Journal of Scientific Research
Banaras Hindu University, Varanasi

Vol. 61, 2017 : 39-60
ISSN : 0447-9483

CaMKIIα EXPRESSION IS DOWN REGULATED IN THE CEREBELLAR CORTEX OF MICE AS A FUNCTION OF PHYSIOLOGICAL AGING

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Abstract

The current study was carried out to find out whether aging affects CaMKIIα expression in the cerebellar cortex of mice and which might be gender-dependent. Accordingly, the cerebellar cortices were dissected out from young (8±2 week), adult (20±4 week) and old (70±5 week) male and female mice. Expression of CaMKIIα was studied by semi quantitative RT-PCR and Western blotting and the CaMKIIα transcriptional machinery was assessed at CaMKIIα promoter-CREB interaction level by EMSA. Our RT-PCR and Western blot data reveal that CaMKIIα expression is significantly decreased in adult and old mice compared to young age in both sexes. The decline in the CaMKIIα expression was associated with significant gradual decline in the level of CaMKIIα promoter-CREB interaction in adult mice compared to young age, however, in old age, the reduced CaMKIIα expression is correlated with increased CaMKIIα promoter-CREB interaction in both male and female mice. The age-dependent decline in the CaMKIIα expression may be implicated in age-dependent decline in LTP leading to decreased motor, motor learning, altered emotional and cognitive functions of the brain during aging with underlying reduced transcriptional machinery in adult age, which is reversed in old mice of both sexes.

Highlights

- Aging leads to gradual decline in CaMKIIα expression in the cerebellar cortex in male and female mice.
- The CREB- CaMKIIα promoter interaction is differentially altered during aging in sex-independent manner
- Decreased CaMKIIα expression from young to adult age corresponds to reduced CREB- CaMKIIα promoter complex and in old age the lowered CaMKIIα level correlates with increased complex formation.

Key words-CaMKIIα, aging, cerebellar cortex, sexual dimorphism, motor learning and memory

Introduction

Aging, a universal and irreversible physiological process, is a known cause of decline in the motor as well as motor learning and memory functions of the brain in addition to impairments in cognitive learning and memory leading to severe change in the quality of elderly human life. According to a recent report in rodents, the motor functions undergoes 7.4% and 13.5% decline in 3-20 months and 20-26 months of age, respectively, as compared to young age [1]. Decline in the gross and fine motor as well

other higher cognitive functions deficits are equally caused due to inception of neurodegenerative disorders and other factors in elderly age. Multiple sex-dependent differences in normal aging or neurodegenerative diseases that range from structural, anatomical, biomolecular and behavioural performances [2], neurogenic properties of neural stem cells (NSCs) [3] in various brain regions including cerebellum have been documented. Sex differences in gene expression and splicing of the transcripts have been detected in the adult human brain [4] and reported to be attributed to various external and internal factors such as sex hormones, natural environment and personal habits, etc. Also, the gender-difference has been documented at the level of brain atrophy with the aging in different regions of the brain including the cerebellum. Cerebellum based motor, motor learning/memory, emotional and cognitive functions have been reported to be differentially regulated in both the sexes [5].

The cerebellum, an important neural structure plays role in the control and coordination of the fine movements and postural adjustments via regulation of acquisition and execution of motor tasks, high-order cognitive functions such as classical conditioning of the eye blink reflex [6], visuospatial recall [7] and procedural learning [8,9] by integrating information from extra-cerebellar brain regions [10] which is dependent on Purkinje cell's long-term depression (LTD) [11]. Purkinje cells receive, process and refine multiple excitatory inputs (glutamatergic) from various extra-cerebellar regions and finally give inhibitory (GABAergic) output to deep cerebellar nuclei (DCN) and thus play a key role in pattern recognition of the motor learning [12, 13]. Over the past few decades, attempts have been made to unravel the mechanisms underlying the functional decline in motor learning as a function of aging and neurodegenerative diseases; however, it is not yet completely understood. Various kinases such as CaMKII, PK-A, B & C, PKG and MAPK and phosphatases (PP1 & PP2B), glutamate (NMDA and AMPA) and GABA receptors and transcription factors (CREB, CBP, C/EBP, NFkB) play important role in above brain functions.

CaMKII, a serine/threonine protein kinase abundantly expressed in cerebral cortex, hippocampus, midbrain, and in brain stem to a lesser extent [14], plays crucial roles in synaptic plasticity and learning and memory. CaMKII activity is required for potentiating hippocampal neurons [15-17] and thus is critically associated with the learning and memory formation in hippocampus and neocortical structures [18]. Its activation in hippocampus and frontal cortex induces LTP whereas in cerebellum it leads to LTD and motor learning. CaMKIIβ, among the four different functional CaMKII subunits (e.g. α, β, γ and δ), is predominantly functional in the hind brain structures including cerebellum, however, the role of CaMKIIα, a molecular memory switch for regulating LTP [19] and LTD [20] is not understood. Further, expression of CaMKIIα is regulated by CREB1, member of a bZIP transcription factor expressed in the neuronal cells, is involved in the growth, division, differentiation and survival of the neurons. Since both CaMKIIα and CREB play role during memory processing, it is speculated that alterations in CaMKIIα expression and alterations in the interaction of CREB and its cognate CRE element in the CaMKIIα promoter are altered in

cerebellum which might be age- and sex-dependent. In the current manuscript, we have analyzed the effects of age and sex on the expression of CaMKIIα and its transcriptional regulatory mechanisms.

Materials and Methods

Animals

Inbred male and female albino mice of AKR strain (75±5 week maximum life span) of three different ages; young (8±2weeks), adult (20±4 weeks) and old (70±5 weeks) were maintained in the mice colony at 25 ± 2°C with 12h light/dark schedule and fed on standard mice pellet and drinking water *ad libitum*. All experiments were performed following the Guidelines of the Institutional Animal Ethical Committee of Banaras Hindu University, Varanasi, India for use of experimental animal. All the experiments were carried out using groups of six mice of each age and repeated 3-4 times. To excise the brain, the mice were sacrificed by cervical dislocation, cerebellar cortices were dissected out on ice and used for RNA and protein isolation and DNA-protein interaction study.

