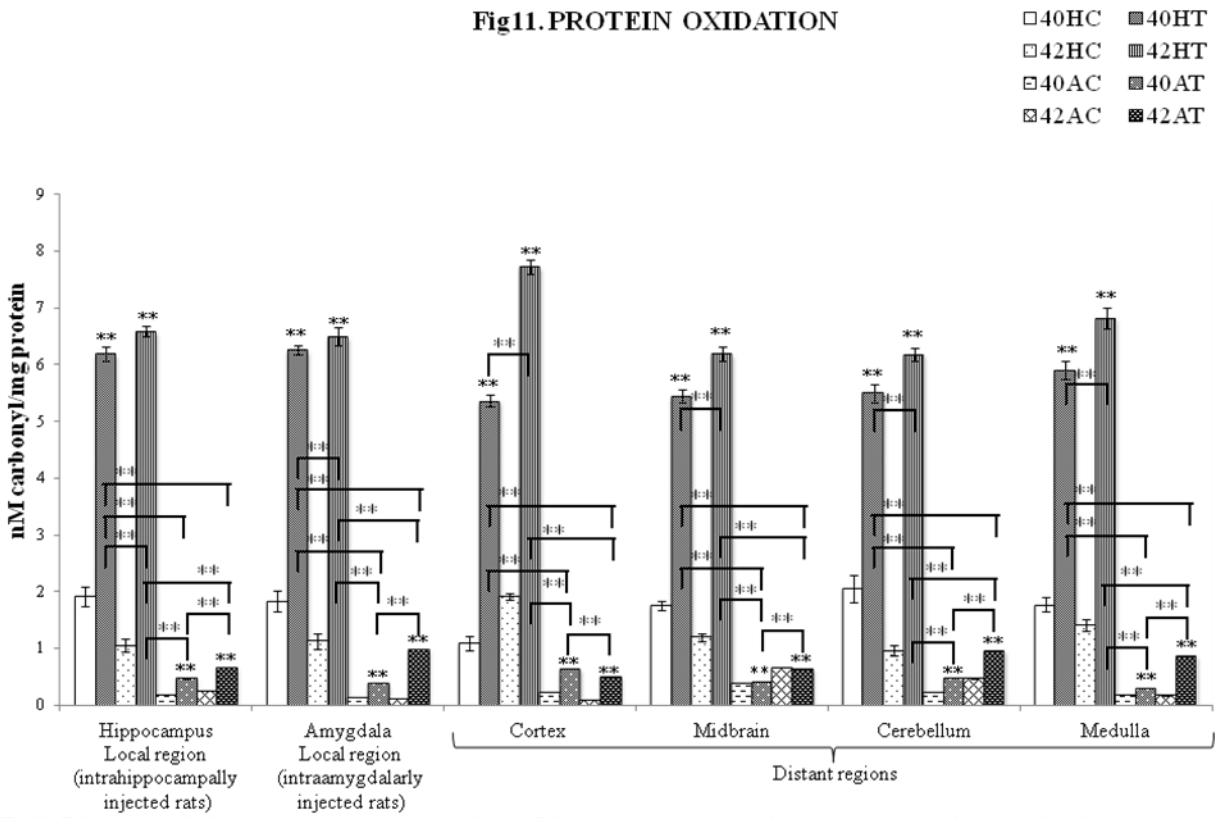


Fig11. Effect of amyloid beta toxicity on protein oxidation. PO levels were significantly increased upon $A\beta_{40}$ and $A\beta_{42}$ administration in both hippocampus and amygdala of the rat brain, at the local site of injection i.e. hippocampus or amygdala, and also at distant regions. Each data point represents the mean±SD of n=6 rats. The symbols on the error bars represent significance of amyloid test values compared to their respective controls. The symbols on top horizontal bars represent the significance level between the various treatment groups. All statistical analysis by Two-way ANOVA. $^{**}P<0.001$, $^{*}P<0.05$, $^{\#}$ Not significant.

