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AGE-DEPENDENT ALTERATIONS IN CaMKIIα EXPRESSION IN DIFFERENT BRAIN REGIONS ARE ASSOCIATED WITH CHANGES IN THE SPATIAL MEMORY IN MALE AND FEMALE MICE

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Abstract

CaMKIIα is implicated in synaptic plasticity and thereby learning, memory and cognition. Though alterations in its expression due to neurological disorders have been reported, information on brain region dependent variation in its expression due to aging and its gender dependence is lacking. Here, we have investigated expression of CaMKIIα in frontal- and parietal cortices and hippocampus of young, adult and old mice of both the sex using immunoblotting and semi quantitative RT-PCR techniques. Our gene expression data reveal that CaMKIIα expression is gradually down regulated in the frontal- as well as parietal cortices whereas it is gradually up regulated in the hippocampus in male mice during aging. Its expression is significantly down regulated in the frontal cortex, up regulated in parietal cortex of the female mice during aging, and increases in adult age followed by its significant decline in old age in the hippocampus of female old mice. Our MWM data on sex- dependent alterations in spatial memory during aging suggests that deficits in spatial memory is associated with the region specific alterations in CaMKIIα expression. Our data is suggestive of the possible implications of CaMKIIα in age- and gender-dependent alterations in memory.

Key words : CaMKIIα, aging, brain, Morris water maze test, learning and memory, cognition

Introduction

Aging is one of the major factors leading to decline in learning, memory and cognition in mammals including humans (1, 2). Existing evidence suggests that the age- related cognitive decline is attributed to the loss of neurons and synaptic changes in cortical or temporal brain structures along with mild neuronal/synaptic loss in the hilus of hippocampus (3, 4). Various hippocampal sub regions, frontal and temporal

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Authors dedicate the article to Prof. M.S. Kanungo, *F.N.A, F.A.Sc., F.N.A.Sc.*, the father of research on aging in India, on the fourth anniversary of his heavenly abode.

cortex have been shown to undergo synaptic remodeling by substantial increase in the dendritic and synaptic complexity with advancing age (5). Alterations in synaptic plasticity have been described to underlie the memory decline during aging (6-9), and in age-dependent neurodegenerative diseases such as Alzheimer's disease (10, 11), Parkinson's diseases and Huntington's disease (12, 13). Though above decline during aging is a common feature among both the genders, large body of reports till date suggests that brain organization (14), cognitive functions i.e., solving spatial and mathematical tasks are sexually dimorphic (15, 16). However, the precise genetic mechanism that underlies the sex-dependent cognitive decline during aging till date remains elusive. Alterations in gene expression may be attributed to the cognitive decline during aging that might be gender-dependent, can explain a possible molecular basis of neuronal loss, associated synaptic alterations and thereby decline in the cognitive function of brain. Although, number of studies on expression of various genes in Alzheimer's disease (AD) brain has been carried out, only limited information is available on the pattern of gene expression as a function of age and sex. Microarray studies have revealed expression of distinct genes in human frontal/prefrontal cortex across the adult life span (17, 18). Also, gene chip based studies on the gene expression has indicated that male brain undergoes more global changes than in female human brain during aging showing a sexually dimorphic pattern (16)**.** But how does expression of genes related to cognition change in various brain regions, which might differ as a function of age and gender, is largely unknown. Existing evidences suggest that development of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor (AMPAR) activation-dependent post synaptic potential (PSP), removal of Mg^{2+} block from the N-methyl-D-aspartate (NMDA) receptor (NMDAR) and thereafter, diffusion of large amount of Ca^{2+} into the post synaptic neurons, and activation of calcium-calmodulin activated protein kinase II (CaMKII) play central role in the development of LTP that underlies the process of memory consolidation (19). Therefore, it is speculated that CaMKII could be one of the candidates and its altered expression might result into sex-dependent differences in learning and memory which might further be modulated due to aging.

CaMKII, a Ca^{2+}-dependent serine threonine protein kinase and predominantly expressed in the forebrain regions, is a heteromultimeric holoenzyme complex. consisting of four subunits- α, β, γ and δ (20). Each subunit is encoded by one of the four distinct genes, called α, β, γ and δ CaMKII genes. The MW of subunits ranges from 54 to 72 kDa. To date, several isoforms for each subunit have been identified (21-23). Each isoform possesses four domains- a catalytic domain, an auto inhibitory or pseudosubstrate domain, a variable domain and an association domain with their distinct function (24). CaMKII α and β isoforms are expressed predominantly (22, 25), or almost exclusively in neural tissues (26, 27), whereas γ and δ isoforms are widely expressed in non-neuronal tissues (22, 25, 26,) and the hind brain regions (28). Although both α and β isoforms are expressed in the forebrain regions depending on the developmental stage, β isoform is particularly expressed in the early developmental

stage of the brain (29) and it promotes motility of filopodia and neuritic branches and enhanced dendritic arbor whereas α subunit is expressed in later stages of the development and contribute to their stabilization (30). Studies from CaMKIIα mutant mice (CaMKII-Asp-286) have shown that these mice possess impaired spatial learning due to failure in achieving LTP and LTD in the visual cortex (31) and LTP in CA1 area of the hippocampus (32), however, contextual learning remains unaffected. This suggests the involvement of separate mechanisms that might underlie during development of hippocampal memory performances (33).

Number of evidence suggests that activation/auto phosphorylation of CaMKIIα at Thr 286 leads to its binding with exaggerated cytosolic Ca^{2+} during glutamate-dependent acquisition, consolidation, storage and retrieval of memory (34), augments with conformational change in the NMDAR and AMPAR upon binding of glutamate, consequent enhancement in their calcium and sodium conductance, respectively (35) and expression of the phosphorylated AMPA receptor on the post synaptic density (PSD) (36, 37) in order to increase in the glutamatergic synaptic strength (38). The phosphorylated CaMKIIα, in turn, has been reported to phosphorylate many post synaptic density (PSD) proteins such as NMDARs, AMPARs, SynGAP, PSD-95, Synapsin1, and MAP2 in Ca^{2+}-independent manner and thus modulate the synaptic plasticity. Evidence from literatures also suggests that the activated CaMKIIα is translocated to the nucleus (39, 40, 41), and catalyzes phosphorylation of CREB, which upon binding with the corresponding promoter sequences regulates the expression of memory associated genes (42). However, pattern of alterations in the expression of CaMKIIα and its association with age and sex is not well understood. Information on alterations in the expression of CaMKIIα in various regions of brain may provide an important basis which might underlie the postnatal aging and sex-dependent differences in the consolidation of learning and memory and thereby cognitive functions of the brain.