Reagents and Chemicals

Analytical grade chemicals were purchased from Sigma Aldrich, USA and gene specific primers (for CaMKIIα and β-actin) and oligos for EMSA study were purchased from Metabion International AG, Germany. Radiolabel α-^{32}P-dCTP was purchased from the Board of Radiation and Isotope Technology (BRIT), CCMB, Hyderabad, India. PVDF membrane was procured from Millipore whereas horseradish peroxidase-conjugated anti-rabbit secondary antibody was bought from Bangalore-Genei (India). All the chemicals and reagents were stored, diluted and used as per manufacturer's instructions.

Isolation of total RNA

Total RNA from cerebellar cortices of mice of various ages was isolated using TRI reagent (Sigma-Aldrich, USA) following the user's manual. Briefly, brain tissues excised from experimental mice were homogenized in 10 volume of TRI reagent using sterilized (DEPC-treated and autoclaved) glass homogenizer and pestle. After homogenization, the homogenates were incubated for 5 min at RT and then centrifuged at 12,000Xg for 10 min at 4^{o}C to remove the insoluble materials. After centrifugation, the supernatant was collected and then incubated for 5 min at RT to dissociate nucleoprotein complexes. Then 0.2X starting volume (200µl) chloroform was added to the starting volume of homogenater. It was then vortexed for 20 sec and incubated for 15 min at RT. The mixture was centrifuged at 12,000Xg for 15 min at 4°C. The colorless aqueous upper phase (containing RNA) was carefully transferred to a separate tube. To the aqueous phase, 0.5X the starting volume (500 µl) isopropanol was added and the resulting mixture was kept at RT for 10 min for RNA precipitation. The precipitated RNA was collected by centrifugation at 12000Xg for 10 min at 4^{o}C and

then washed with 75% ethanol. The pellet was dried at RT for 10 min and dissolved in DEPC treated water.

Semi-quantitative RT-PCR

Reverse transcription

For reverse transcription of RNA, 2.0 μg of DNA-free total RNA was mixed with 200 ng of random hexamer primers (MBI Fermentas) and the reaction volume was made to 11.0 μl with sterile DEPC-treated water. The reaction mixture was incubated at 70° C for 5 min in thermal cycler (MJ Mini^{TM} Personal Thermal Cycler, Bio-Rad, USA) and immediately chilled on ice. Thereafter, 2 μl of 5× reaction buffer, 2 μl of 10 mM dNTP mix and 20 U of RNase inhibitor (Ribolock™, MBI Fermentas, USA) were added, and the volume was made up to 19 μl with DEPC-treated ATDW . The tube was incubated for 5 min at 25° C and 1.0 μl (200 U) of MMuLV reverse transcriptase (New England BioLabs, NEB, USA) 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 the tube at 70° C for 10 min, and after chilling on ice the tube was directly used for the PCR reactions or stored at -80° C.

PCR amplification

For PCR reaction, 2 μl of cDNA was used and PCR was carried out using a thermal cycler (MJ Mini, Bio-Rad). The following primer pairs were used: CaMKIIα forward primer—5'-CGGAGGAAACAAGAAGAACG-3', reverse primer—5' GGGT GATCTGACAGGGAGAA-3', β-actin forward primer—5'-ATCGTGGGCCGCTCT AGGCACC-3', reverse primer—5' CTCTTTGATGTCACGCACGATTTC-3'. PCR condition includes 1 min of initial denaturation at 94°C, 32 cycles (94°C for 45 s, 60°C for 45 s, 72°C for 1 min) for CaMKIIα and 26 cycles for β-actin followed by a final extension at 72°C for 10 min.

Native polyacrylamide gel electrophoresis (Native-PAGE)

The PCR amplified products of the CaMKIIα and β-actin genes were resolved by 8% native poly-acrylamide gel electrophoresis (Native-PAGE) in 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, gels were stained in ethidium bromide solution, visualized in UV transilluminator and photographed.

Immunoblot analysis of CaMKIIα protein

Preparation of tissue lysate and SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

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). Protein was estimated by Bradford method

using BSA as standard [21]. To perform the SDS-polyacrylamide gel electrophoresis (SDS-PAGE) experiment, the cerebellar cortical tissue lysate was resolved on a denaturing polyacrylamide gel [22]. The resolving gel was prepared containing 12% acrylamide: bisacrylamide mix (29.2:0.8), 390mM Tris-Cl (pH-8.8), 0.1% SDS, 0.1% APS and TEMED and allowed to polymerize. The stacking gel was cast onto resolving gel containing 5% acrylamide: bisacrylamide mix (29.2:0.8), 125mM Tris-Cl (pH-6.8), 0.1%SDS, 0.1% APS and TEMED. For western blotting 25 μg of crude sample was denatured in 1X Laemelli gel loading buffer containing 50 mM Tris-Cl (pH-6.8), 1% SDS, 1% β-ME, 10 % glycerol and 0.1% bromophenol blue in boiling water bath for 5 min. The samples were cooled on ice and loaded along with prestained protein marker (MBI Fermentas). Electrophoresis was carried out in gel running buffer containing 250 mM glycine, 25 mM Tris and 0.1% SDS. For proper stacking, the samples were run at 15 mA in stacking gel and resolved at 30 mA in resolving gel. Electrophoresis was carried out till the bromophenol blue dye migrated to the bottom of the gel. After electrophoresis, the gel was stained in staining solution (50% methanol, 40% water, 10 % acetic acid and 0.05% wt/vol Coomassie brilliant blue (R 250) and destained in 10% methanol and 10% acetic acid mixture quality check and photographed. A fresh gel was run for direct processed for the Western blotting experiment.

Immunoblotting and detection

The gel was incubated for 10 min in transfer buffer containing 192 mM glycine, 25 mM Tris, 0.05% SDS and 20% methanol after electrophoresis. The PVDF (0.45μ Millipore) membrane was processed for transfer by dipping in 100% methanol for 15-20 sec. and then placed in water for 5 min and then in transfer buffer for 5 min. The gel and the membrane were sandwiched between prewetted Scotch-Brite pads, foams and Whatman 3M filter paper in electroblotting apparatus. Thereafter, the proteins were transferred onto the membrane overnight at 4°C with constant power supply of 50V. The protein transferred onto the membrane was visualized by Ponceau S staining (0.5% Ponceau S in 1% acetic acid) and then destained in deionized water. The membrane was blocked in 5% non-fat milk in TBS pH -7.4 for 4 hrs at RT. The blot was then incubated with rabbit anti-CaMKIIα (1:10000 dilution, Abcam, UK), β-actin (1:15000 dilution, HRP-conjugated, Sigma-Aldrich, USA) and in 5% non fat milk and 0.1 % Tween-20 in TBS pH - 7.4, overnight at 4 °C. After three repeated washes with 1xTBST (0.1% Tween-20) for 5 min. each, the CaMKIIα blot was incubated with HRP-conjugated goat anti-rabbit IgG (1:2500 dilution, Bangalore Genni, Bangalore) in TBS pH - 7.4 containing 5% non-fat milk and 0.1 % Tween 20 for 4 hr at RT, washed with TBST pH-7.4, immunoreactive proteins were detected using ECL super signal West Pico Kit (Pierce Biotechnology) on X-ray film, and their expression level was measured by densitometry. β-Actin was used as internal control.