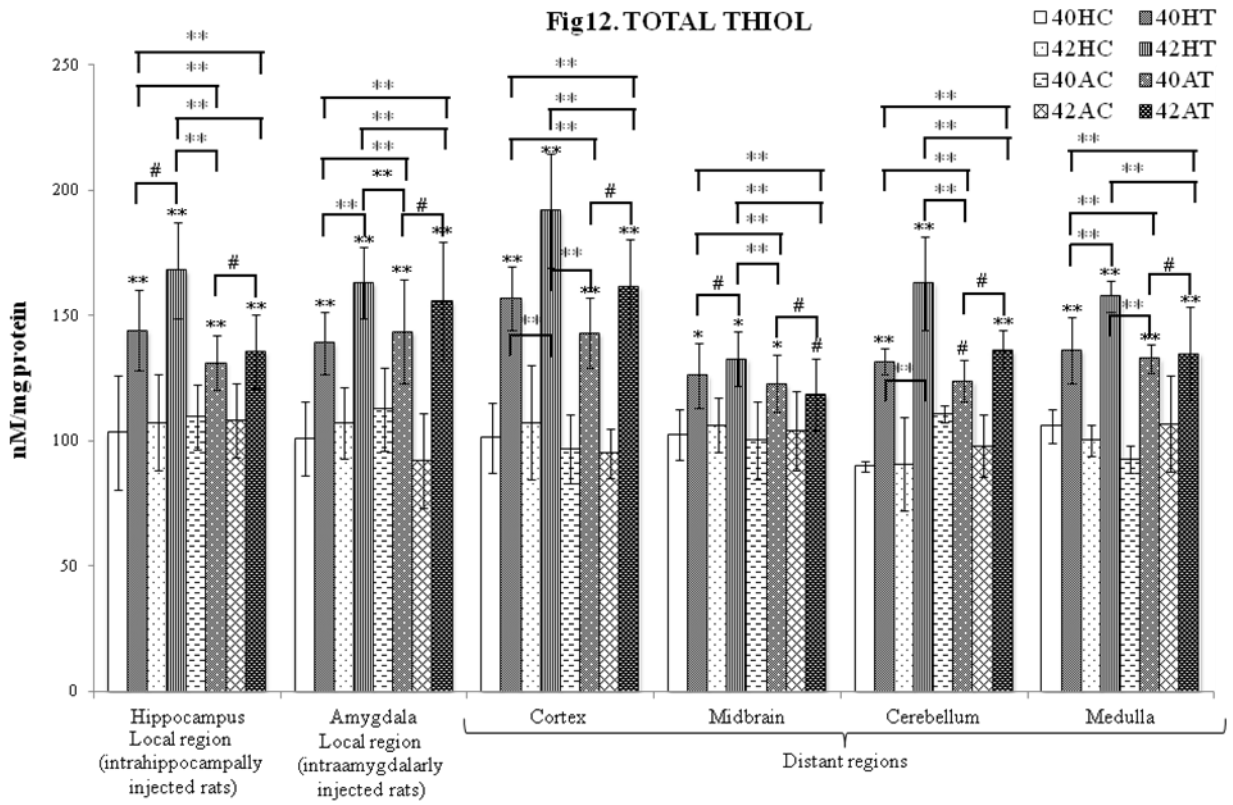


Fig12. Effect of amyloid beta toxicity on total thiol levels. TT levels are increased upon $A\beta_{40}$ and $A\beta_{42}$ administration in both hippocampus and amygdala of the rat brain, at the local site of injection i.e. hippocampus or amygdala, and also at distant regions. Each data point represents the mean ±SD of n=6 rats. The symbols on the error bars represent significance of amyloid test values compared to their respective controls. The symbols on top horizontal bars represent the significance level between the various treatment groups. All statistical analysis by Two-way ANOVA. $^{**}P<0.001$, $^{*}P<0.05$, $^{\#}$ Not significant.

Tables:-

Table-1: Details of animal housing and ages of animals during the behavioral experiments.