Therefore, in the current communication, we have studied expression of CaMKIIα in different regions of young, adult and old mice of both the sexes using Western blotting and semi quantitative RT-PCR techniques and correlated with our findings with the age- and sex-dependent alterations in spatial learning and memory. Our data are novel as the CaMKIIα expression has been found to vary among different regions of brain as a function of age and sex, which may be implicated in alterations in the synaptic plasticity which might be underlying the age- and sex-dependent differences in the memory function of the brain.

Materials and methods

Animals and isolation of different brain regions

Inbred male and female mice of AKR strain (young, 8±2 weeks, adult, 20±4 weeks and old, 70±5 weeks) were used for the present study. They were fed standard

mice feed and water was supplied *ad libitum*. Mice were maintained at 25±2° C and under 12 hr light/dark cycle. They were sacrificed by cervical dislocation, their brain cases were opened and various brain regions such as frontal- and parietal cortices and hippocampus were removed, cleaned of adherent blood, blotted dry between folds Whatman 1M filter paper carefully, dipped in the liquid nitrogen and then stored at -70° C or used immediately for the experiment. All the experimental procedures were carried out following the guidelines of Institutional Ethical Committee of Banaras Hindu University. 5-6 mice were grouped together each age group and the experiments were repeated thrice.

Reagent and antibodies

Analytical grade and molecular biology grade chemicals were used for all the experiments. They were purchased from Sigma, USA or Merck India. Antibodies against CaMKIIα and β-Actin were purchased from Santa Cruz Biotechnology Inc, USA and Sigma, USA, respectively. The HRP-conjugated secondary antibody was obtained from Bangalore Genei, India. CaMKIIα and β-Actin specific primers were custom synthesized by Imperial Biomed, USA.

Isolation of total RNA from frontal and parietal cortices and hippocampus

Total RNA was isolated from various brain regions of young, adult and old age mice using TRI reagent® (Sigma, USA) as described in manufacturer's manual. Briefly, the pooled brain tissues were separately homogenized in 10 volume of the TRI reagent using glass homogenizer and pestle. Thereafter, the homogenates were separately centrifuged at 12,000Xg for 10 min. at 4^{o} C to remove the insoluble materials. The supernatant was incubated for 5 min at RT to dissociate nucleoprotein complexes. Then 0.2 times the homogenate volume of chloroform was added to each tube, vortex mixed for 20 sec and incubated for 15 min at RT. The homogenate was centrifuged at 12,000Xg for 15 min at 4^{o} C. Colorless upper phase (containing RNA) was carefully drained into separate tubes. Thereafter, half the volume of isopropanol (in respect to homogenate vol.) was added to each tube and incubated at RT for 10 min. The precipitated RNA was collected as pellets by centrifugation at 12,000Xg for 10 min at 4^{o} C, washed with 75% ethanol, dried at RT and dissolved in DEPC-treated water. To ensure purity of isolated RNA, the RNA preparation was treated with DNase I before quantitation to remove the contaminating DNA. Thereafter, the RNA content was determined by measuring the absorbance at 260 nm.

Semi quantitative RT-PCR

Reverse transcription

For reverse transcription of RNA, 2.0 μg of RNA was mixed with 200 ng of random hexamer primer in sterile DEPC-treated water in the reaction volume of 11.0 μl. It was incubated at 70°C for 5 min and chilled on ice and thereafter 10X reaction buffer (750mM KCl, 500mM Tris-HCl, 30mM MgCl2, 100 mM DTT), 2.0 μl; 10 mM

dNTP mix, 2.0 µl; RNase inhibitor (human placenta) 0.5 µl (20 units); Deionized water, 3.5 µl were added. The tube was then incubated for 5 min at 25°C and 1.0 µl of MMuLV reverse transcriptase (RevertAid^{TM} H Minus, 200 units) was added. Further, the tube was incubated for 10 min at 25°C and then at 42°C for one hour. The reaction was terminated by heating at 70°C for 10 min, and after chilling on ice the tube was stored at -70°C or directly used for the PCR reaction.

Polymerase chain reaction

For PCR reaction, 1/10th of the reaction volume, i.e. 2 µl of the cDNA was taken and PCR was carried out using a thermal cycler (MJ Mini, Bio-Rad). The PCR conditions and specific primers used for amplification as mentioned in table 1. The PCR amplified CaMKIIα and β-actin cDNA were resolved on 8% native polyacrylamide gel (Native-PAG) containing 0.5X TBE buffer. The samples were mixed with 6X loading dye containing 30% glycerol, 0.25% bromophenol blue and 0.25% xylene cyanol. After electrophoresis was over, gels were stained in ethidium bromide solution, visualized under UV-Transilluminator and the images were captured by digital camera.

Tissue lysate preparation

Various brain region tissues were homogenized in 50 mM Tris-Cl, pH- 7.4, containing 0.2 % triton X-100, 5mM EDTA, 5mM EGTA, 2 mM PMSF, 5 mM benzamidine, 2 mM βME and protease inhibitor cocktail (Sigma Aldrich) as described by Prasad and Singh, 2014 (43). Protein content of the tissue lysate was determined by Bradford method using BSA as standard (44).

SDS-Polyacrylamide gel electrophoresis

The tissue lysate proteins were resolved on a 12% SDS-Polyacrylamide gel containing acrylamide: bisacrylamide (29.2:0.8), 390 mM Tris-Cl, pH 8.8, 0.1% SDS, 0.1% APS and TEMED with stacking gel containing 5% acrylamide: bisacrylamide mix (29.2:0.8), 125 mM Tris-Cl, pH 6.8, 0.1% SDS, 0.1% APS and TEMED following the procedure of Laemmli, 1970 (45). Briefly, 25 µg of the lysate sample was denatured in 1X Laemmli gel loading buffer (50 mM Tris-Cl (pH-6.8), 1% SDS, 1% β-ME, 10 % glycerol and 0.1% bromophenol blue) on boiling water bath for 5 min, cooled immediately on ice and loaded in parallel with pre stained protein marker (MBI Fermentas). After electrophoresis was over, the gel was stained in 0.05% Coomassie brilliant blue R-250 in 50% methanol, 40% water, 10 % acetic acid medium. Thereafter, the gel was adequately destained for quality check, and then photographed. A fresh gel was run and directly processed for Western blotting without staining with CBB-R250.