Electrophoretic mobility shift assay of CaMKIIα promoter-CREB interaction

The electrophoretic mobility shift assay (EMSA) technique was used to examine the transcriptional regulatory mechanism of CaMKIIα gene expression in aging brain. Due to unavailability of the characterized and functional promoter sequence of

CaMKIIα in mouse genome, it was obtained through the BLAST alignment of previously characterized rat CaMKIIα promoter sequence [23] with the 5'UTR of mouse CaMKIIα gene sequence (C57BL/6J mouse, chromosome 18) through NCBI data base. In addition, to increase the functional significance of CREB in regulation of CaMKIIα promoter in human brain aging, the newly obtained putative mouse CaMKIIα promoter was further BLAST aligned with human CaMKIIα promoter sequence. The BLAST alignment result of I.0 Kb sequence of mouse CaMKIIα promoter with corresponding human sequence is mentioned in result section (Fig. 1). In order to find the various putative regulatory *cis*-acting elements on CaMKIIα promoter sequence, different transcription factor binding sites prediction tools were used which showed multiple trans-factor interaction sites such as MZF1, CREB1/CREB, C/EBP, SRY, TATA, GATA-1, v-Myb, GATYA-2. Total 1.0 kb sequence of mouse CaMKIIα promoter region ranged between -851 upstream and TSS to +149 bp downstream in which 20-mer long CRE sequence was located at –861 to -842 positions. The designed oligos corresponding to CRE-element of CaMKIIα promoter used in electrophoretic mobility shift assays (EMSA) were custom synthesized from the Metabion International AG (Martinsried, Germany). The sequence of oligos of the CRE-elemnt of CaMKIIα (20-mer) used in the experiments is as follows: 5' TGGGTCTTACGACTGCCTTA 3' (Upper strand/antisense strand) and 3' AATGCTGACGGAATGACTCTGTCT 5' (Lower strand/sense strand). This oligomer corresponds to –861 to -842 bp of mouse CaMKIIα gene promoter containing CRE element (description is given in result section).

Preparation of nuclear extract

Nuclear extract was prepared from cerebellar cortices obtained from mice of male & female belonging to vanous age groups according to the procedure of Dignam et al., 1983 [24] with minor modifications. Briefly, a 10% of the tissue homogenate was prepared in buffer A in a glass homogenizer using Teflon pestle at ice temperature. The homogenate was centrifuged at 1000xg for 15 min at 4° C and the pellet was collected. The pellet was then suspended in buffer A containing 0.3M sucrose and then 0.2% triton X-100. An equal volume of buffer B was added containing 1.8M sucrose and mixed gently. Thereafter, it was centrifuged at 25,000xg for 30 min at 4° C. After completion of centrifugation, the crude nuclear pellet was washed by suspending the pellet in buffer A containing 0.3M sucrose and centrifuged at 1,000xg for 10 min at 4°C. The pellet thus obtained was suspended in the same solution and an aliquot was observed under microscope every time till clear nuclei devoid of any tissue or membranous debris was seen in the nuclear preparation. An aliquot of the nuclear suspension was disrupted in 2M NaCl and 5M urea to estimate the DNA content of the nuclei by measuring A_{260}. The nuclear pellet was suspended in buffer C 20 mM HEPES-KOH, pH-7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF) containing 2μg/ml protease inhibitor cocktail (Sigma-Aldrich) and was kept on ice for 30 min and stirred intermittently to lead to nuclear lysis in order to obtain nuclear extract. The chromatin was pelleted by centrifugation at

25,000xg for 40 min at 4° C. The supernatant was aliquoted and snap frozen in liquid N_2. The nuclear proteins were quantitated according to Bradford method (1976).

Oligonucleotide annealing and Native-PAGE analysis

The complementary oligos for CRE element of CaMKIIα promoter were annealed in 50µl reaction volume having equimolar concentration in 1xTNE buffer. The oligos were denatured at 95° C in a water bath for 15 min and then cooled down gradually at RT overnight. The annealed oligos were stored at -20° C for further use in EMSA experiments. Further, the annealed oligos for the CRE element of CaMKIIα gene promoter were resolved in 20% non-denaturing polyacrylamide gel (29:1 acrylamide:bis-acrylamide) electrophoresis in 1X TBE (pH 8.0) to check the quality and integrity of annealing. After electrophoresis, the gel was stained in 0.5 µg/ml ethidium bromide solution and photographed.

End filling of annealed oligos and spun column chromatography

The annealed ds-oligos were labeled by end-filling using Klenow fragment, exo- of DNA polymerase. The 5' overhang was filled in a reaction volume of 20µl having 100 ng of ds-oligos, 2.0 µl of 10x reaction buffer, 2.5 µl of 2 mM dNTP mix (without dCTP), 50 µCi α^{32}P-dCTP and one unit of Klenow enzyme for 15 min. at 30° C. The reaction was stopped by heating at 70° C for 10 min. To separate the labeled oligos from unlabeled and free nucleotides in the above reaction mix spun column chromatography was used. A Sephadex G-50 column containing Sephadex G-50 slurry was prepared in 1 ml insulin syringe. The slurry was equilibrated by 0.1x TE buffer, pH-8.0 and centrifuged at 1600Xg for 5 min in a swinging bucket rotor. Total volume of the labeled oligos was made up to 100 µl and loaded on to the column. It was again centrifuged at 1600Xg for 5 min. The eluate containing labeled oligos was measured for radio activity using a Beckman LS counter (Beckman LS 1801) and stored at -80^{o} C for further experiments.

