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Differential activation of Gsk-3β in the cortex and the hippocampus induces cognitive and behavioural impairments in middle-aged ovariectomized rat

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ABSTRACT

Glycogen synthase kinase-3 (Gsk-3β) aberration act as a crucial pathogenic factor in several neurological conditions. However its role in menopause associated behavioural impairments is still not unclear. The present study was designed to understand the role of Gsk-3β in the progression of neurobehavioural impairments in middle-aged ovariectomized (ovx) rats. The animals showed a significant impairment in spatial and recognition memory, along with anxiety and depression-like behaviour following 22 weeks of ovx. The genomic expression of *ERα, ERβ, Nrf2, HO-1, TNFα*, and *IL-6* was altered in both the cortex and the hippocampus of ovx rats. Protein expression of *p*-Gsk-3β(Ser^9) was significantly downregulated in the cortex after ovx. However, the hippocampus showed a surprisingly opposite trend in the levels of *p*-Gsk-3β(Ser^9) as that of the cortex. Differential activation of Gsk-3β and its downstream proteins such as β-catenin and *p*-mTOR were also altered following ovx. The study concluded that differential activation of Gsk-3β, along with oxidative stress and neuroinflammation in the cortex and the hippocampus, leads to the induction of cognitive and behaviour impairments in ovx rats.

1. Introduction

Menopause is a normal aging process in women characterized by decreased or permanent cessation of sexual hormones, especially estrogen. Community-based global studies have shown the induction of menopause in women at the age of 45–55 years [1]. A study showed an average onset of menopause among women from 50.1 to 52.8, 43.8–53, 50.5–51.4, and 42.1–49.5 years in Europe, Latin America, North America, and Asia, respectively [2]. The sex hormone also regulates normal brain physiological functions like, neurogenesis, synaptic plasticity and cognition [3]. The available literature indicates that estrogen prevents neuronal damage against β-amyloid toxicity, oxidative stress, excitotoxicity, and mitochondrial failure [4]. Risk of age-related neurodegenerative conditions such as Alzheimer's disease (AD) and vascular dementia, mood disorders like anxiety and depression increase following menopause [5,6].

Oxidative stress plays a major role in the aging process through abnormal production of free radicals, such as reactive oxygen species (ROS) that interfere with normal cell signalling cascades. Unlike other organs, the brain requires a high amount of oxygen for normal physiological functions, making it more vulnerable to oxidative stress. Nuclear factor erythroid 2 (NFE2)-related factor 2 (Nrf2) is a redox-sensing transcription factor that scavenges ROS by initiating transcription of genes that consist of antioxidant response elements in their promoter [such as heme oxygenase 1 (*HO-1*) and NAD(P)H: Quinone oxidoreductase-1 (*NQO1*)] and glutamate-cysteine ligase catalytic subunit (*Gclc*) [7]. The activity of endogenous antioxidants such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) decline following menopause, thereby making women more prone to oxidative stress [8]. Oxidative stress initiates numerous destructive processes in the neurons and damages biological macromolecules (DNA, proteins, and lipids), leading to emotional oscillations and other age-linked neurological conditions. Different studies have suggested the developing of neurological disorders in women during menopause due to oxidative stress [9].

Another major event that leads to neurobehavioral impairments in women during menopause includes neuroinflammation [10]. An increase in the proinflammatory mediators during aging, also known as "Inflamm-aging," instigates the brain to alter neuroinflammatory response [11]. A few studies have shown exacerbation of the inflamm-aging process in women following menopause. Expression of proinflammatory cytokines such as tumor necrosis factor-alpha (TNFα) and interleukin 6 (IL-6) in

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women were found to be elevated in blood after menopause [12]. A systemically elevated level of pro-inflammatory cytokine breaches the blood-brain barrier (BBB), starts local neuroinflammatory events, and initiates of degeneration in neuronal and non-neuronal cells that finally ends up with neurobehavioral impairments [11]. Edel. et al. (2017) showed that increasing the systemic TNFα level induces acute cognitive impairment in the rats suffering from progressive neurodegenerative disorder by enhancing the transcription of the same gene in the hippocampus.

Similarly, an elevated level of IL-6 in the astrocyte-derived exosome from the plasma of patients who have sporadic amyotrophic lateral sclerosis indicated the involvement of inflammation in the progression of neurodegenerative diseases [13]. A population-based study also showed the association of elevated levels of TNFα and IL-6 in the gradual development of sickness behaviour in AD [14]. Moreover, the genomic expression of microglial reactivity-related genes was elevated in the frontal cortex region of the rat and human brain following the menopause [15]. These shreds of evidence suggested that neuroinflammation triggers the neurodegenerative events, and estrogen deprivation after menopause further worsens the condition. Several groups have validated estrogen's anti-inflammatory effects in various neuroinflammatory models [16].

Glycogen synthase kinase-3 (Gsk-3) is a serine/threonine kinase that consists of two isoforms α and β, which are highly expressed in the brain, and controls important cellular and neurophysiological processes such as neurogenesis, neurotransmission, and circadian rhythm [17]. Gsk-3 plays a crucial role in numerous signalling cascades, so its activity is strongly regulated by phosphorylation of its active or inhibitory residue, *i.e.,* Ser^{9} and Tyr^{216} for Gsk-3β [7]. Alteration in the normal activity of Gsk-3β is associated with impairment in the reconsolidation process of memory formation [18], development of the anxiety-like [17], and depression-like behaviour [19]. Gsk-3β overexpression in mice showed hyperlocomotion, whereas knock-down of GSK-$3\beta^{+/-}$ resulted in depression-like behaviour [17], suggested an important contribution of the same protein in the maintenance of cognition and behaviour. Expression of the active form of Gsk-3β was upregulated in the brain of young ovariectomized (ovx) rats but was downregulated following the estrogen treatment [20]. β-Catenin is a dual function protein that participates in adhesion between two cells and acts as a transcription factor via the Wnt signalling pathway. β-Catenin is a direct downstream substrate of Gsk-3β, which modulates its activity. Alteration in the Gsk-3β/β-catenin signalling pathway trigger cognitive [21] and behaviour impairment [22].

Moreover, a study also showed that activation of Gsk-3β results in the downregulation of β-catenin in the young rat's brain after ovx, suggesting the relationship of estrogen deprivation and Gsk-3β signalling impairment [20]. Mammalian target of rapamycin (mTOR) is another indirect downstream target of Gsk-3β. Downregulation of mTOR signalling pathway associated with cognitive impairment in the mice model of AD, while treatment with Gsk-3β inhibitor, lithium chloride improves the same by upregulating the mTOR signalling [23]. Numerous evidence suggested that activation of Gsk-3β enhances oxidative stress and neuroinflammation, resulting in the initiation of neurodegenerative events [7,24,25]. Interestingly, Gsk-3β was found to be upregulated after ovx in the cerebrum of aged rats, making them more susceptible to blood-brain barrier damage during an ischemic stroke [26]. Some preclinical and clinical studies favour the use of exogenous estrogen after menopause to overcome the associated neurobehavioral conditions [27]. However, estrogen use is also linked with atrophy of the brain, reducing the volume of cortex and hippocampus [28], thus increasing the risk of dementia [29]. The available literature also supports the association of cognitive deficits and behavioural impairments in females after menopause [4].