Western blotting and signal detection

The gel obtained from above was incubated in transfer medium containing 192 mM glycine, 25 mM Tris, 0.05% SDS and 20% methanol for 10 min and the proteins

from the gel were immobilized onto PVDF (0.45μ Millipore) membrane at 4° C for overnight at constant power supply of 50V by wet transfer method. The transferred protein onto the membrane was visualized by staining of the gel in Ponceau-S (0.5% Ponceau S, 1% acetic acid and deionized water). The membrane was blocked in 5% non-fat milk in PBS pH -7.4 for 4 hrs at RT. The blot was then incubated with rabbit anti- CaMKIIα (1:10,000 dilution, Abcam, UK), β-actin (1:15,000 dilution, HRP-conjugated, Sigma-Aldrich, USA) and in 5% nonfat milk and 0.1 % Tween-20 in PBS pH 7.4, overnight at 4° C, washed with 1x PBST (0.1% Tween-20). The CaMKIIα blot was incubated with HRP-conjugated goat anti-rabbit IgG (1:2,500 dilutions, Bangalore Genie, Bangalore) in PBS pH 7.4 containing 5% nonfat milk and 0.1 % Tween 20 for 4 hr at RT. After washing with PBST pH 7.4, immunoreactive proteins were detected by enhanced chemiluniscence (ECL) method using Super Signal West Pico Kit (Pierce Biotechnology, USA) on X-ray film. The signals were captured and their intensity was measured by alpha imager densitometric scanning. β-Actin was used as internal control.

Morris Water Maze (MWM) test for spatial learning and memory

To analyze the age- and sex-dependent alterations in spatial learning and memory, Morris water maze (MWM) spatial navigation test paradigm was used (46). The behavioral tasks which included both the training and testing procedures were strictly conducted between 08:00 A.M. and 10:00 A.M. Before experimentation, mice were habituated for 1 hr in a dim room to adjust to the environment. During the training phase, the mice were placed at the far end of the illuminated compartment facing away from the door. Mice belonging to different experimental groups were subjected to MWM test in a circular water tank (76 cm high × 106 cm diameter) filled with water (up to 30 cm deep) maintained at room temperature (23 ± 2° C) that was made opaque with nontoxic white paint. The surface of the hidden plexi glass platform (8 × 8 cm) was 1 cm below the water surface. Four trials each of 90s/day with an inter-trial interval of 5min were conducted for 7 successive days with the same platform location. There were four possible locations for the platform. One of these platform positions was assigned to each mouse as the correct location during the training. Latency to reach the platform was recorded during the training procedures for 7 consecutive days. When the distance between the mouse and the wall of the pool was less than 8 cm, the mouse was considered to be at the perimeter. Behavioral performances of both male and female mice belonging to different age groups for 7 consecutive days (four training trials/ day) were recorded and represented as line graphs to show the age-and sex-dependent alterations in acquisition of spatial memory. Again, all subjects were tested for a probe short-term memory-retention test (probe test A) after 2hrs of final training trial on the same day and their escape latencies were recorded. Then after, all age groups of mice were kept idle for two days in their respective cages under standard housing conditions to assess the retrieval of spatial memory on day 10 (probe test B or probe retrieval test without platform to assess long-term memory retention which is expressed in terms of track path followed to locate and reach up to the platform, total

distance travelled (in meters), time spent in the platform quadrant (in sec.), platform crossings in no. of times), respectively. Behavioral performances of mice were recorded by camera and analysis was performed using ANY-maze behavior software (Stoelting Co., USA). After 30 min of the test, mice were sacrificed by cervical dislocation. Whole brain from experimental and control mice was removed quickly, briefly chilled on ice, and the desired brain tissues were taken out from each brain and pooled. They were then processed further for isolation of RNA and proteins. Semi-quantitative RT-PCR and Western blotting studies were done as described earlier.

Statistical analysis

All the experiments were repeated three times (n= 6-7/age/experiment). The gels and X-ray films were photographed by a digital camera (Nikon Corporation, Japan). The bands were analyzed and quantitation was done using computer-assisted densitometry (AlphaEase FC^{TM} software, Alpha Innotech Corporation, CA). For RT-PCR and Western blots, the signal intensity of the band was measured after normalization with β-Actin and expressed as relative densitometric value (RDV). Results were presented as mean ± SEM of data obtained from three independent sets of experiments. Statistical analysis of the molecular and behavioural data was performed using One-way ANOVA followed by Tukey's post hoc test using standard SPSS 11.5 software. The $P<0.05$ values were taken as statistically significant.

Results

Optimization of PCR amplification cycle number

To obtain the optimum quantity of CaMKIIα and β-actin transcripts, PCR amplification was performed separately for 10-40 and 15-40 cycles, respectively. For CaMKIIα, the 599 bp amplicon was detected from 20th cycle and it was found to be linear till 35th cycle. In regard to β-actin, the 543 bp amplicon was detectable from 25th cycle and linearity was observed till 40th cycle. Therefore, the optimum cycle numbers were chosen as 24 (Fig. 1a) and 26 (Fig. 1b) for semi quantitative analysis of CaMKIIα and β-Actin, respectively.

Semi quantitative reverse transcriptase-polymerase chain reaction analysis

Alterations in the level of CaMKIIα transcript in frontal cortex

Our semi-quantitative RT-PCR data reveal that the level of CaMKIIα gene expression in the frontal cortex is highest in 8 week (young) mice of both the sexes. Thereafter, with advancement of age, its level is significantly down regulated in 20 week male as well as female (adult) mice ($P<0.05$) (Fig. 2a & b). The expression of CaMKIIα is further decreased significantly in the frontal cortex of 70 week (old) mice in the sexes ($P<0.05$).

Alterations in the level of CaMKIIα transcript in parietal cortex

The RT-PCR data reveal that the level of the CaMKIIα transcript is highest in the parietal cortex of 8 week (young) male mice and its level is significantly and gradually down regulated in the 20- (adult) and 70 week (old) age ($P<0.05$) (Fig. 3a & b). However, the pattern of its expression in female mice is opposite i.e. the level of the CaMKIIα transcript is lowest in 8 week female mice and thereafter, its level is significantly up regulated in 20- and 70 week female mice ($P<0.05$).