Electrophoretic mobility shift assay (EMSA)

Electrophoretic mobility shift assay was carried out following the protocol of Liu et al. (1999) [25]. Approximately 0.1 - 0.2 ng (approximately 5,500 cpm) labeled ds-oligos were taken per reaction. In 40 µl reaction mixture, 10 µg of nuclear protein was taken in presence of the binding buffer (5 mM HEPES, pH-7.9, 10% glycerol, 25 mM KCl, 0.05 mM EDTA, 0.125 mM PMSF), 1µg of poly-dI/dC and 0.1ng of radio labeled probe. The mixture was incubated for 20 min at 22° C. The reaction was terminated by adding 5 µl of 5X loading dye (6% sucrose, 2 mM Tris-Cl, pH-8.0, 0.05% bromophenol blue, 0.05% xylene cyanol FF). The native polyacrylamide gel was pre-run for 1 hour at 100V. The samples were loaded onto 5% non denaturing polyacrylamide gel (acrylamide: bisacrylamide 29:1) and 0.5X TBE buffer with constant 100V power supply. After resolving, the gel was transferred to Whatman 1M

filter paper and fixed in 10% acetic acid and 10% methanol for 15 min. It was then covered with saran wrap and dried for 45 min at 80° C. The gel was exposed to the intensifying screen in cassette and signals for the radiolabelled DNA-protein complexes were captured in the computer-assisted Typhoon FLA 7000 Phsophor Imager (GE Healthcare Life sciences, UK). Later, the signal images were scanned using AlphaImagerTM 2200 Software (Alpha Innotech Corporation, CA) for quantitation of the interactions.

The specificity of interaction of CRE *cis*-element of CaMKIIα promoter and nuclear proteins was analyzed by the following types of gel mobility shift assays: (i) In order to determine the concentration of nuclear proteins that bind to DNA, a control reaction was carried out with varying concentrations of nuclear extract ranging from 5 to 15 μg. The optimum concentration of nuclear proteins was used for the rest of the experiments, and (ii) in the control reaction mixture titrations with i) 10-, 50-, 100-, and 500-fold of cold competitor ds-DNA, ii) non specific cold competitor *E.coli* DNA (heterologous *E. coli* sheared DNA sheared for analyzing the specificity of binding of nuclear proteins.

Statistical analysis

All the experiments were repeated thrice (n = 6/ age/experiment). The gels and X-ray film exposures were photographed using a digital camera (Nikon Corporation, Japan). The bands were analyzed and quantitated using computer-assisted densitometry (AlphaEase FC^{TM} software, Alpha Innotech Corporation, CA). For RT-PCR and western blots, the signal intensities of bands of interest were measured after normalization with β-actin and expressed as relative densitometric value (RDV). EMSA bands were also analyzed by similar procedure excepting use of any control. In this case, the absolute scan value or integrated density value (IDV) was used for interpretation of the DNA-protein binding reactions. Results represent the mean ± SEM of data obtained from three different sets of experiments. Statistical analysis was performed using one-way ANOVA followed by Turkey's post hoc significant test through standard SPSS 11.5 software. $P<0.05$ was taken as statistically significant (95% confidence interval).

Results

Effects of age and sex on the expression of CaMKIIα transcript in cerebellar cortex

Semi-quantitative RT-PCR of CaMKIIα gene in the cerebellar cortices of male and female mice (Fig. 2 a & b) of various ages shows that its expression was highest at 8w mice in both the sexes. Thereafter, its expression is significantly declined with advancing age, both at 20w and 70w age with least expression level in old age. The down regulation of the CaMKIIα expression both in male and female mice were significant compared to that in both young and adult age ($P<0.05$).

Effects of age and sex on the expression of CaMKIIα protein in cerebellar cortex

The Immunoblot data on the analysis of CaMKIIα protein in the cerebellar cortices of both male and female mice of different ages (Fig. 3 a & b) reveals that its expression followed the same pattern of expression in both male and female as it was at transcript level. Its expression was highest at 8w mice. Beyond young age, its expression gets significantly declined with increasing age, both at 20w and 70w age. However, in contrast to transcript expression of CaMKIIα expression in female cerebellar cortex, at protein level, the expression of CaMKIIα at 70-week was not significant compared to that of 20w mice ($P<0.05$).

Electrophoretic mobility shift assay of CREB-CaMKIIα promoter interaction

The promoter sequences and location of several *cis*-acting elements have been predicted (using bioinformatics tools) in rat and human CaMKIIα genes but the same are not available in case of mouse CaMKIIα gene. In addition, only few CaMKIIα promoter cis-sequences gene involved in the transcriptional regulation of rat and human CaMKIIα genes, such as RA element (retinoic acid response element) etc., have been experimentally validated till now. Therefore, to carry out the EMSA study, the CRE-element of mouse CaMKIIα promoter was deduced by the BLAST alignment of mouse CaMKIIα genomic sequence with the rat CaMKIIα promoter sequence. Thereafter, to correlate its significance value in case of human, the 1.0 kb putative mouse CaMKIIα promoter region that ranged from -851 upstream and TSS to +149 bp downstream was subjected to alignment with human genome using NCBI BLAST tool. The BLAST result for the query of mouse CaMKIIα promoter sequence with that of known human promoter database was shown in ESM1, the Software found the high similarity with the region at the 5' flanking region of CaMKIIα gene. Accordingly, the detailed map of the mouse CaMKIIα promoter was drawn out through in silico analysis using TFSEARCH transcription factor binding search tool. It depicted many upstream sequences for various transcription factors such as MZF1 (-944 bp), CREB1/CREB (-842 bp), C/EBP (-819 bp), SRY (-712 bp), TATA (-316 bp), GATA-1 (-150 bp), v-Myb (-116 bp), GATA-2 (-47 bp). The organization of CaMKIIα promoter with cis-elements is shown in (Fig. 4). Out of the above, the CREB (-842 bp) cis-acting elements was preferentially selected to study its role in the transcriptional regulation of CaMKIIα gene in cerebellar cortices of both the sexes during aging..

Annealing of radio labeled CaMKIIα-CRE oligos

Accordingly, the oligos for consensus CREB (-842) sequence of CaMKIIα promoter were custom synthesized, labeled with α^{32}p-dCTP and were analyzed for annealing before EMSA. The annealing experiments for CREB (-842) sense and antisense oligos showed that they were perfectly annealed (Fig. 5a).