Furthermore, abnormal activation of Gsk-3β is linked with neurobehavioral impairment [18] in neurodegenerative disorders. However, its involvement in the developing of cognitive and behavioural impairment in females after menopause is still not clear. Removal of both the ovaries through a surgical procedure in the rat is a well-established model that mimics the human post-menopausal conditions [30]. Hence, the present study was designed to investigate the role of Gsk-3β in the development of cognition and behaviour impairment following menopause by using the middle age rat model of ovx.

2. Materials and methods

2.1. Experimental animals and care

The experiment was performed in female Sprague Dawley rats of 10 months' age. All the rats were housed in standard cages (maximum 3 rats/cage), except for 1 week to take post-operative care (described in a later section). The animals were kept in the animal house of CSIR-IHBT at 25 ± 2 °C with constant humidity of 50–60% and light/dark cycle of 12 h. The animals were provided *ad libitum* access to food and water. All the experimental procedure on rats was performed between 09.00 h and 16.00 h. The Institutional Animal Ethics Committee of the CSIR-IHBT approved the experimental protocol. The animal care was done as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Fisheries Animal Husbandry and Dairying, Department of Animal Husbandry and Dairying, Government of India.

2.2. Surgery and experimental protocol

A total of 15 female Sprague Dawley rats were used in the present study. Randomly, 8 females were subjected to ovariectomization (ovx) procedure to induce postmenopausal conditions and associated neurobehavioral impairments, as described earlier [31], with slight modification. However, the rest of the 7 female rats were used as sham control (*sham* group) in the study. Briefly, each animal was anesthetized using ketamine 50 mg/kg (Ketapil, Psychotropics India Ltd.) and xylazine 20 mg/kg (Sigma Aldrich, USA) cocktail intraperitoneal (*i.p.*), fur on the surgical area was removed, the surface was cleaned and sterilized. A small incision was made in the abdomen just near the right side of the rat's 5th nipple. The left uterine horn and the ovaries were located. A knot with an absorbable suture (SOLUS-910®, 3–0, LNW 2437–910, Lotus, India) was made between an ovary and the distal part of the uterine tube. The ovary was then excised. The procedure was repeated for the second ovary. The wound was closed using sterile absorbable suture for peritoneal and muscle layer and non-absorbable (NYLUS®, 3–0, LNW 3328, Lotus, India) for the skin. After suturing, the surgical area was disinfected using povidone-iodine. A similar surgical procedure was applied on the animals of *sham* group ($n = 7$), except removal of ovaries. After surgery, animals received analgesic (Diclofenac 10 mg/kg, *i. p.*) and streptomycin (20 mg/kg, *i. p.*). Animals were also rehydrated using saline (1.5ml/rat, *s. c.*). The animals were allowed to recover after surgery and housed individually in the post-operative room for 1 week after that, re-grouped in their home cage. One animal subjected to ovx procedure did not recover after the surgery. The rest of the animals were kept for the following 22 weeks to develop the postmenopausal conditions, considered as *ovx* group ($n = 7$). Thereafter, cognitive and behaviour functions were accessed in a sound-attenuated room by an observer blinded to the groups (Supplementary 1, Fig. 1). After 24 h of completing the behavioural studies, all the animals were sacrificed by decapitation, the brain was separated to isolate the hippocampus and the cortex, and stored separately at −20 °C. The uterus was also isolated to calculate the adjusted uterus weight by dividing the uterus weight by the animal's body weight and multiply by the average body weight of the group to confirm a successful ovx procedure [32].

2.3. Morris water maze test (MWM)

Morris water maze test was performed to analyse the spatial cognitive functions of the animals. Briefly, the water maze consisted of a large

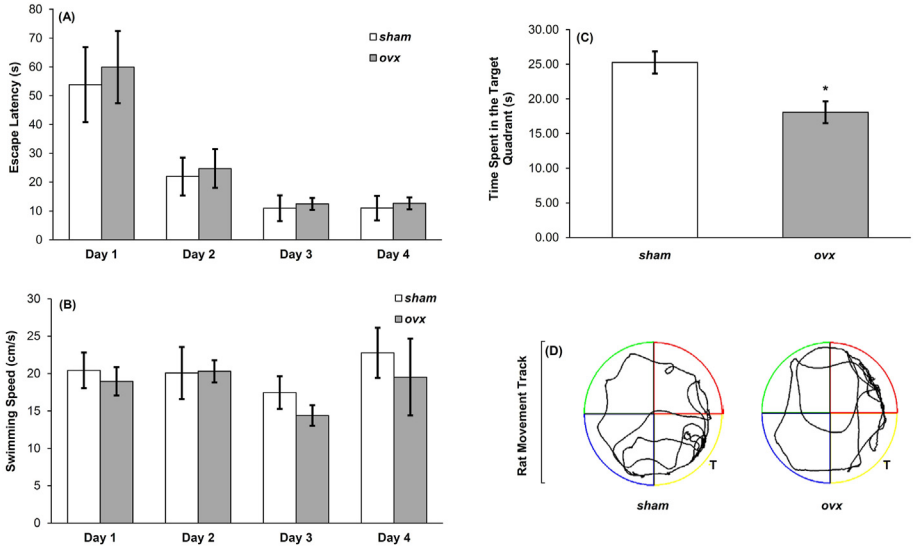

Fig. 1. Effect of ovx on spatial learning and memory as (**A**) escape latency from day 1st to day 4th; (**B**) swimming speed from day 1st to day 4th; (**C**) time spent in the target quadrant on day 5th and; (**D**) representative animal movement track on day 5th in MWM. $n = 7$ in each group. Statistical analysis was performed using the repeated measure of the ANOVA for escape latency and swimming speed, and by unpaired student's t-test for time spent in the target quadrant. *P < 0.05 as compared to *sham*; ***sham*:** Group subjected to surgery; ***ovx*:** Group subjected to ovariectomy, (**s**): seconds and (**T**): Targeted quadrant.

round black circular tank filled with clean water (25 ± 2 °C) to a depth of 30 cm. A camera (Sony color and Lente, CAMCOLORPAL), attached with a video tracking software (SMART V3.0., Panlab, Barcelona) was fitted at the tank's top centre. All extra-maze visual clues in the room remained fixed throughout the experiment. The maze was divided into 4 equal quadrants virtually (Q1 to Q4) using the software. A small transparent platform having a diameter of 12 cm was placed in the centre of Q4 (named as target quadrant), 1 cm beneath the water surface. The test was performed for a total duration of 5 days. Each rat received alternate 4 trials for acquisition/learning with 5 min inter-interval daily for the first 4 days. The position of the submerged platform was fixed in each trial, reorganizing the starting quadrant. During each trial, an individual rat was placed gently in the centre of a quadrant, facing the tank wall, and allowed to locate the submerged platform with 2 min upper cut-off time. In case the rat failed to locate the submerged platform within the cut-off time, it was guided towards the platform and further allowed to rest for the 20 s to imbibe external cues. During the trials, escape latency to locate the hidden platform and swimming speed was recorded. A probe trial was performed on day 5 of the test, for which the platform was removed, and rats were allowed to swim for 1 min. The total time spent in the target quadrant was recorded as an index of memory [33].