Alterations in the level of CaMKIIα transcript in hippocampus

Our RT-PCR data reveal that the level of CaMKIIα transcript is lowest in the hippocampus of 8 week male mice whereas it is significantly up regulated in 20- and 70 week mice ($P<0.05$) (Fig. 4a & b) . In female mice, its level is significantly higher in the adult age ($P<0.05$) compared young mice, which thereafter, undergoes significant decline in 70 week age ($P<0.05$).

Immunoblot analysis of CaMKIIα protein expression

Alterations in the level of CaMKIIα protein in frontal cortex

Immunoblot data on the level of CaMKIIα protein shows that its pattern is similar in the frontal cortex of both male and female mice of different ages as was observed in the pattern of its transcripts (Fig. 5a & b). It reveals that the expression of *CaMKIIα protein* is highest in the frontal cortex of 8 week (young) mice and its expression is significantly down regulated in 20- and 70 week old mice. The level of *CaMKIIα protein is significantly lower in the 70 week (old)* male *mice than in the adult mice* ($P<0.05$) (Fig. 5a). Our immunoblot data further reveals that the pattern of the *CaMKIIα protein* expression is similar during aging in female mice.

Alterations in the level of CaMKIIα protein in parietal cortex

Data obtained from the immunoblot analysis on the *CaMKIIα protein* shows the sex-dependent dimorphic patterns of the expression of the CaMKIIα protein. In male, its expression is highest in the parietal cortex of young mice (Fig. 6a) whereas its level was least in the female mice (Fig. 6b). With advancing age, its expression is significantly down regulated in adult male mice and it is gradually up regulated with the increasing age in female mice ($P<0.05$).

Alterations in the level of CaMKIIα protein in hippocampus

The immunoblot data on the expression of *CaMKIIα protein* reveals that level of CaMKIIα expression is lowest in the hippocampus of male young age mice. Thereafter, its expression is significantly and gradually up regulated in adult age mice (20 week) in both male and female mice ($P<0.05$). However, it expression is significantly up regulated in 70 week old male mice ($P<0.05$) (Fig. 7 a & b) whereas it undergoes significant down regulation in 70 week old female mice ($P<0.01$).

Age-dependent alterations in spatial learning and memory

Effect of aging and sex on acquisition of spatial memory

MWM data recorded for analyzing the age- and sex-dependent alterations in acquisition of spatial memory expressed in terms of escape latency i.e. the time to reach the platform (in sec.) showed that on day 1 of learning trials, young, adult and old mice belonging to both male and female sex groups exhibited higher escape latencies which was highest in case of old mice of both the sexes as compared to young and adult age groups. With repeated training trials for 7 consecutive days, the platform-searching behavior is gradually and significantly improved and on 7^{th} day, mice belonging to all the three age groups exhibited nearly similar learning potentials in both the sexes (Fig. 8a & b). However, female mice of all the three age groups performed better in acquisition of spatial memory as compared to their age-matched male groups (Fig. 8b).

Analysis of effect of aging and sex on short-and long-term retrieval of spatial memory

Effect of age- and sex on short-term retention of spatial information (expressed in terms of escape latencies) was evaluated on the same day after 2hrs of final acquisition trial by removing the platform from the MWM apparatus on the probe trial (Probe test A) (Fig. 8c). Thereafter, the long-term retention of spatial information was evaluated by conducting the second probe test (probe test B) after 2 days (on 10^{th} day) of the initial probe test A and was expressed in terms of track path followed to locate and reach up to the platform (Fig. 9a), total distance travelled (in meters) (Fig. 9b), time spent in the platform quadrant (in sec.) (Fig. 9c) and platform crossings (in no. of times) (Fig. 9d). Our data related to the retentions of both short-term and long-term of spatial memory in MWM as evaluated through probe tests A & B, respectively, revealed that the retention/retrieval of memory in both the sexes is severely compromised from adult to old age during aging.

The track reports (Fig. 9a) and total distance travelled to reach the platform (in meters) (Fig. 9b) belonging to different age groups of mice showed that young mice of both the sexes spent most of the time in or around the target quadrant therefore they travelled least distance to reach the platform whereas male and female mice of adult and old age groups spent their most of the time in other quadrants in relation to the target quadrant therefore they travelled longer distance to reach the platform which was further higher in old mice compared to adult mice. The total time spent in target quadrant (in sec.) was also highest in case of young age and is gradually decreased from adult to old age with old mice exhibited minimum time spending in platform quadrant. Surprisingly, female old mice spent more time in platform quadrant as compared to their age-matched male old mice (Fig. 9c). Again, the number of platform crossings was recorded maximum for young age mice that significantly decreased up to adult age in both the sexes. Thereafter, it was further decreased in old male mice but again increased in female old age groups (Fig. 9d).

Discussion

Expression of genes related to learning, memory and cognition, and many brain functions are altered during development and aging and they exhibit sexually

dimorphic patterns. However, alteration in the expression of CaMKIIα, known as a memory molecule (47) and which plays critical role in synaptic plasticity during memory development, in respect to sexually dimorphic behavioral pattern that differ as a function of aging is not well established. In the current study, we have characterized pattern of its expression in prefrontal cortex, parietal cortex and hippocampus of young, adult and old mice of both the sexes to understand whether aging has effects on its expression in these brain regions, and the effects are sexually dimorphic and which might have possible correlation with age- and gender-dependent variation in the acquisition/consolidation of memory in above brain regions.

