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Lithium therapy subdues neuroinflammation to maintain pyramidal cells arborization and rescues neurobehavioural impairments in ovariectomized rats

Anil Kumar Rana[1,2] · Supriya Sharma[1,2] · Vikram Patial[1,2] · Damanpreet Singh[1,2]

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Abstract

Oestrogen deprivation as a consequence of menopause alters the brain neuronal circuit and results in the development of neurobehavioural symptoms later. Hormone replacement therapy to some extent helps to overcome these abnormalities but is associated with various adverse events. Lithium therapy is being used to manage multiple neuropsychiatric disorders and is reported to maintain structural synaptic plasticity, suppress neuroinflammation, and promote adult neurogenesis. The present study examined the effect of lithium treatment on the neurobehavioural impairments in ovariectomized rat model mimicking clinical postmenopausal condition. A protective effect of lithium treatment was observed on the reconsolidation of spatial and recognition memory along with depression-like behaviour in ovariectomized rats. The Golgi-Cox staining revealed increased dendritic length and spine density in the pyramidal neurons of the CA1 region of the hippocampus, layer V of the somatosensory cortex, and layer II/III of the prefrontal cortex in the treated group. A significant reduction in pro-inflammatory markers, *Il2*, *Il6*, and *Il1b,* was observed in the hippocampus, somatosensory cortex, and prefrontal cortex following lithium treatment. mRNA expression studies of *Gfap* and *Pparg*, along with histopathological analysis, suggested reactive astrogliosis to be a major contributor of neuroinflammation in ovariectomized rats that was normalized following lithium treatment. Further, the treatment inhibited Gsk-3β activity and maintained the normal level of β-catenin, CREB, and BDNF. The results revealed a defensive role of lithium against ovariectomy-induced neurobehavioural impairments, thus suggesting it to be a potential therapeutic agent for managing postmenopausal neurological symptoms.

Keywords Brain-derived neurotrophic factor · cAMP response element-binding protein · Glycogen synthase kinase 3 (Gsk-3) · Hippocampus · Inflammatory cytokines · Mammalian target of rapamycin

Introduction

Middle-aged women are always at a higher risk of developing neurobehavioural conditions than coeval men due to gradual oestrogen depletion as a consequence of menopause onset [1]. Oestrogen regulates reproductive functions and engages in memory formation, behavioural functions, neuroprotection, and synaptic plasticity [2, 3]. The transition from the reproductive to non-reproductive stage in women is proposed to be a prodromal phase of neurodegenerative disease [1]. Dendritic spines are small protrusions present in the neuronal dendrites of the hippocampus, prefrontal cortex, and neocortex regions that are highly sensitive to oestrogen fluctuations [2, 4]. Several preclinical studies have supported the reduction in spine density in the apical dendrite of pyramidal neuron of layer V in the female rodents following ovariectomy. These alterations were in the different neocortical regions, apical dendrite of the CA1 region of the hippocampus, and apical and basal dendrite of pyramidal neurons in the prefrontal cortex [5–7].

Neuroinflammatory events are the major contributors to postmenopause-associated neurological conditions [1]. Some preclinical studies showed increased expression of interleukins (IL-1β, IL-6, and TNF-α) in the brain of middle-aged ovariectomized (*ovx*) rats that ultimately led

✉ Damanpreet Singh
dsinghpharmacology@gmail.com; damanpreet@ihbt.res.in

[1] Pharmacology and Toxicology Laboratory, Dietetics and Nutrition Technology Division, CSIR-Institute of Himalayan Bioresource Technology, 176061, Palampur, Himachal Pradesh, India

[2] Academy of Scientific and Innovative Research (AcSIR), Ghaziabad 201002, India

to disruption of neuronal circuits and interfered with normal cognitive and behaviour processes [3, 8]. These pro-inflammatory cytokines are also known to play a crucial role in the pathogenesis of several neuropsychiatric conditions [9]. Astrocytes are a type of glial cells that, despite being devoid of electrical impulse, participate in several physiological processes of the brain [10]. The available information suggested that reactive gliosis and its inflammatory properties significantly promote and sustain neuronal death in neurodegenerative diseases [11]. Oestrogen deprivation affects the neuronal circuit and enhances the blood–brain barrier (BBB) permeability in the middle-aged *ovx* rats [12]. In rodents, chronic BBB damage is associated with the accumulation of blood-derived neurotoxic proteins such as thrombin, fibrin, hemosiderin, haemoglobin, plasmin, and iron, which leads to neuronal damage, either directly via neurotoxins or by neuroinflammation [13].