2.4. Novel object recognition test (NORT)

Recognition or non-spatial memory of the rats was accessed using NORT. The test was started the next day following completion of MWM in a black square box (65 L x 65 B × 45 H cm) with an opened top. A camera (HD Logitech C525) was attached to the tracking software fitted at the box's top centre. The test procedure involved three separate phases, habituation, familiarization, and recognition. During the habituation phase, each rat was placed in centre of the box without objects and allowed to explore for 5 min at 2-time points with 4 h inter-interval time. The familiarization phase was carried out 24 h after habituation. Each rat was allowed to explore two identical plastic objects (O and O*) for 4 min placed in the adjacent corner of the box, 10 cm far away from the box wall. The recognition phase was started 4 h after the familiarization phase. Object O* was replaced with new object R, and rats were allowed to explore for 3 min individually, and recording was done to calculate the object exploration time. Two virtual zones were made around the objects using the tracking software, and time spent in each zone was calculated to find the object exploration time using event count mode. Novel object preference index and object discrimination ratio was calculated from the object exploration time by using equations $T_R/(T_R + T_O)$ x 100 and $(T_R\text{-}T_O)/(T_R + T_O)$, respectively, where T_R and T_P indicate time spent to explore object R and O, respectively [34].

2.5. Assessment of behavioural impairments

The forced swim test (FST) was done to observe depression-like behaviour in the rats by recording the immobility period. The test was performed 1 h after open field test in a Plexiglas cylinder of 50 cm height, filled with clean water up to 30 cm (25 °C ± 2). A camera (HD Logitech C525) attached to the video tracking software was fitted in front of the cylinder. Each rat was placed gently in the cylinder and allowed to swim for 6 min. The total duration of immobility was recorded using the software's event count mode.

OFT was performed 1 h before FST to analyse anxiety-like behaviour and locomotion to avoid false positive/negative results in the later test. OFT was performed in a Plexiglas box with transparent walls, open-top, and grey coloured floor (100 L x 100 B × 35 H cm). The camera linked with video tracking software was fitted at the top centre of the box. The box was divided into 25 squares (5 × 5) of equal size, virtually using the software. Each rat was placed in the middle of the box, and locomotion was recorded for 5 min [34]. Mean central square entries and mean total square entries were calculated to analyse anxiety-like behaviour and locomotion, respectively.

2.6. Quantitative real time PCR analysis (qRT-PCR)

The genomic expression of the targeted genes was analysed through qRT-PCR (Step One Plus Real-Time PCR system, USA). Total RNA from the cortex and the hippocampus was isolated by a Trizol reagent method (Sigma Aldrich, USA). Chloroform (0.2 mL) was mixed with the tissue homogenate, followed by centrifugation at 12,000 g for 15 min in a cooling centrifuge (Sigma 3–18 K, USA). The aqueous layer was pipetted out, and the remaining part was stored at 4 °C for further analysis. RNA was isolated by precipitating the aqueous layer using isopropanol, followed by centrifugation at 12,000 g for 10 min in the cooling centrifuge. The obtained pellet was washed twice with 75% ethanol and finally dissolved in nuclease-free water and stored at −20 °C. The quality and quantity of total RNA were measured by NanoDrop™ (Thermo Fisher Scientific, USA). Total RNA (2 μg) was reverse transcribed to cDNA as per manufacturer guidelines (Applied Biosystems, USA) for qRT-PCR. Primer 3 software was used to design primers of targeted genes (Supplementary

1, Table 1). *GAPDH* was used as an internal control for qRT-PCR analysis. Further, to reduce sampling error, each sample was repeated three times technically, and the mean value was considered. The relative fold change between the *ovx* and *sham* groups was calculated using 2^ΔΔCT [35].

2.7. Western blotting

The total protein was isolated from the cortex and the hippocampus using a Trizol reagent method. After RNA isolation from the supernatant, DNA in interphase and protein layer was left in the residue. To avoid the nucleic acid contamination in the total protein sample, DNA was isolated first by precipitation using absolute ethanol/1 ml TRIzol reagent, incubated at room temperature for 3 min, and centrifuged at 2000×*g* for 5 min at 4 °C. Phenol-ethanol supernatant was then collected, and protein was isolated by precipitation using 1.5 mL of isopropanol/mL of Trizol reagent. It was incubated at room temperature for 10 min and centrifuged at 12,000×*g* for 10 min in a pre-cooled centrifuge. The supernatant was then discarded. The protein pellet was washed thrice with 0.3 M guanidine HCl in 95% ethanol/mL of Trizol, incubated at room temperature for 20 min, centrifuged at 7500×*g* for 5 min at 4 °C. In the last washing step, absolute ethanol was used instead of guanidine HCl, and the pellet was air-dried, finally resuspended in rehydration buffer, and stored at 4 °C for 48 h. The protein concentration was determined by Bradford assay, and 25 μg protein was used for further expression analysis. Protein denaturation was done in 1X Laemmli buffer, followed by separation on SDS-polyacrylamide gel electrophoresis and transferred onto 0.45 μm PVDF membrane (Amersham™ Hybond™, GE Healthcare, Germany). The membrane was blocked in 5% non-fat dried milk (Bio-Rad Laboratories, USA) for 1 h at room temperature, washed thrice in PBST and probed overnight at 4 °C with primary antibodies [Gsk-3β (1:1500,# MA5-15109, Invitrogen, USA), *p*-Gsk-3β (Ser^9) (1:1500,# MA5-14873, Invitrogen, USA), β-catenin (1:4000,# PA5-19469, Invitrogen, USA), mTOR (1:1000,# orb 14604, Biorbyt Ltd., UK), *p*-mTOR (Ser^{2448}) (1:1000,# SC-293133, Santa Cruz)]. After incubation, the membrane was washed with PBST and further incubated with secondary antibody conjugated with HRP specificity against rabbit (1:10000) and mouse (1:50000) at room temperature for 3 h. Finally, the membrane was washed with PBST, and bands were visualized using Clarity ECL-HRP substrate (Bio-Rad Laboratories, Hercules, USA) under Azure cSeries (Azure Biosystem California, USA). The protein bands were analysed through ImageJ software, β-tubulin (1:2000, # PA5-16863, Thermo Fisher Scientific, USA) used as an internal control.

2.8. Statistical analysis

All the results were expressed as mean ± standard error. The repeated measure of analysis of variance (ANOVA) was performed to analyse escape latency and swimming speed in the MWM. Shapiro-Wilk test was applied to assess data normality. The student's t-test analysed the data following a normal distribution. In contrast, the Mann-Whitney *U* test was used for the parameters where the data was not normally distributed. Cohen's d did an effective size analysis for the parametric test. In contrast, $r = Z/\sqrt{N}$ (Z = Standardized value for the U-value, r = Correlation coefficient, N = Total number of the samples) for nonparametric test and Eta square (η2) was calculated for the repeated measure of ANOVA. The statistical significance of data was considered at $P < 0.05$.

3. Results

3.1. Effect of ovx on uterine weight

The uterine weight was used as a marker of the peripheral activity of estrogen. Ovariectomization in *ovx* group showed a significant ($P < 0.001$, $r = 0.83$) decrease in the uterine weight/body weight ratio (0.159 ± 0.024) after 22 weeks of surgery, as compared to *sham* group (0.493 ± 0.034) when compared using Mann-Whitney *U* test. There were no changes observed in body weight between any of the experimental groups.