Our data suggests that the expression of CaMKIIα gene is gradually down regulated in the frontal cortex of male as well as female mice as the age of mice increases from young age to adulthood, and from adult to old age and this effect is similar in both the sexes. This age-dependent decline in the level of CaMKIIα may be correlated with decline in age-dependent gradual decline in the acquisition of information, decline in the formation of the frontal cortex associated short term learning and memory and taking immediate decision and execution of memory based tasks (31, 48). This decline in learning, memory and cognition may be attributed to low abundance of AMPA receptor on the post synaptic density due to decline in the CaMKII-dependent phosphorylation of its GluR1 subunits (49) which might not be adequately able to excite the NMDA receptor (50, 51) and thereby decline in its conductance on the post synaptic membrane, synaptic strength and LTP (52, 53, 54). Gradual decline in the expression of CaMKIIα in parietal cortex from young to old age may be associated with age-dependent decline in the consolidation and retrieval of episodic memories. Our finding on decline in the level of CaMKIIα in the parietal cortex may be correlated with functional MRI based report on the role of parietal cortex in its partial contribution in the development of episodic memory (55-58). However, our finding on the up regulation of CaMKIIα during aging in the female mice contradicts with our data on its age-dependent down regulation in male mice. This indicates a gender-dependent differential role of CaMKIIα in the female mice as a function of age. It is in alliance with number of publications on sex biased function of the brain as some of the brain functions exhibited by female subjects do not decline due to aging. Thus the up regulation of CaMKIIα in the parietal cortex of female mice might accordingly be associated with its function influenced by female sex steroid hormones. Further, as parietal cortex has been shown to be critical for retrieval process of memory (59) and transient storage (60), diminished expression of CaMKII in male parietal cortex may be responsible for reduced capability in retrieving and storing of memory traces whereas in the female increased expression may suggest the disturbance in this capacity of these processes. More importantly, age-associated difference in expression pattern of CaMKIIα in selective brain regions may suggest sexual dimorphism in brain functions. In contrast to young, reduction in proper induction of LTP and LTD has been shown in slices prepared from visual cortex of adult CaMKIIα mutant mice, further confirming the diminished activity of CaMKIIα with advancing

age, especially in cerebral cortical regions (6). Also, age-dependent decline in the expression of CaMKIIα may be correlated with decline in its activity due to an altered imbalance between CaMKIIα and CaMKIIβ, which might underlie the age- and gender- dependent alterations in the synaptic remodeling and hence cognitive ability of brain.

Our data clearly indicates that the level of CaMKIIα significantly increases in hippocampus in adult age compared to its level in young irrespective of the gender factor, however, its expression further rises in male old mice whereas it is significantly down regulated in female old mice. Though the pattern of its expression in hippocampus in old mice is sex-dependent, but its abundance in the hippocampus might be attributed to its gender dependent involvement in memory processes. Similar predominant expression of the CaMKIIα in CaMKIIα-GFP line has been found (61). CaMKII activity has been shown to affect ion channel function by phosphorylating the GluR1 subunit of the AMPA receptor (62) and the NR2B subunit of the NMDA (N-methyl-D-aspartate) glutamate receptor (63) and thereby enhance the LTP in the hippocampus by way of phosphorylation of tubulin, microtubule associated proteins (MAPs) and Tau, and causes outgrowth in differentiating neurons by altering the neuronal cytoskeletal organization (64). Therefore, above age-dependent variation in the pattern of CaMKIIα expression in the hippocampus may be attributed to age-dependent alterations in memory consolidation and strengthening of synapse (LTP) in both the genders which may further differ in old age in respect to gender as observed in our study. Rise in the expression of CaMKIIα in adult age may be correlated with reshaping of synaptic plasticity in both sexes compared to young age. Decline in its level in female old mice may be correlated with a distinct patterns of synaptic plasticity that might be estrogen mediated. Further, age-dependent enhanced expression of CaMKIIα in hippocampus in either sex may be correlated with increased intracellular Ca^{2+} that might lead to over excitation of hippocampal neurons and may further disturb the process of acquisition, consolidation and conversion of short-term memory into long–term memory sex-dependent manner in old age due to high Ca^{2+}-induced excitotoxicity. It may also be speculated that LTP induction might be normal in adult age but it may not be properly maintained due to abnormally enhanced activity of CaMKIIα in the hippocampus in older age and it might be correlated with abrupt dendritic branching and proteasomal degradation of synaptic target proteins required for structural plasticity and neuronal connectivity. Thiagarajan et al (65) has shown that enhanced synaptic plasticity up regulates CaMKIIα expression and down regulates CaMKIIβ in cultured hippocampal neurons. Up regulation of CaMKIIα in the aging male hippocampus and female parietal cortex may be correlated with enhanced LTP/ synaptic activity in these brain regions in older age, however, this enhanced synaptic activity may lead to abnormal synaptic connectivity and thus memory formation, however, it needs to be established. Gradually increased expression of CaMKIIα from young to adult age in the hippocampus of male as well as female mice might be correlated with a positively controlled neural network during development of synaptic

plasticity in both the genders which gets further altered in sex-dependent manner i.e. its level continues to be increased in old male mice whereas it is significantly down regulated in female old mice. This age- and sex-dependent variation in the expression of CaMKIIα might be correlated with age- and sex-dependent variation in the synaptic plasticity. However, on the contrary, level of hippocampal CaMK has been reported to be unaffected in the age range of four to 12 months in senescent accelerated mice (SAM) strain (66). Our data pertains to the gender-dependent differential expression pattern of CaMKIIα during normal aging mice which might be correlated with altered consolidation of information and memory retention as a function of normal aging. However, its correlation with above finding needs a carefully designed study.

Our data on the age-dependent differential patterns of CaMKIIα expression is important in the light of significance of calcium homeostasis during development of synaptic plasticity. Calcium is known for its role in LTP and LTD by regulating the release of neurotransmitters from presynaptic terminals and in the regulation of activity-dependent gene expression, synaptogenesis, dendritic spine development, synaptic plasticity and processes contributing to the primary neuronal function in information processing, storage and retrieval. Tightly controlled calcium homeostasis is required to support normal brain physiology and to maintain neuronal integrity and long-term cell survival (67). Any disturbance in normal physiological (intracellular or extracellular) concentration of calcium may be correlated with disturbance in normal brain function and may lead to neurodegeneration (68). Modest impairment in Ca^{2+} homeostasis in the brain has been shown to have profound effects on normal brain functioning (69). Therefore, disturbance in the Ca^{2+} homeostasis may affect the role of CaMKII which might retrospectively affect activities of voltage-gated calcium (VGC) as well as NMDA (N-methyl D-aspartate) glutamate receptor channels (70). Hippocampus has been reported to be particularly susceptible for Ca^{2+}-dependent neurotoxicity in older age due to decreased expression of Calretinin and Calbindin-28 (calcium binding proteins) and reduced Ca^{2+} buffering capacity of mitochondria (71). While NMDAR-dependent LTP is decreased in CA1 area of hippocampus in aged rats (72), VSCC- dependent LTP was found to be increased (73). Globally dysregulated calcium signaling due to enhanced L-type Ca^{2+} currents has been implicated in aging and neurodegenerative diseases (74, 75). Enhanced Ca^{2+} influx in aged neurons has been proposed to be a likely candidate to play a role in impaired functioning and enhanced susceptibility of neurons to neurotoxic influences during aging and it has apparently attributed to increased activity/ conductance (76) of VSCCs and not to the reduction in inactivation mechanism (77, 78).