	Age of Animal (Rats) (in weeks)	**Timeline of Experiments**	**Housing of Animals**
Pre-surgery	3rd onwards (post weaning)	Day0	In groups of two
Surgery	24	Day01	Individually
Post-surgery	24th – 25th	Day01-07 (Recovery)	
Morris water maze	25	Day08	Individually
Open field test	26	Day15	Individually
Light-dark chamber test	27	Day20	Individually
Post light-dark experiment	27	Day21 onwards (After Light-dark exp.)	Group of two
Three chamber social behavior	27	Day23	Group of two
Sacrifice (for biochemical assays)	28	Day25	--

Table-1: Ages of animals at the beginning of the experiment (day of surgery is taken as Day1), and ages when they were tested in various behavioral paradigms. 25 days post surgery, the animals were sacrificed and their brains were isolated.

Table-2: Percentage oxidative stress [(Test-Control)/Control]

	Control Level ($A\beta_{1-40}$)	**Exp. Level ($A\beta_{1-40}$)**	**%**	**Control Level ($A\beta_{1-42}$)**	**Exp. Level ($A\beta_{1-42}$)**	**%**
HIPPO			**$A\beta_{1-40}$**			**$A\beta_{1-42}$**
PO	1.91±0.17	6.19±0.13**	**224**	1.05±0.12	6.59±0.10**	**528**
TT	103.42±22.93	144.10±16.11*	**39**	107.31±19.12	168.14±19.24**	**69**
SOD	12.12±0.35	9.83±0.36**	**-19**	12.37±0.29	7.39±0.32**	**-40**
GPx	13.85±0.20	4.22±0.01**	**-70**	14.97±0.11	2.37±0.07**	**-84**
GR	10.86±0.51	2.82±7.4**	-74	8.14±0.62	2.08±0.32**	-74
LP	0.13±0.0031	0.67±0.0051**	415	0.14±0.0011	0.62±0.0029**	343
AMYG						
PO	1.23±0.13	5.30±0.05**	**331**	1.40±0.12	6.39±0.22**	**356**
TT	109.60±12.88	131.23±11.05*	**20**	108.28±14.54	135.73±14.95*	**27**
SOD	11.93±0.23	10.92±0.39**	**-8**	11.88±0.22	8.31±0.35**	**-30**
GR	7.63±0.10	4.82±0.13**	**-37**	8.72±0.16	2.66±0.16**	**-69**
GPx	15.61±0.13	6.93±0.24**	-56	14.92±0.10	4.07±0.23**	-73

Table-2: Percentage oxidative-stress at the local site of intrahippocampal or intraamygdalar injection of $A\beta_{1-40}$ or $A\beta_{1-42}$ in the rat brain. AB_{1-42} seems to be more toxic than AB_{1-40} as it produces quantitatively greater oxidative stress as compared to $A\beta_{1-40}$ and with amygdalar administration. PO- Protein oxidation, TT- Total thiol, SOD- Superoxide dismutase, GPx- glutathione peroxidase, GR- glutathione reductase, LP- lipid peroxidation. All the values are expressed as mean±S.D. of n=6 rats. Significance of amyloid tests compared with their respective controls: **P<0.001, *P<0.05, #not significant.

Conclusion:-

In conclusion, the results from the present study showed that different brain regions have varied vulnerability amyloid associated toxicity; in our study hippocampus was more vulnerable to amyloid associated toxicity than amygdala. Also, amyloid associated toxicity leads to elevated oxidative stress in the injected sites i.e. amygdala and hippocampus, and the oxidative stress spreads from the injected sites to the distant brain regions (cortex, midbrain, cerebellum and medulla), where quantitatively very high levels of oxidative stress parameters were attained. The study also provides novel information on the effects of $A\beta_{40}$ and $A\beta_{42}$ toxicity on social behavior; both $A\beta_{40}$ and $A\beta_{42}$ injected intrahippocampally or intraamygdalarly impaired emotional state and social behavior. The behavioral anomalies produced after intrahippocampal injections were more severe than those produced by intraamygdalar injection. Compared with $A\beta_{40}$, $A\beta_{42}$ generated higher levels of oxidative stress and produced more severe behavioral deficits indicating that $A\beta_{42}$ is more toxic than $A\beta_{40}$.

Acknowledgements:-

The author Neha Mishra is thankful to the University Grants Commission for providing financial assistance through Junior Research Fellowship and Senior Research Fellowship.

Disclosure statement:-

None of the authors report any potential or actual conflict of interest.

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