3.2. Effect of ovx on cognitive functions

The escape latency was used as a measure of the memory consolidation process in the MWM. There was day-wise significant reduction $F_{(1.38,\ 16.63)} = 25.418$, $P < 0.001$, $\eta2 = 0.67$) in escape latency within *ovx* and *sham* group, but no alteration was observed between the groups ($F_{(1.38,\ 16.63)} = 0.064$, $p = 0.87$, $\eta2 = 0.005$), indicating that memory consolidation process was unaffected post ovx (Fig. 1 A). Furthermore, swimming speed was also recorded to find out the sensorimotor deficit in both the experimental subjects (Fig. 1 B). There was no significant difference was observed in swimming speed at any day within the groups ($F_{(1.74,\ 20.99)} = 1.33$, $p = 0.28$, $\eta2 = 0.10$) and between the groups ($F_{(1.74,\ 20.99)} = 00.166$, $p = 0.82$, $\eta2 = 0.01$).

However, on day 5, there was a marked alteration in spatial memory reconsolidation in the probe trial, indicated by lesser time spent by *ovx* group ($P = 0.007$, $d = 1.727$) in the target quadrant as compared to *sham* group. MWM results showed no alteration in the learning process in both the experimental groups, but memory formation was significantly altered following the ovx in the *ovx* group (Fig. 1C and D).

NORT was performed to analyse the recognition memory functions. A significant decrease in the discrimination ratio was observed in *ovx* group ($P \leq 0.001$, $d = 2.6053$) compared to the *sham* group (Fig. 2B). Furthermore, preference index toward the novel object was also determined in NORT, which showed that rats of *ovx* group exhibited significantly ($P = < 0.001$, $d = 2.6053$) less preference toward novel object as compared to *sham* group (Fig. 2A). These observations indicated that animals in *ovx* group failed to recognise the distinction between novel and familiar objects.

3.3. Effect of ovx on behaviour parameters

We first assessed the animals' locomotor activity to avoid false-positive results by calculating total square entries. The results revealed no change in total square entries among *sham* and *ovx* groups ($P = 0.180$, $d = 0.762$), indicating all rats were normally walking during the experiment (Fig. 3A). Further, to assess the anxiety-like behaviour, central square entries were calculated. It was observed that *ovx* group showed significantly ($P = 0.036$, $d = 1.247$) less central square entries than the *sham* group (Fig. 3B).

Further, to analyse the depression-like behaviour, the immobility period of rats in FST was calculated. There was a significant ($P \leq 0.001$, $d = 2.421$) increase in immobility time in *ovx* group as compared to *sham* group (Fig. 3C). Behaviour variables revealed a significant enhancement in the anxiety-like and depression-like behaviour following the ovx in *ovx* group.

3.4. Effect of ovx on the genomic expression of estrogen receptors

The effect of systemic estrogen depletion on the gene expression of estrogen receptor alpha (*ERα*) in the cortex and the brain's hippocampus regions was performed by using qRT-PCR. In the cortex ($P \leq 0.001$, $d = 3.588$) (Fig. 4A) and the hippocampus ($P = 0.048$, $d = 2.762$) (Fig. 4 A1), a significant decrease in the *ERα* gene expression was observed in *ovx* group as compared to *sham* group. Similarly, the estrogen receptor beta (*ERβ*) gene showed significant downregulation in the cortex of *ovx* group ($P = 0.001$, $r = 0.89$) (Fig. 4B) in comparison to *sham* group. However, there was significant upregulation of *ERβ* gene expression in the hippocampus region of *ovx* group ($P = 0.002$, $d = 2.105$) compared to *sham* group (Fig. 4B1).

3.5. Effect of ovx on the expression of oxidative stress-related genes

Nrf2 is a transcription factor that participates in the transcription of

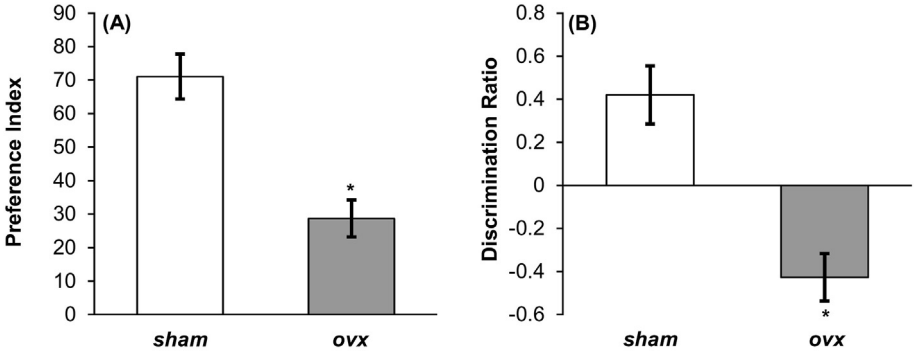

Fig. 2. Effect of ovx on recognition memory as (**A**) percentage preference index in NORT and (**B**) discrimination ratio $n = 7$ in each group. Statistical analysis was performed using unpaired student t-test. *P < 0.05 as compared to *sham*. ***sham*:** Group subjected to surgery and ***ovx*:** Group subjected to ovariectomy.

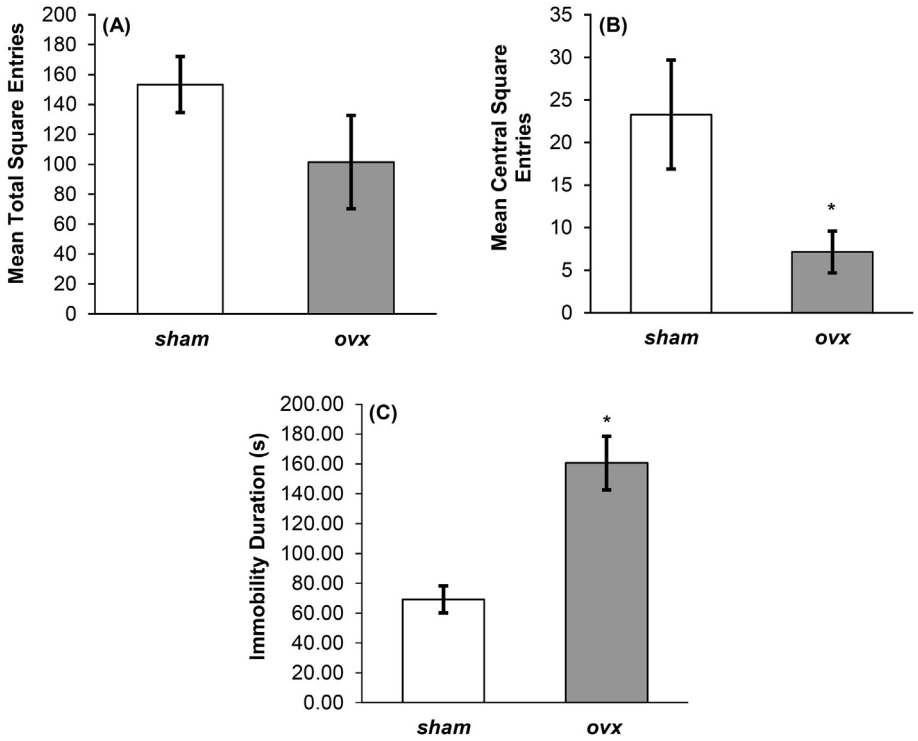

Fig. 3. Effect of ovx on (**A**) locomotor activity (mean total square entries) (**B**) anxiety-like behaviour (mean central square entries) in OFT and (**C**) depression-like behaviour (immobility duration). $n = 7$ in each group. Statistical analysis was performed using the unpaired student t-test. *P < 0.05 as compared to *sham*. ***sham*:** Group subjected to surgery and ***ovx*:** Group subjected to ovariectomy and (**s**): seconds.

genes required to overcome oxidative stress. There was no change in *Nrf2* gene expression in the cortex (P = 0.063, d = 1.0952) (Fig. 4C) and the hippocampus (P = 0.296, d = 0.583) (Fig. 4C1) region of *ovx* group as compared to *sham* group. Heme oxygenase 1 (HO-1) is another protein that participates in combating oxidative stress. In the present study, there was significant up-regulation of *HO-1* gene expression in the cortex (P = 0.007, d = 1.745) (Fig. 4D) and the hippocampus (P = 0.023, d = 1.398) (Fig. 4D1) region of the *ovx* group as compared to sham group. That indicates ovx enhances the oxidative stress in both the studied regions of the brain.