Despite its association with PSD proteins in regulation of synaptic strength, CaMKII is known to be linked with ubiquitin-proteasomal system at synapses (79) where autophosphoryalted CaMKII has been shown to enhance binding affinity of proteosomes and it serves as a scaffolding factor for proteasomal machinery in an activity-dependent manner. It helps in the accumulation of proteasomes at spines and mediates degradation of polyubiqutinated proteins at spines by phosphoryalting

proteasome subunit Rpt6 on Serine 120. Down regulation of expression/ phosphoryaltion of CaMKIIα during aging may be viewed as normal aging phenomenon which might be accountable for decline in LTP, synaptic plasticity and thus learning and memory whereas enhanced expression/phosphoryaltion may lead do degenerative changes due to pathophysiological aging or may be a coping up mechanism to deal with this anomaly. The phenomenon of CaMKIIα translocation from soluble to particulate fraction is known to be under dynamic and precise control after electrical stimulation and is neuronal region-specific (80). When coupled with NMDA receptors at the post synaptic density (PSD), it is involved in the translation of electrical signal into induction of LTP whereas when in the cytosol, it may function as a signaling molecule regulating transcription of genes associated with long-term memory. Reduced CaMKIIα in the frontal cortices of both the sexes may indicate their reduced involvement in the acquisition of new information and also suggest lack of its effector function for memory consolidation in this brain region and suggest that cortical functions are more compromised during brain aging as compared to hippocampus. The gender-and age-related differential expression of CaMKIIα in various brain regions may be correlated with gender and age-dependent differences in the patterns of memory development.

To test whether the age- and the brain region/tissue- dependent variation in male and female mice is linked with similar changes in the spatial learning and memory, we performed MWM test on the young, adult and old mice of both the sexes. Our MWM test data suggested that the learning ability of both male and female mice all ages is affected during aging. It also showed that female mice of all three ages were able to learn better. Thereafter we questioned whether, the memory retrieval process, an index of the intactness of memory trace storage, is affected by age and might that be sex-dependent using the MWM test paradigm as mentioned above, however, during this investigation, the platform was removed and memory retrieval aspects at how early they locate the platform quadrant (without platform) (measured in terms of length they travel for the same, their stay time in the platform quadrant and how often they cross the platform quadrant. Taken together, based on the above parameters, our data suggested memory retrieval ability of mice of both sexes decline as a function of age and the performance of female mice is better than in the male mice i.e. they show sexually dimorphic pattern of their cognitive abilities. In general, this decline in the memory retrieval (cognitive function of the brain) is correlated with age-dependent alterations in the expression of CaMKIIα which varies in respect to major memory forming brain regions/tissues. Our data provides a link with well documented gender differences in cognitive functions during aging, however, Geinisman et. al. (2004) (83) has reported that spatial memory is not affected by chronological aging. This gender- and region-dependent distribution of CaMKIIα may be one of the neuromodulatory mechanisms leading to change in the synaptic plasticity affecting memory and cognition and could be important in the field molecular biology of cognitive aging.

Disclosure Statements

To the best of their knowledge and belief, authors do not have any personal, potential and actual conflict of interest related to present research. SP is the Ph.D. supervisor of VND and is Professor in the Department of Zoology, Banaras Hindu University. MSK (posthumous) was Professor Emeritus in the same Department to whom VND was associated initially and later he joined SP for his current research work.

Acknowledgement

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Legend to Figures:

Fig. 1. Determination of linearity of PCR amplification for CaMKIIα and β-actin. Integrated density value of the PCR products obtained for different number of cycles is presented as a function of PCR cycles. Optimal number of cycle selected is shown by *asterisk.* (a) PCR product of 599 bp for CaMKIIα mRNA. *M* stands for marker; 100 bp DNA ladder. (b) PCR product of 543 bp for β-actin mRNA.

Fig. 2. RT-PCR of CaMKIIα gene in the frontal cortex of male (a), and female (b) mice of various ages. *M* denotes marker; 100 bp DNA ladder. *W* denotes age in weeks. Histogram represent cumulative data expressed as mean ± SEM obtained from three different sets of experiments conducted for male and female frontal cortex. * Significance from 8-week group; ** Significance from 20-week group; $P<0.05$.

Fig. 3. RT-PCR of CaMKIIα gene in the parietal cortex of male (a), and female (b) mice of various ages. Histogram represent cumulative data expressed as mean ± SEM obtained from three different sets of experiments conducted for male and female parietal cortex. * Significance from 8-week group; ** Significance from 20-week group; $P<0.05$. *W* denotes age in weeks.

Fig. 4. RT-PCR of CaMKIIα gene in the hippocampus of male (a), and female (b) mice of various ages. *W* denotes age in weeks. Histogram represent cumulative data expressed as mean ± SEM obtained from three different sets of experiments conducted for male and female hippocampus. * Significance from 8-week group; ** Significance from 20-week group;$P<0.05$.

Fig. 5. Immunoblot showing 54 kDa band of CaMKIIα protein in the frontal cortex of male (a), and female (b) mice of various ages. Histogram represent cumulative data expressed as mean ± SEM obtained from three different sets of experiments conducted for male and female frontal cortex. * Significance from 8-week group; ** Significance from 20-week group; $P<0.05$. *W* denotes age in weeks.

Fig. 6. Western blot of CaMKIIα protein in the parietal cortex of male (a), and female (b) mice of various ages. Histogram represent cumulative data expressed as mean ± SEM obtained from three different sets of experiments conducted for male and female parietal cortex. * Significance from 8-week group; ** Significance from 20-week group; $P<0.05$. *W* denotes age in weeks.