Optimization of nuclear extract protein concentration

Before carrying out specific DNA-protein interaction in different experimental conditions, the pilot experiment carried out for finding the optimum nuclear protein in

the reactions for CREB binding sequence, revealed 5 μg nuclear proteins as optimal protein concentration out of 0, 5, 10 and 15 μg total proteins used separately. This experiment was carried out with the nuclear proteins prepared from 20-week mice. Also, this experiment yielded two complexes C1 and C2 corresponding to CREB and CAAT-enhancer binding protein (C/EBP), respectively as reported in literatures. Although, age-dependent alterations in the interaction of both CREB and C/EBP bound complexes were recorded but only the upper complex i.e. CREB complex is included and discussed in the study which might be likely to be affected due to experimental conditions. The concentration concluded from above study was used for further experiments in aging mice brain (Fig. 5b).

Analysis of specificity of interaction of CREB with its cognate sequence

The experiments carried out to examine whether the binding of the transcription factor CREB with their cognate promoter sequence from homologous and heterologous system using molar excess of cold competitor (non-radiolabelled cognate oligos) and the sheared *E. coli* DNA, respectively revealed that the increasing concentrations of cold competitor removed all CREB from the nuclear extract used for the binding reaction and was not available to interact with the same radio labeled oligo. This led to run the radio labeled oligo in polyacrylamide gel without any retardation and no labeled complex was seen as was seen in case without any cold competitor. This indicated that the CREB binding with its corresponding sequence was specific (Fig. 6a). Further, from the other experiments using increasing concentrations of *E. coli* DNA in the binding reaction, the results obtained clearly revealed that the intensity of the retarded band was not affected. This indicated that CREB binding with its cognate sequence was specific (Fig. 6b).

Effects of age on the interaction of CREB with its cognate promoter sequence in cerebellar cortex of male and female mice.

EMSA with CRE element of CaMKIIα promoter for both male and female cerebellar cortices again showed formation of two complexes, CREB and C/EBP as was discussed in earlier results. From these two complexes, CREB was noted as the major complex in all the ages (Fig. 7 a & b and Fig. 8 a & b). EMSA data showed that the level of interaction of CREB to CaMKIIα promoter in cerebellar cortices of both the sexes with the advancing age was significantly reduced at 20w age ($p < 0.05$) in comparison to that of 8w age. However, in 70w old mice, the binding of CREB was again up regulated to the level that was highest as compared to that of previous age ($p < 0.05$) (Fig. 8a & b). The association graph plotted between age-related modulations in CREB binding to CaMKIIα promoter and mRNA levels of CaMKIIα in both male and female cerebellar cortices revealed that both aspects were positively correlated up to adult age but formed negative correlation at old age (Fig. 7c & 8c).

Discussion

We have studied expression of the CaMKIIα in the cerebellar cortex of young, adult and old male and female mice to understand whether this undergoes any age- and sex-dependent alterations and which might be related to age-dependent decline in the motor as well as motor learning and memory functions of the brain and might show sexually dimorphic patterns. Since CaMKIIα expression and its transcriptional machinery are intricately involved in the development of LTP leading to acquisition, consolidation and retrieval of memory in the basic memory related areas such as pre frontal and parietal cortices and hippocampus and its implications in the motor learning and memory in cerebellar cortex which might differ from young to old age, and might be sexually dimorphic as has been reported in most of cognitive brain dysfunctions, we thought to undertake above study.

We observed that the level of CaMKIIα expression was highest in the cerebellar cortex of young mice which may be correlated to proper induction of LTD in cerebellar cortices of both the sexes, a prerequisite for motor learning in the cerebellum and CaMKIIα may be associated in this process. Our data coincides with the requirement of CaMKIIα-dependent LTD for elimination of the climbing fibers that is typically completed after about three weeks of birth and reported to be delayed in juvenile mutant αCaMKII-/- mice to provide more fine-tuned instructive signals to drive the induction of motor skill learning [26]. Also, the highest expression of CaMKIIα in young mice may be attributed to the high-threshold calcium conductance in early adulthood which may help in the developmental maturation of PCs [27] and in the maintenance of homeostatic balance to protect neurons from death due to fluctuating concentration of Ca^{2+}. The decline in the level of CaMKIIα in cerebellar cortices of adult and old ages of male and female may be attributed to the sexual difference in the expression of CaMKIIα between males and females, however, the slight variations in the extent of expressions of CaMKIIα between both the sexes may underlie the sex differences in cellular functioning of the CaMKIIα. As CaMKIIα has been implicated in motor learning, the significant reductions in CaMKIIα expression in cerebellar cortices of male and female mice with the advancing ages may suggest a general and consequential decline in motor performance and adaptability in adult and old mice correlated with reduced LTD, subsequent possible induction of LTP and impaired plasticity and certain other forms of motor attributes as a function of both age and sex [26] and could be correlated with volumetric loss of the cerebellar cortices during aging particularly in the PC layer for about 25.63 % in cat [28]. Since CaMKIIα is mainly located in the PCs in Purkinje cell layers, the decreased expression of CaMKIIα might be attributed to the loss of Purkinje cell layer in the cerebellar cortex [29]. Our finding can be correlated with its implications in the reduced CaMKIIα autophosphorylation, reduced kinase activity which might be attributed to increased CaMKII-mediated inhibition by increased PDE1 [30] and uncoupling of Homer3 from its molecular partners at PSD and this in turn may affect molecular architecture and synaptic plasticity in adult and old mice [31]. leading to decline in the LTP. A recent study has

shown that neurodegeneration of PC (Purkinge cells) is associated with the reduced expression of CaMKIIα in DNA excision repair cross-complementing group 1 (Ercc1) knock-out mice which is a well accepted model of normal aging [32].

The reduced expression of CaMKIIα in older adults may suggest impaired activation of CaMKIIα in the cerebellum, especially in PC leading to subsequent conversion of LTD into LTP as has been observed with adult αCaMKII-/- knockout mice [26]. Reduction in CaMKIIα level with advancing age may lead to impaired phosphorylation of GluR1 subunit of AMPA receptor, its trafficking to post synaptic density and thereby the conductance of AMPA receptor, subsequent reduction in the PSP development and NMDA receptor and thus decline in LTP in cerebellar cortical neurons and thus may disturb motor learning [33].