3.6. Effect of ovx on the expression of inflammation-related genes

There was a significant upregulation of *TNFα* gene expression in the cortex (P = <0.001, d = 2.659) (Fig. 4E) and the hippocampus (P = 0.002, d = 2.150) (Fig. 4E1) of *ovx* group in comparison to *sham* group. Similarly, the expression of the *IL-6* gene was also found to be significantly upregulated in the cortex (P = 0.013, d = 1.566) (Fig. 4F) and the hippocampus (P = 0.048, d = 1.177) (Fig. 4 F1) of *ovx* group when compared to the *sham* group.

3.7. Effect of ovx on p-Gsk-3β protein expression

The cortex (P = 0.004, d = 1.917) showed significant downregulation of total Gsk-3β protein expression in *ovx* group compared to the *sham* group (Fig. 5 E). However, there was no change in the expression for the same protein in the hippocampus (P = 0.394, d = 0.4723) of *ovx* group compared to *sham* group (Fig. 6 E). Inactivation of Gsk-3β depends on the phosphorylation of Ser^9. We found a significant downregulation of *p*-Gsk-3β(Ser^9) in the cortex of *ovx* group (P ≤ 0.001, d = 2.4354) in comparison to *sham* group (Fig. 5 F). Surprisingly, *p*-Gsk-3β(Ser^9) in the hippocampus showed a significant upregulation trend in *ovx* group (P = 0.023, d = 1.3965) when compared with *sham* group (Fig. 6 F).

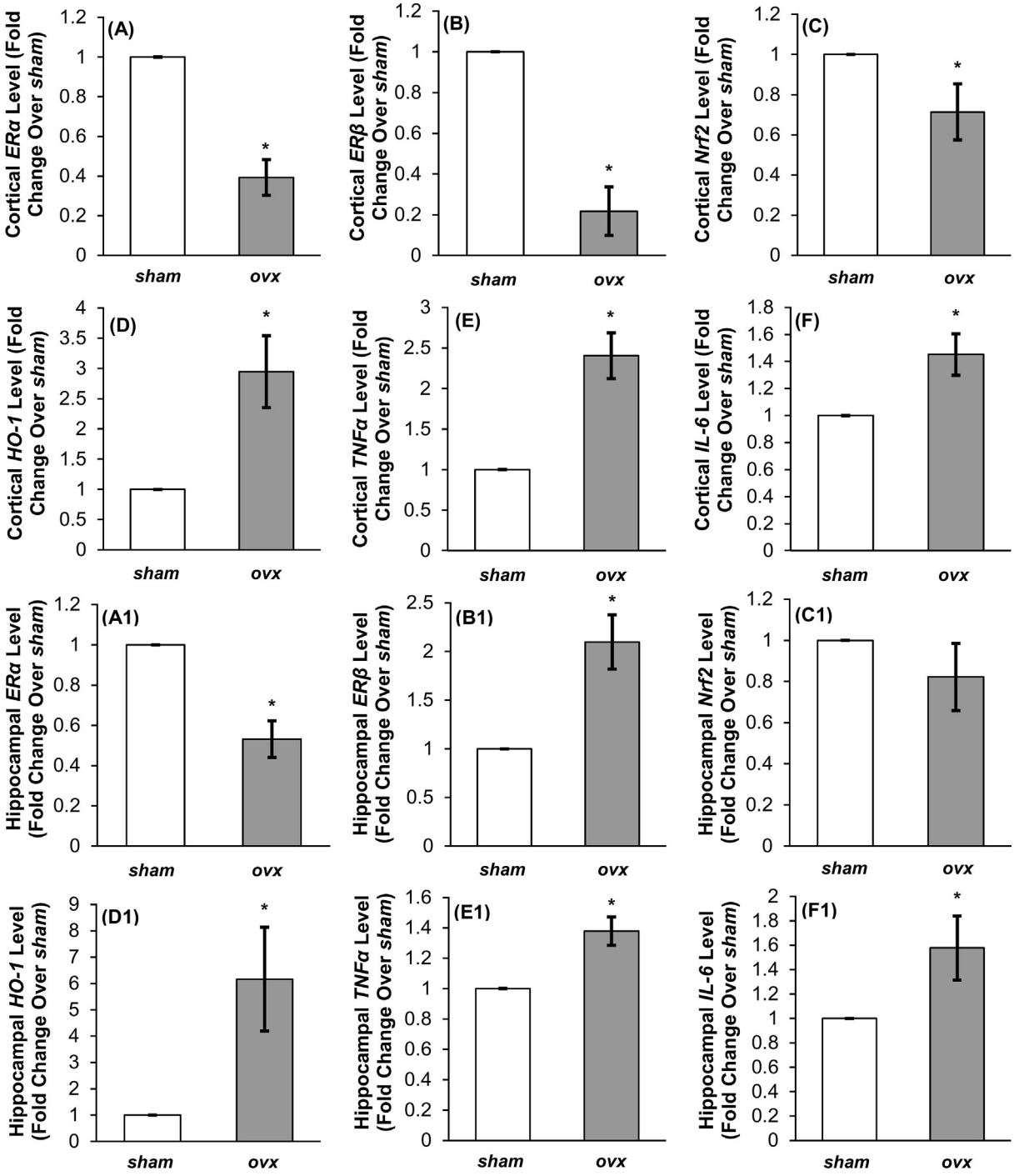

Fig. 4. Effect of ovx on gene expression as (**A**) *ERα* in the cortex, (**B**) *ERβ* in the cortex, (**C**) *Nrf2* in the cortex (**D**) *HO-1* in the cortex, (**E**) *TNFα* in the cortex (**F**) *IL-6* in the cortex (**A1**) *ERα* in the hippocampus, (**B1**) *ERβ* in the hippocampus, (**C1**) *Nrf2* in the hippocampus (**D1**) *HO-1* in the hippocampus, (**E**) *TNFα* in the hippocampus and, (**F**) *IL-6* in the hippocampus. $n = 7$ in each group. Statistical analysis was performed using Mann-Whitney *U* test for non-parametric data (*ERβ* in the cortex) and unpaired student t-test for parametric data (all the remaining parameters). *$P < 0.05$ as compared to *sham*. ***sham*:** Group subjected to surgery; ***ovx*:** Group subjected to ovariectomy; ***ERα*:** Estrogen receptor alpha; ***ERβ*:** Estrogen receptor beta; ***Nrf2*:** Nuclear factor erythroid 2 (NFE2)-related factor 2; ***HO-1*:** Heme oxygenase-1; ***TNFα*:** Tumor necrosis factor alpha and ***IL-6*:** Interleukin 6.

3.8. Effect of ovx on β-catenin protein expression

There was a significant downregulation of total β-catenin protein expression in the *ovx* group's cortex ($P \leq 0.001$, $d = 2.8228$) compared to *sham* group (Fig. 5 D). Interestingly, insignificant change in β-catenin level was found in the hippocampus ($P = 0.121$, $d = 0.8923$) among both the experimental group animals (Fig. 6 D).