Fig. 7. Western blot of CaMKIIα protein in the hippocampus of male (a), and female (b) mice of various ages. *W* denotes age in weeks. Histogram represent cumulative data expressed as mean ± SEM obtained from three different sets of

experiments conducted for male and female hippocampus. * Significance from 8-week group; ** Significance from 20-week group; $P<0.05$.

Fig. 8. Sex-dependent alterations in acquisition of spatial memory as expressed in terms of escape latency. Line graphs show age-and sex-dependent alterations in acquisition of spatial learning during a 7-day learning regime in Morris water maze (MWM) in (a) male and (b) female mice, respectively. Analysis of age- and sex-dependent alterations in retrieval of short-term spatial memory in young, adult and old male and female mice (c) evaluated on the same day after 2hrs of final acquisition trial by removing the platform from the MWM apparatus and expressed in terms of escape latency i.e. time (in sec.) taken by mice to reach the platform during MWM test (Probe test A).

Fig. 9. Analysis of age- and sex-dependent alterations in retrieval of long-term spatial memory by MWM task on day 10 without platform (probe test B conducted on day 10 without platform). (a) Track reports (b) total distance travelled to reach to the platform (in meters) (c) total time spent in platform quadrant (in seconds) belonging to different age groups of mice (d) platform crossings (number of times). M denotes male mice and F denotes female mice. *Significance from male young age group, **Significance from 20w male group; #Significance from female 8w group; ##Significance from female 20w age group; @ Significance from inter-sex age-matched group; $P<0.05$.

Figures

Fig. 1

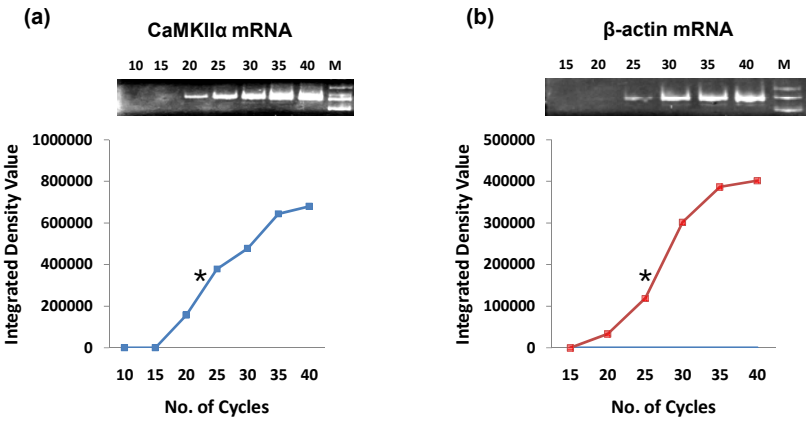


Fig. 2

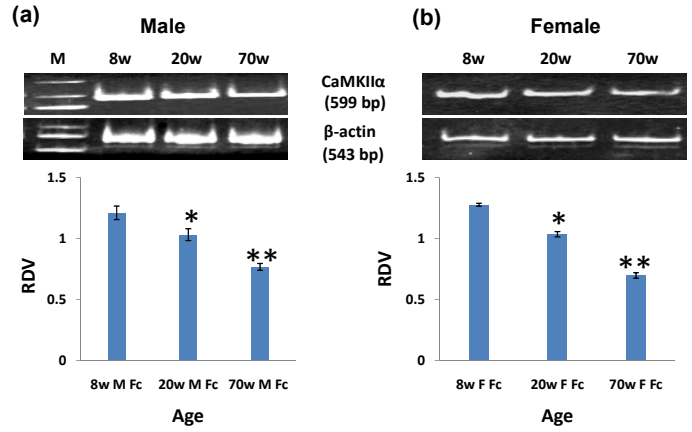
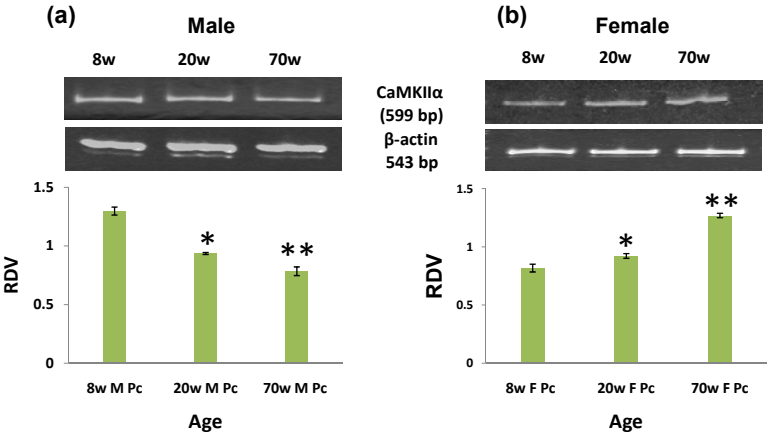


Fig. 3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CaMKIIα
(599 bp)
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70w M Pc
Age
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Female
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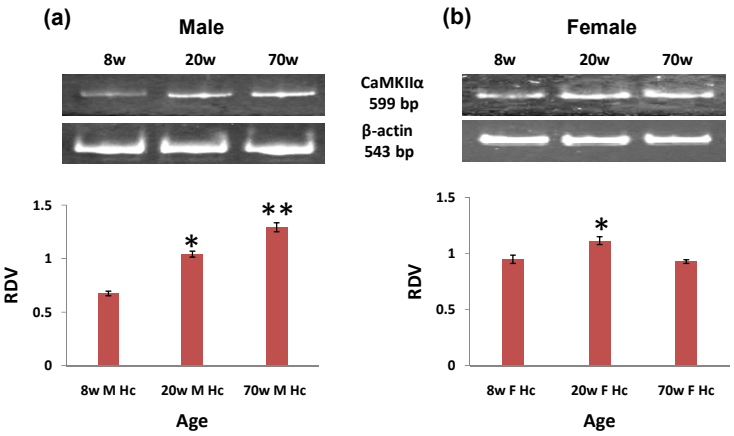
70w F Pc
Age