As our data indicated that age-dependent gradual decline in the expression of CaMKIIα, we thought to examine whether this results due to age-dependent variation in the CaMKIIα transcriptional machinery or it is because of the mRNA or protein over that might be operating during aging. We, therefore, addressed the first aspect related to alterations in CaMKIIα gene transcription due to aging process. For this we studied the interaction of CREB, a bZIP transcription factor associated with the regulation of CaMKIIα gene transcription, with the CRE sequence in the CaMKIIα promoter using annealed ^{32}P-labeled oligo as decribed in the methodology section using electrophoretic mobility shift assay (EMSA). Our EMSA data, which also revealed a significant decline in the level of complex (C1, CREB complex), an index of the transcriptional activity, in adult age compared to young age, corresponded to our RT-PCR and Western blot data. This indicated that alterations in this process leads to decline in the expression of CaMKIIα up to adult age in both the sexes and may be correlated with the underlying decline in LTM formation in adult age. However, in mice of both the sexes, the level of the CREB-CaMKIIα promoter complex in old age exceeds its level in the young and adult mice and is retrospectively correlated with declined CaMKIIα expression in the cerebellar cortex as evident from the correlation graph (Fig. 4c and 5c). Our EMSA data also depicted formation of the other less retarded complex (C2, the C/EBP complex) due to some degree of structural similarities with CRE sequence but since the experiments were carried out with a designed CRE sequence, the relevance of variation in the C/EBP complex is out of context until we characterize the same separately [34- 36]. However, C/EBPs are also reported to have both constitutive and cAMP-inducible activities during regulation of CaMKIIα expression and they have been suggested to be considered as a cAMP-responsive nuclear regulator [37, 38]. Decline in the level of CREB complex in adult age, which is directly correlated with reduced CaMKIIα expression and increase in its level that is in reverse relation with the CaMKIIα expression in old age of both male and female mice may result the age-dependent differential expression of the CREB. Further, the observed variation may likely be due to intramolecular differential CREB phosphorylation of CREB^{Ser133} vs CREB^{Ser142}), which might affect its binding with CaMKIIα promoter and leads to its age-dependent expression. As per our data that shows an age-dependent variation in

the CaMKIIα transcriptional system which might reflect of a phenomenon of decline in motor as well as motor learning and memory functions of the cerebellar cortex during aging in both the sexes.

Conclusions

CaMKIIα, a Ca^{2+}-Calmodulin dependent kinase peesent in cerebellum and other parts of the brain, undergoes age-dependent decline in its level during aging in both male and female mice and is attributed to age-dependent differential alterations in the CREB –dependent CaMKIIα transcriptional machinery

Acknowledgements

The present work was supported by Board of Research in Nuclear Sciences (BRNS) (2009/37/SS/BRNS/3298) and partial financial assistance from Indian Council of Medical Research (ICMR), Govt. of India, UGC-CAS Program in Zoology, UGC-UPE (Focus area II) and DBT-ISLS to SP. VND thanks ICMR and UGC for his JRF and SRF.

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Legend to Figures:

Fig. 1. Sequence of mouse CaMKIIα gene Promoter obtained from nBLAST between active human CaMKIIα gene promoter (Wang et. al. 2008) with mouse 1000 bp 5' flanking region of CaMKIIα gene.

Fig. 2. Semi quantitative RT-PCR analysis of CaMKIIα gene in the cerebellar cortex of male (a) and female (b) mice of various ages. Histogram represent data expressed as mean ± SEM obtained from three indenedent sets of experiments. * Significant from 8w age; ** Significant from 20w age group; $P<0.05$. M, Male; F, female; CBL, cerebellar cortex; w denotes age in week.

Fig. 3. Immunoblot analysis of CaMKIIα protein in the cerebellar cortex of male (a) and female (b) mice of various ages. Histogram represent data expressed as mean ± SEM obtained from three indenedent sets of experiments. * Significant from 8w age; ** Significant from 20w age group; $P<0.05$. M, Male; F, female; CBL, cerebellar cortex; w denotes age in week.

Fig. 4. Schematic representation of the putative promoter of mouse CaMKIIα gene showing various 5' upstream cis-acting elements and transcription factors binding sites. CRE-cAMP-response element; C/EBP-CCAAT-enhancer binding protein.

Fig. 5. Annealing of CRE oligos and optimization of nuclear extract concentration (a) 20% native polyacrylamide gel electrophoretic analysis of annealed ds oligos of CRE (-842 bp). Ss- sense strand oligo, as- anti-sense strand, ds- double strand oligo. (b) EMSA of DNA fragment containing CRE (-842) element of CaMKIIα promoter with increasing concentrations of nuclear extract (NE) isolated from whole cerebral cortex of adult mice. NE was incubated with α^{32}P-dCTP -labeled ds DNA of 20-mers CRE (-842). C1-complex1 (CREB); C2-complex2 (C/EBP). NE, nuclear extract.

Fig. 6. EMSA for the for the competitive binding of specific radio labeled CRE (-842) of CaMKIIα with increasing molar excess of non-radioactively labeled oligo. of DNA fragment (a) and with non-specific competitor (sheared *E.coli* DNA) (b). 5 μg of NE was incubated with ^{32}P-labeled dsDNA of 20- mer CRE (-842). F-free DNA. NE, nuclear extract.

Fig. 7. EMSA of CREB-CaMKIIα promoter sequence interaction in the cerebral cortex of aging male mice. (a) EMSA of CREB (-842) showing DNA-protein complex formed between CREB (-842) and CREB present in the cerebellar cortex nuclear extract of male mice (b) Histogram showing data expressed as mean ± S.E.M. obtained from three different sets experiments. F, free probe; w, week; *, significant from 8w age group; *#, significant from 20w age group; $p<0.05$. **(c)** Association graph between age-dependent alterations in CaMKIIα

mRNA expression and CREB-CaMKIIα promoter complex. Y, young; A, adult; O, old; M, Male; CBL, cerebellar cortex.

Fig. 8. EMSA of CREB-CaMKIIα promoter sequence interaction in the cerebral cortex of aging female mice. (a) EMSA of CREB (-842) showing DNA-protein complex formed between CREB (-842) and CREB present in the cerebellar cortex nuclear extract of male mice (b) Histogram showing data expressed as mean ± S.E.M. obtained from three different sets experiments. F, free probe; w, week; *, significant from 8w age group; *#, significant from 20w age group; $p<0.05$. **(c)** Association graph between age-dependent alterations in CaMKIIα mRNA expression and CREB-CaMKIIα promoter complex. Y, young; A, adult; O, old; F, Female; CBL, cerebellar cortex.