3.9. Effect of ovx on the p-mTOR expression

In the cortex, no change was observed in total mTOR protein expression in both the experimental groups ($P = 0.209$, $r = 0.38$) (Fig. 5

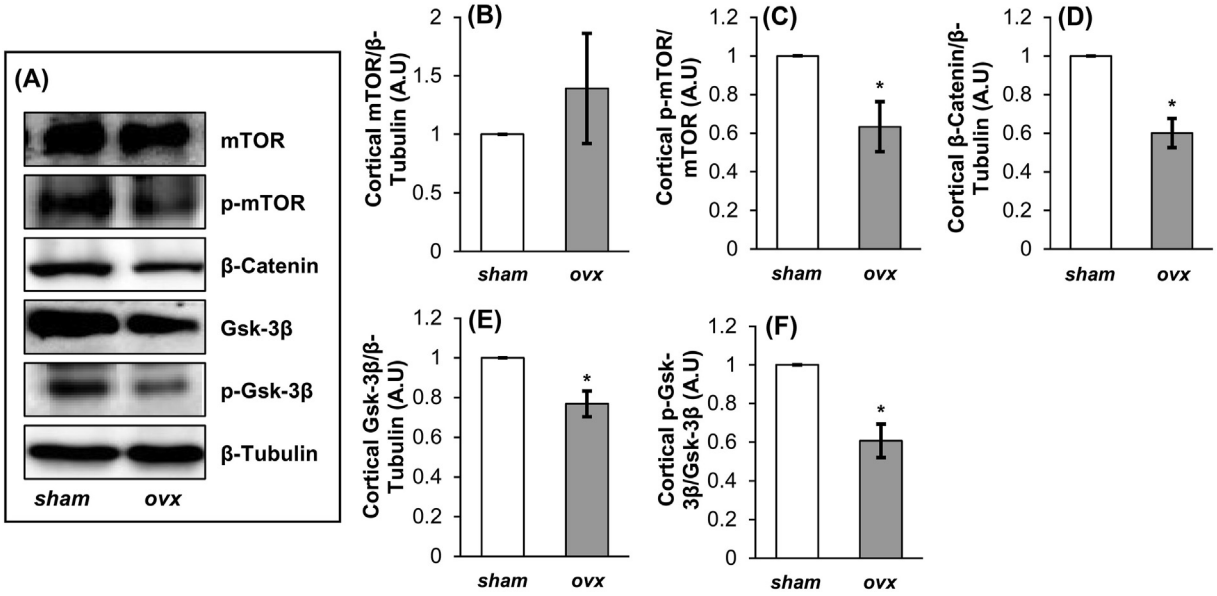

Fig. 5. Effect of ovx on protein expression in the cortex indicating (**A**) representative Western blot image of mTOR, *p*-mTOR, Gsk-3β, *p*-Gsk-3β, β-catenin and β-tubulin. Graphic representation of relative fold change in relation to *sham* group of (**B**) mTOR (**C**) *p*-mTOR (**D**) Gsk-3β (**E**) *p*-Gsk-3β (**F**) β-catenin. $n = 7$ in each group. Statistical analysis was performed using Mann-Whitney *U* test for non-parametric data (mTOR expression) and unpaired student t-test for parametric data (all the remaining parameters). *P < 0.05 as compared to *sham*. ***sham*:** Group subjected to surgery; ***ovx*:** Group subjected to ovariectomy; **mTOR:** mammalian target of rapamycin; ***p*-mTOR:** phosphorylated-mammalian target of rapamycin; **Gsk-3β:** Glycogen synthase kinase-3 beta; ***p*-Gsk-3β:** phosphorylated- Glycogen synthase kinase-3 beta, **β-catenin:** beta catenin; **β-tubulin:** beta-tubulin and; **A.U.**: Arbitrary unit.

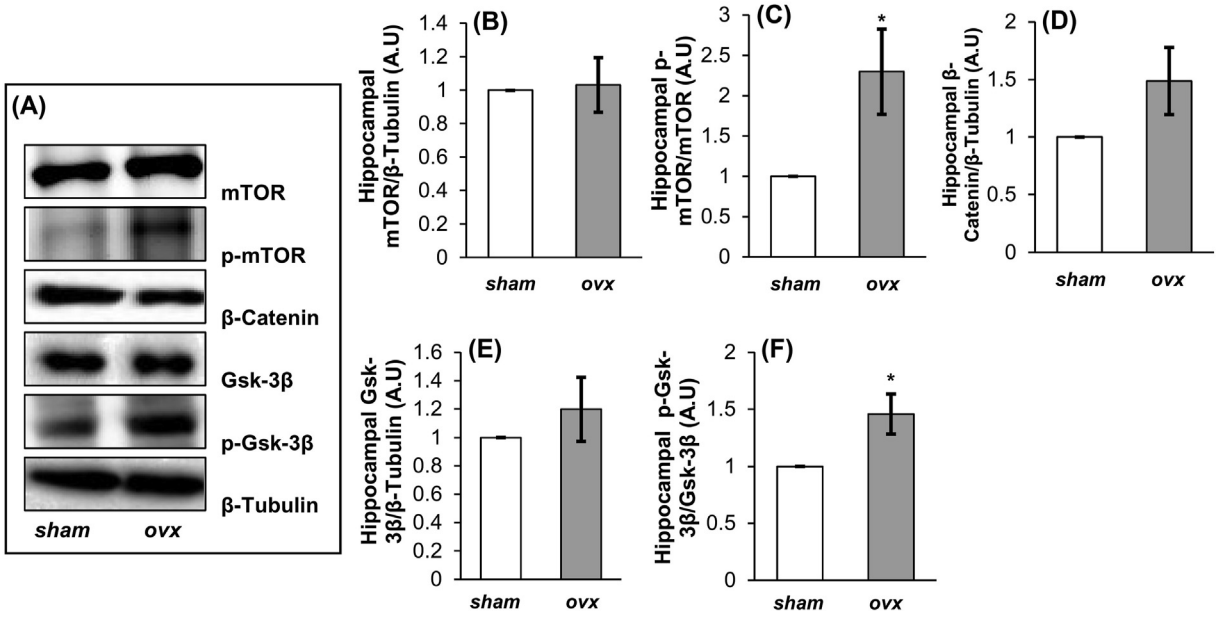

Fig. 6. Effect of ovx on protein expression in the hippocampus indicating (**A**) representative Western blot image of mTOR, *p*-mTOR, Gsk-3β, *p*-Gsk-3β, β-catenin and β-tubulin. Graphic representation of relative fold change in relation to *sham* group of (**B**) mTOR (**C**) *p*-mTOR (**D**) Gsk-3β (**E**) *p*-Gsk-3β (**F**) β-catenin. $n = 7$ in each group. Statistical analysis was performed using unpaired student t-test. *P < 0.05 as compared to *sham*. ***sham*:** Group subjected to surgery; ***ovx*:** Group subjected to ovariectomy; **mTOR:** mammalian target of rapamycin; ***p*-mTOR:** phosphorylated-mammalian target of rapamycin; **Gsk-3β:** Glycogen synthase kinase-3 beta; ***p*-Gsk-3β:** phosphorylated- Glycogen synthase kinase-3 beta, **β-catenin:** beta catenin; **β-tubulin:** beta-tubulin and; **A.U.**: Arbitrary unit.