Fig. 4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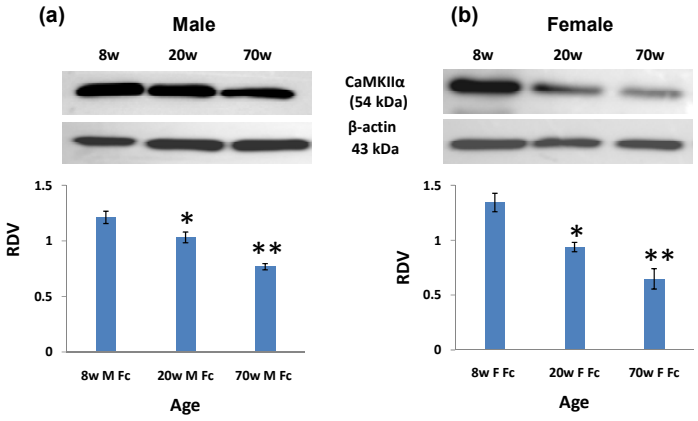
70w F Hc
Age

Fig. 5


(a)
Male
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(54 kDa)
β-actin
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20w M Fc
70w M Fc
Age
(b)
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RDV
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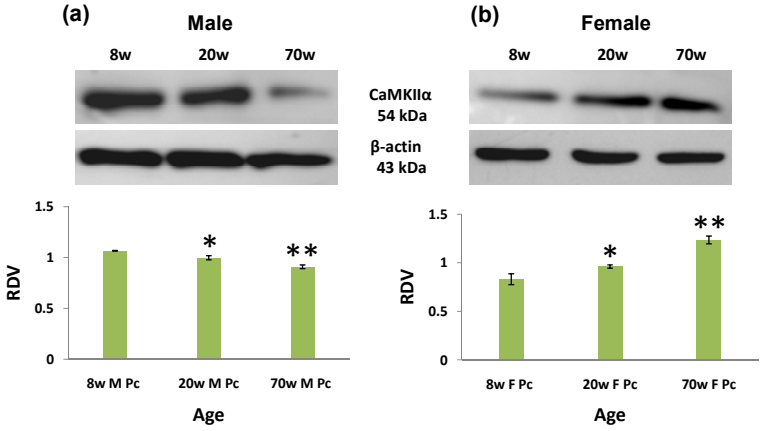
70w F Fc
Age

Fig. 6


(a)
Male
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CaMKIIα
54 kDa
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Age
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8w F Pc
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70w F Pc
Age

Fig. 7

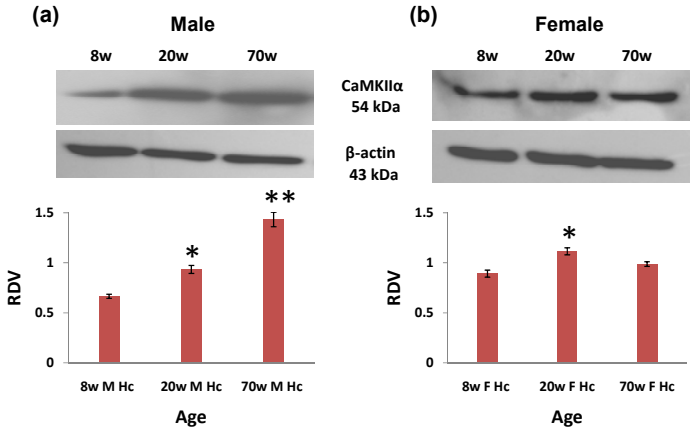
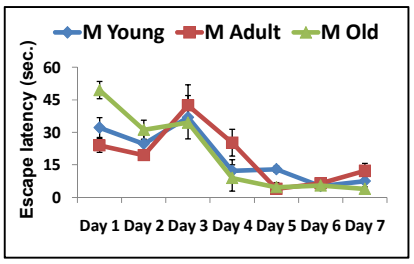
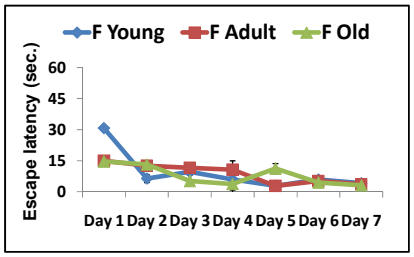


Fig. 8 (a)



(b)



(c)

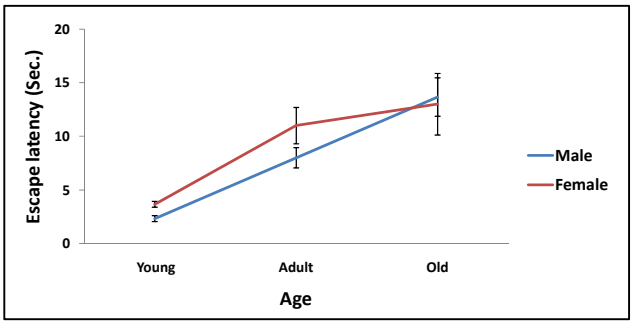


Fig. 9 (a)

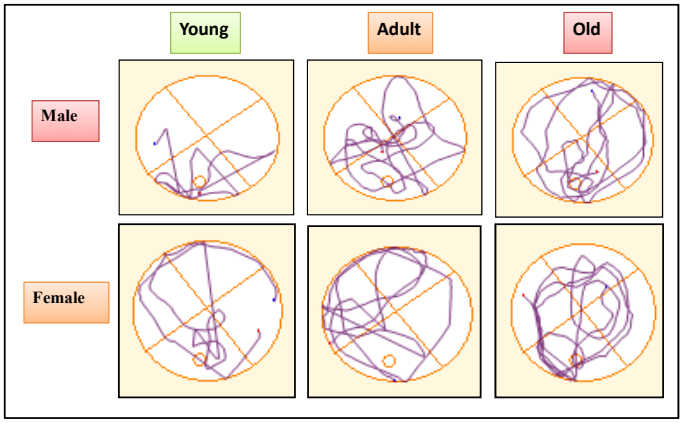
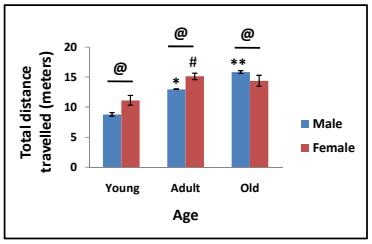
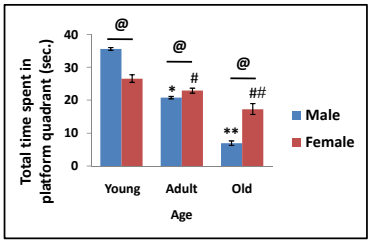


Fig. 9

(b)



(c)



(d)