Fig. 1

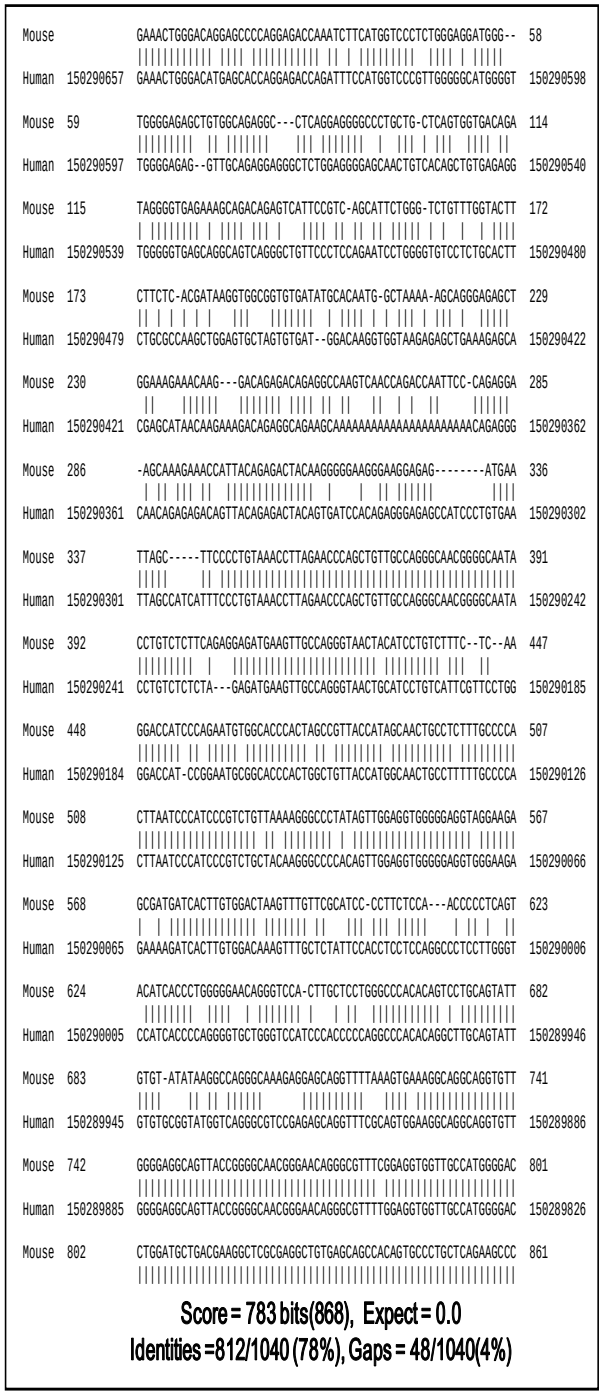

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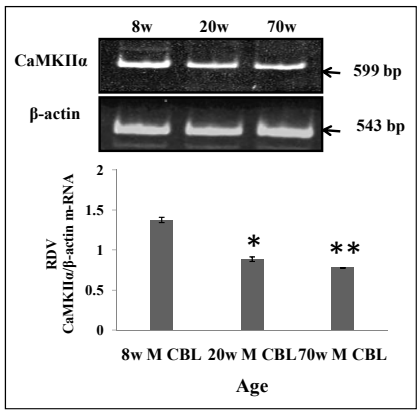
Fig. 2. (a)



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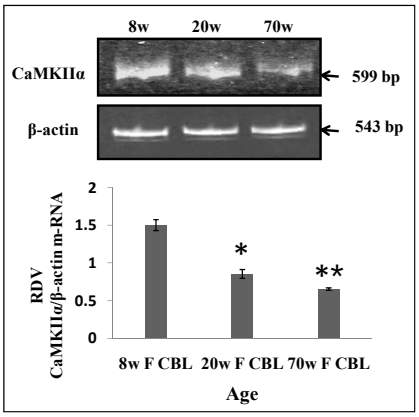
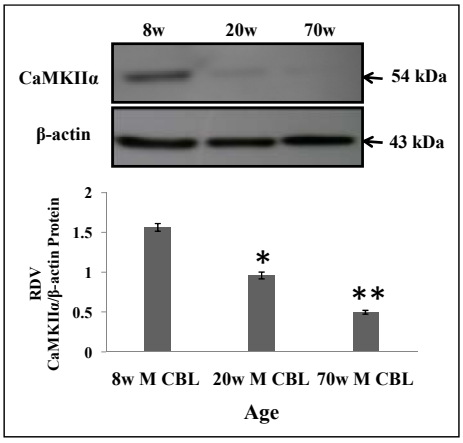


Fig. 3. (a)



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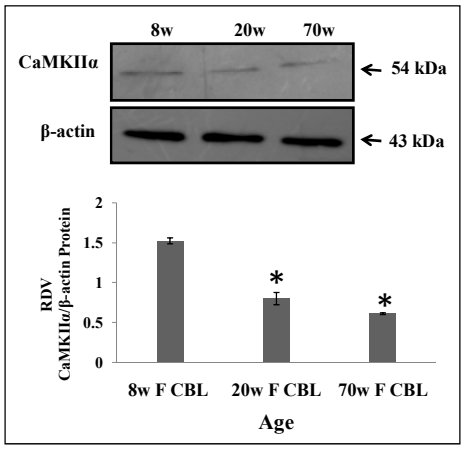


Fig. 4

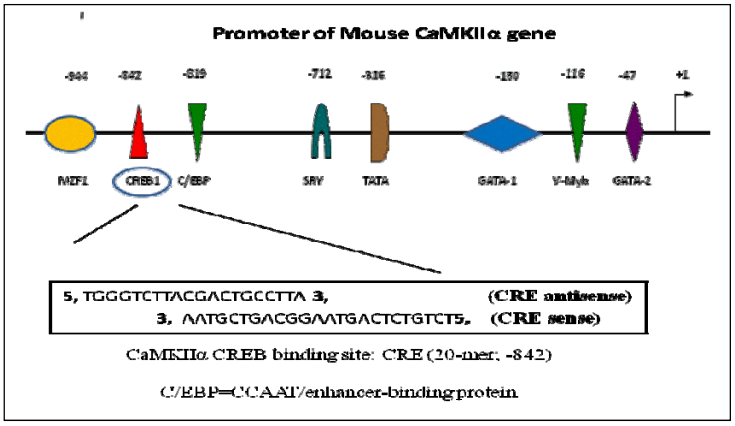
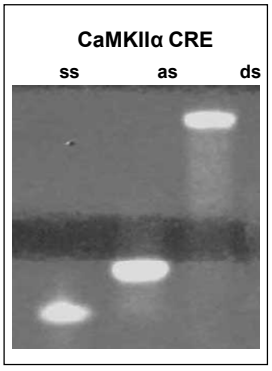