B). However, *ovx* group (P = 0.015, d = 1.5159) showed significant downregulation of *p*-mTOR expression compared to *sham* group (Fig. 5C). Similar to the cortex, no change was observed (P = 0.852, d = 0.1022) in total mTOR protein expression in hippocampus of *ovx* group as compared to sham group (Fig. 6 B). Surprisingly, hippocampus showed a significant upregulation of *p*-mTOR protein expression in *ovx* group (P = 0.013, d = 2.1419) as compared to *sham* group (P = 0.030, d = 1.311) (Fig. 6C).

4. Discussion

Neurobehavioral impairments greatly affect the quality of post-menopausal women's life. Continuous research is in progress to identify putative molecular targets for improving the functionality and well-being

after menopause. Gsk-3β is gaining wide attention as a potential target for the management of neurological disorders. The present study is a first attempt towards investigating the role of Gsk-3β in neurobehavioral impairment after ovx in middle-aged female rats. The results showed a significant impairment in spatial, and recognition memory with the induction of anxiety and depression-like behaviour in *ovx* group. The genomic expressions of *ERα*, *ERβ*, *Nrf2*, *HO-1*, *TNF-α,* and *IL-6* in the cortex and the hippocampus were altered after ovx. Furthermore, differential expression of *p*-Gsk-3β and their downstream proteins such as β-catenin and mTOR was observed in both the studied regions of the brain.

Women at the middle age experience a dramatic downfall of the systemic circulating sexual hormone during menopause. The cognitive function starts to decline in middle age, and it doubles in women after menopause [36]. Similarly, in the present study, the rats of *ovx* group spent less time in the target quadrant of MWM in comparison to *sham* group, thus supporting the earlier findings showing spatial memory impairments [4]. Retrieval of previous life events is a normal process associated with recognition memory that falls under the subcategory of declarative memory. A clinical study showed impairment of recognition memory in women following menopause [37]. In our study, recognition memory was assessed by NORT, indicated an alteration in short-term recognition memory in *ovx* group. The discrimination index in NORT is used to determine the time spent by a rat in discriminating familiar *vs* novel objects [38]. The discrimination index of *ovx* group in our study was negative, suggesting that rats spent comparatively more time with a familiar object than the *sham* group.

Along with cognitive impairment, women are also exposed to neuropsychiatric conditions such as anxiety and depression during and/or after menopause affecting the quality of life [39]. In the present study, anxiety-like behaviour in rats was assessed using OFT. There was no change in the total ambulatory distance among both the groups that indicated no alteration in total locomotion. However, central square entries were significantly lowered in *ovx* group compared to *sham* group, thus suggesting the induction of anxiety-like behaviour, thus supporting the previous finding [40]. Rather than anxiety, women are also more susceptible to depression after menopause than men of any age [41]. Untreated depression cases tend to develop some psychological and physical problems, triggering suicide at a later age. Hence, to study depression-like behaviour, FST was performed in the present study. There was a significant increase in depression-like phenotype in *ovx* group characterized by increased immobility time compared to *sham* group. The study finding was also supported by some previous reports [42].

Estrogen acts by binding to estrogen receptors (ERs), initiating transcription of genes via estrogen response element in their promoter, and finally modifying cellular behaviour. The estrogen action not only depends on its presence in the serum but also on the type of organ and distribution of ERs [43]. In the brain, ERs regulate reproductive functions and participate in other neuroendocrine events such as memory formation, cognition, and behaviour. ERα is reported to participate in the formation of short-term memory [4]. In our study, significant downregulation in the *ERα* expression in the cortex and the hippocampus of *ovx* group was observed. Qu et al. [4] also reported the downregulation of ERα expression and impaired spatial memory following the 8 weeks of ovx in young rats associated with the reduction in the neuronal population and synaptic plasticity. Other than memory formation, *ERα* also contributes to behaviour development. Furuta et al. [44] observed that downregulation of *ERα* following the postpartum induces anxiety and depression-type behaviour in rats, suggesting a relationship of systemic estrogen on the regulation of ER. Similarly, in the present study, decreased genomic expression of *ERα* in *ovx* group was observed along with anxiety and depression-like behaviour.

Similarly, there was also a marked decrease in the expression of *ERβ* in the *ovx* group's cortex. Our results were in line with a previous report indicating the induction of anxiety-like behaviour with *ERβ* gene knockout [45]. Moreover, decreased expression of ERβ is also associated with depression-like behaviour via the serotonergic system alteration in mouse brain of following ovx [46]. Likewise, in our study development of depression-like behaviour with a decrease in *ERβ* gene expression was observed in the *ovx* group. Surprisingly, the hippocampus showed significant upregulation of *ERβ* gene expression in *ovx* group. These alterations in the genomic expression of ERs can be linked with the level of locally synthesized estrogen [47]. A study conducted by Prange-Kiel et al. [48] also showed upregulation of ERβ and downregulation of ERα in an estrogen-free *in-vitro* culture of the adult rat hippocampus. Increased expression of ERβ in the hippocampus was found to be linked with AD [49], a neurodegenerative disease that is also associated with cognition and behavioural alterations.

Oxidative stress is the primary key factor responsible for cognitive and behavioural impairments in neurodegenerative disorders. Nrf2 is a transcription factor known to protect against oxidative stress via initiating the transcription of glutathione S-transferase (GST), *HO-1*, SOD, and CAT [7]. HO-1 plays an important role in oxidative stress by converting the heme into biliverdin, which further changes into antioxidant molecule bilirubin [50]. In contrast to protection, the upregulation of *HO-1* participates in the oxidative stress via enhancing the Fe^{2+} accumulation in the mitochondria, further leading to macroautophagy [51]. The relation between oxidative stress and neurobehavioural impairment was very well reported [52]. Overexpression of HO-1 and their co-localization with neurofibrillary tangles, senile plaques, and corpora amylacea were observed in the cortex and the hippocampus of patients affected with mild cognitive impairment of AD [51]. Similarly, we also observed an upregulation in the expression of *HO-1* and altered cognitive impairments in *ovx* group. Thus, indicated that the sex hormone deprivation after menopause interferes with the genomic expression of genes related to the antioxidant pathway. Interestingly, the genomic expression of *Nrf2* remained unchanged in both the regions of the brain in all experimental groups, while alteration in the cognitive functions was observed only in *ovx* group. Likewise, impairment in spatial memory without alteration in the genomic expression of *Nrf2* has also been reported in a mouse model of mid cerebral hypoperfusion [53], a kind of neurological disorder that progressively develop dementia.

Neuroinflammatory events are also altered in the brain during aging, characterized by poorly regulated or increased production of pro-inflammatory mediators [11]. Previous studies have reported the upregulation of *TNFα* in women after oophorectomy [54]. Kireev et al. [55] also showed an upregulation of TNFα expression in aged ovx rats' brains. The elevated level of the TNFα in mice following surgery seems to be associated with cognition decline via triggering the cytokine cascade [56]. Moreover, Taniguti et al. [57] have showed that the elevated level of TNFα in the hippocampus and the pre-frontal cortex of mice following lipopolysaccharide injection enhances depression-like behaviour via interfering the BDNF signalling. Similarly, we also observed that the upregulation of *TNFα* in *ovx* group enhanced the cognitive and behaviour impairment. E_2-inhibitory element or TNF-responsive element (TNF-RE), mapped from −125 to −82 regions in the promoter of *TNFα*, has been explored as a responsible factor for the downregulation of its expression in the presence of estrogen [58]. Estrogen is the major gonadal steroid hormone, and its loss in menopause may be responsible for the upregulation of *TNFα* expression following ovx and associated with neurobehavioural impairments. IL-6 is another important pro-inflammatory cytokine that also participates in the pathogenesis of various neurological disorders such as AD, Parkinson's disease (PD), and multiple sclerosis. In our study, a significant upregulation of *IL-6* gene in both the studied regions of the brain in *ovx* group was observed as compared to *sham*, indicative of estrogen deficit mediated inflammation. Kireev et al. [55] also showed a significant elevation of *IL-6* expression after ovx in rats. NF-IL6 and Nf-κb are the positive regulatory factors that regulate the *IL-6* genomic expression. In the presence of estrogen, both the transcription factors lose their DNA binding ability, finally leading to the down-regulation of *IL-6* gene expression [59]. Similarly, in the present study,

deprivation of estrogen in ovx group may be the reason for a significant upregulation of *IL-6* in both the studied regions. Moreover, the elevated level of TNFα and IL-6 has been associated with cognitive impairments [60], and depression-like behavioural in ovx rat [61]. A similar relation was also observed in our study.

Gsk-3 is a serine/threonine kinase that plays a crucial role in maintaining learning and memory functions. Several other reports have suggested active participation of Gsk-3β in the development of the neurobehaviour impairment in neurological disorders [18,62]. Spatial memory was found to be altered in a streptozotocin-induced rat model of AD [63]. The brain samples from AD patients and transgenic mice showed abnormal activation of Gsk-3β, which enhanced the accumulation of Aβ and tau proteins and reduced the spine density in neurons [64] and finally end with the development of severe dementia. Activation of Gsk-3β plays a crucial role only in maintaining the reconsolidation process of spatial memory rather than the learning process. Kimura et al. [18] showed increased expression of *p*-Gsk-3β(Ser^{9}) in the hippocampus of mice to be responsible for the impairment in the reconsolidation process of spatial memory during the probe trial MWM. Still, they did not affect the consolidation process. Likewise, in this study, increased *p*-Gsk-3β(Ser^{9}) expression was observed in the hippocampus region of the brain in *ovx* group may be the reason for the spatial memory impairment after ovx in middle-aged female rats.

Apart from memory formation, Gsk-3β also participates in developing mood disorders such as depression and anxiety. Martin et al. [19] reported that Lithium chloride mediated inhibition of Gsk-3β improved depression-like behaviour by rescuing spine density in the pyramidal neuron of the *Dixdc1 KO* mice. Another study also revealed that activation of Gsk-3β through amphetamine in the mice cortex increased the anxiety-like behaviour assessed through OFT [17]. Similarly, downregulation of *p*-Gsk-3β(Ser^{9}) protein expression in the cortex of *ovx* group observed in our study might be responsible for depression-like and anxiety-like behaviour. Numerous evidence suggested the inactivation of Gsk-3β by estrogen through various pathways in the acute phase, but the reciprocation of the same in the reported chronic studies is still not clear [65]. Since it is known that aggravated Gsk-3β can lead to various neurological disorders [62], there might be a chance that exogenous estrogen therapy is providing symptomatic relief only from post-menopause associated neurobehavior impairment.

Total β-catenin expression was analysed to confirm the activity of Gsk-3β in both regions of the brain. β-Catenin is the key protein of Wnt/β-catenin pathway that is subjected to proteasomal degradation via the ubiquitination process initiated after Gsk-3β activation [66]. Several studies support the downregulation of β-catenin to be associated with neurobehavioral alteration in neurological ailments [67,68]. Chronic unpredicted stress in rat alter their performance in MWM as well as enhances the depression-like behaviour. Further, molecular analysis revealed that activation of Gsk-3β downregulates the cytosolic level of β-catenin, and interfere with the cytosolic to nuclear translocation of the same protein [21]. It indicated that activated Gsk-3β interacts with β-catenin signalling, resulting in neurobehavior impairment. Similarly, we found a significant downregulation of total β-catenin protein expression in *ovx* group, which also supported the activation and participation of their upstream regulator Gsk-3β in the development of cognitive and behavioural alteration.

The mTOR is another serine/threonine kinase that contributes to the translation process, normal cell growth, and endocrine resistance. mTOR signalling participates in numerous of neurophysiological processes such as axonal sprouting, dendritic spine growth, axonal regeneration, and myelination [69]. Tuberous sclerosis complex (TSC1 and TSC2), an upstream inhibitor of mTOR signalling pathway is repressed during the normal condition by their upstream kinases, such as protein kinase B and extracellular signal-related kinase [70]. The first report of phosphorylation of TSC1/2 at Ser^{1345} via AMPK was described by Inoki et al. [70]. It acts as a substrate for Gsk-3, which further initiates TSC1/2 complex activity via phosphorylation on the $Ser^{137/1341}$ site, leading to mTOR inhibition. In our study, there was a significant downregulation of *p*-mTOR (Ser^{2448}) in the cortex of *ovx* group. Surprisingly, in the hippocampus, results were opposite to the cortex.

The transforming activity of mTOR in neurological disorders such as epilepsy, anxiety, and depression are directly associated with cognitive behaviour alteration [33,71]. The altered *p*-mTOR (Ser^{2448}) expression in both the brain regions suggested that differential activation of Gsk-3β might cause the induced neurobehavioral alterations after ovx in the middle-aged female rats. Some clinical studies also suggest that women undergoing surgical menopause are at higher risk of developing cognitive and behaviour impairment than the women proceeding naturally [72]. A study suggested that estrogen withdrawal from the body when ovaries are more functional is the main reason for cognitive and behaviour alteration following ovx [73]. Observations of the present study and the literature also indicated that systemic estrogen is a crucial neuroendocrine hormone that directly affects cognitive processing and behaviour development. A sudden decline in systemic estrogen levels may disrupt the body homeostasis, resulting in oxidative stress, neuroinflammation and impaired Gsk-3β activity that ultimately triggers the neuronal events in case of estrogen deprivation.

5. Conclusion

The present study concluded that there was a differential activation of Gsk-3β in the brain of middle-aged female ovx rats. Oxidative stress and neuroinflammatory events were also provoked in the brain after ovx that indirectly altered the activity of Gsk-3β. Differential activated Gsk-3β altered the expression of downstream proteins and induced neurobehavioral alterations. Based on the results, it can be correlated that Gsk-3β is a potential target for the management of cognitive and behaviour impairments in postmenopausal women, provided its role is further validated in future preclinical and clinical studies.

Declaration of competing interest

None declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cpnec.2020.100019.

List of abbreviations

BBB	Blood brain barrier
CAT	Catalase
CO	Carbon monoxide
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
FST	Forced swim test
GPx	Glutathione peroxidase
Gsk-3	Glycogen synthase kinase-3
Gsk-3α	Glycogen synthase kinase-3 alpha
Gsk-3β	Glycogen synthase kinase-3 beta
IL-6	Interleukin 6

MDA	Malonaldehyde
MWM	Morris water maze test
NORT	Novel object recognition test
Nrf2	Nuclear factor erythroid 2 (NFE2)-related factor 2
OFT	Open field test
Ovx	Ovariectomy
qRT-PCR	Quantitative real time polymerase chain reaction
ROS	Reactive oxygen species
RT	Room temperature
SOD	Superoxide dismutase
TNF-α	Tumor necrosis factor alpha

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