Promoter of Mouse CaMKIIα gene
-944
-842
-819
-712
-816
-130
-116
-47
+1
MZF1
CREB1
C/EBP
SRY
TATA
GATA-1
V-Myb
GATA-2
5, TGGGTCTTACGACTGCCTTA 3, (CRE antisense)
3, AATGCTGACGGAATGACTCTGTCT5, (CRE sense)
CaMKIIα CREB binding site: CRE (20-mer; -842)
C/EBP=CCAAT/enhancer-binding protein

Fig. 5 (a) **(b)**


CaMKIIα CRE
ss
as
ds

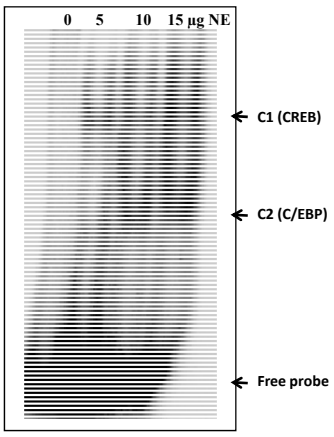

0 5 10 15 µg NE
C1 (CREB)
C2 (C/EBP)
Free probe

Fig. 6 (a) **(b)**

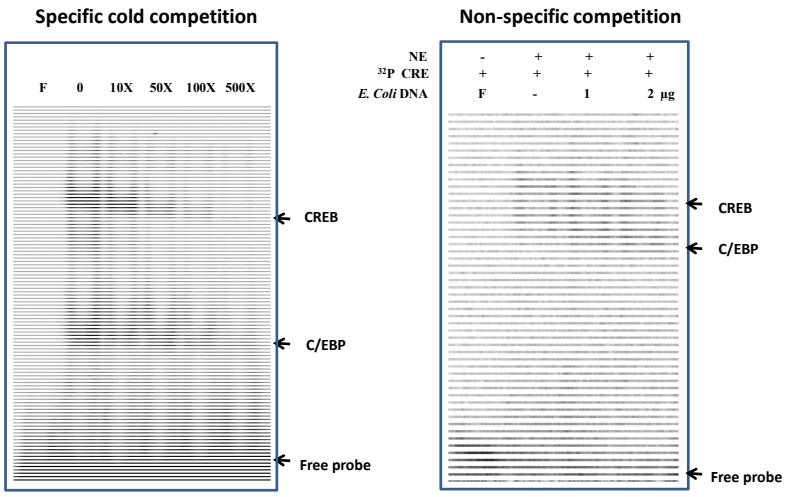
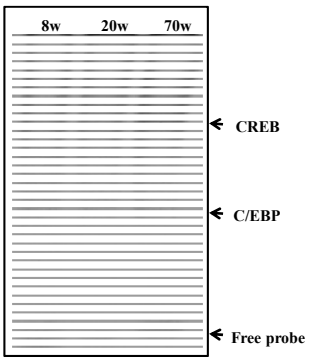
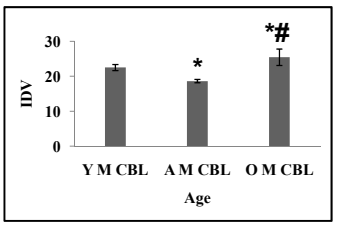


Fig. 7



(a)



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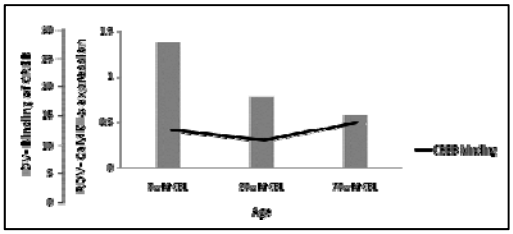
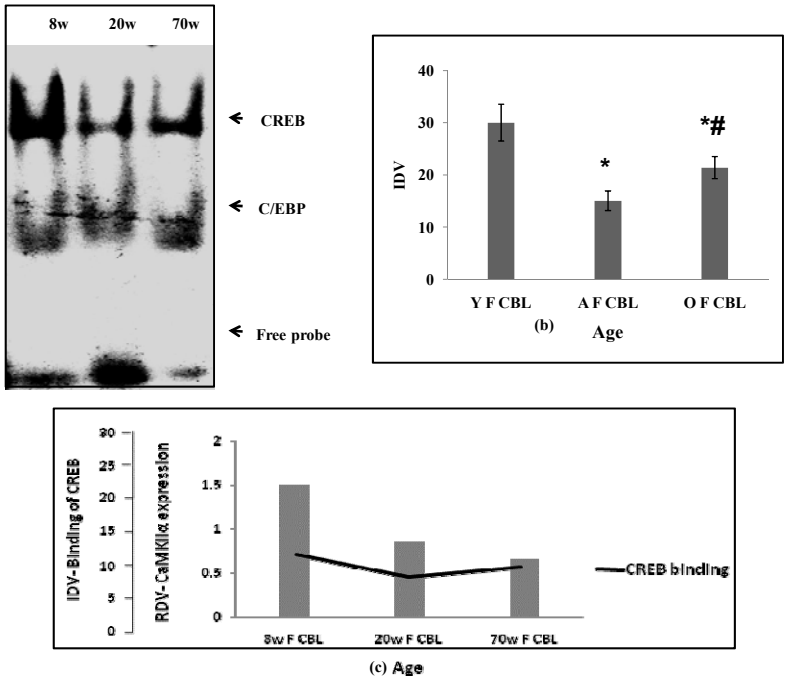


(c)

Fig. 8


8w
20w
70w
CREB
C/EBP
Free probe
IDV
40
30
20
10
0
*
*#
Y F CBL
A F CBL
O F CBL
(b)
Age
IDV-Binding of CREB
RDV-CaMKIIα expression
30
25
20
15
10
5
0
2
1.5
1
0.5
0
8w F CBL
20w F CBL
70w F CBL
CREB binding
(c) Age