The beneficial effect of exogenous oestrogen is reported to combat postmenopause-linked neurobehavioural symptoms by enhancing structural synaptic plasticity and reducing neuroinflammation [8]. However, studies have shown the risk of dementia associated with exogenous oestrogen treatment due to the atrophic changes in the brain, resulting in cortex and hippocampus volume reduction [14, 15]. Chronic treatment of oestrogen following a critical window period in middle-aged *ovx* rats enhanced the BBB permeability [16], thus suggesting a potential risk of the therapy towards neurodegenerative diseases. Therefore, it is important to identify molecules or drugs that replace the need for exogenous oestrogen and minimize postmenopausal conditions.

Lithium, a gold standard drug in psychiatric medicine, is mainly used to treat bipolar disorder and major depression [17]. Some preclinical studies suggested the neuroprotective effect of lithium via inhibiting autophagy, neuroinflammation, and enhancing structural synaptic plasticity [18, 19]. Furthermore, lithium has been reported to regulate various signalling cascades, including glycogen synthase kinase 3 (Gsk-3), cAMP response element-binding protein (CREB), and brain-derived neurotrophic factor (BDNF) [17]. Our previous study showed that differential activation of Gsk-3β is involved in the development of neurobehaviour impairments in middle-aged *ovx* rats [3]. Activated Gsk-3β is also known to engage in the prolonged stimulation of neuroinflammatory process, which eventually leads to neurodegenerative diseases [20]. Moreover, activated Gsk-3β also interferes with the mammalian target of rapamycin (mTOR) signalling, resulting in impairment of the cognition process [3], which was improved following lithium treatment [21]. Therefore, in the present study, we hypothesized that treatment of lithium might suppress postmenopause-associated neurobehaviour symptoms via inhibiting neuroinflammation and maintaining the structural synaptic plasticity in the discrete brain regions. We used middle-aged *ovx* rat model that mimics postmenopausal conditions to study the effect of lithium.

Materials and Methods

Experimental animals and care

The available literature shows that around 10-month-old female rats start experiencing irregularity in their oestrous cycle, indicating initiation of reproductive cessation [22]. Therefore, the study was conducted on 10-month-old Sprague Dawley female rats. The rats were housed in standard polycarbonate cages (three animals/cage) at temperature of 25 ± 2℃, relative humidity of 50–60%, and 12 h day/night cycle in the animal house of CSIR-IHBT. Food and water were provided *ab libitum* to the animals throughout the experiment. All the experimental procedure on animals were performed during the day cycle, and the protocol was duly approved by Institutional Animal Ethics Committee (IAEC) established by CPCSEA, Ministry of Fisheries, Animal Husbandry and Dairying, Government of India.

Ovariectomy and experimental protocol

The surgical procedure for ovariectomy in SD female rats was performed following anaesthesia with ketamine and xylazine (detailed in Supplementary Information, Supplement 1). A separate group of rats ($n = 8$) indicated as *sham-N* served as sham and received similar surgical procedure, except removal of ovaries. All the animals were kept for 12 weeks' post-surgery to develop the postmenopausal symptoms [3]. Successfully recovered *ovx* rats were randomly divided into two groups ($n = 8$/group). An *ovx* rats group (*ovx-V*) received normal standard chow diet (Golden feeds, New Delhi, India) for 10 weeks, whereas the other group (*ovx-LI*) was provided with a chow diet containing lithium carbonate (2.4 g/Kg of chows) during the same period [23]. The therapeutic concentration of lithium in the serum was maintained by 2.4 g of lithium carbonate per kg of chow to achieve 0.97 ± 0.20 mM, equivalent to 0.6–1.2 mM in humans [24]. It has been observed that long-term lithium therapy is required to maintain its therapeutic concentration in the brain to cure psychiatric conditions [25, 26]. A previous preclinical study has shown that treatment of lithium for 10 weeks increases the learning process in rats [27]. Therefore, 10 weeks' administration of lithium was selected in the current study. Following the treatment, the animals were subjected to neurobehavioural tests. All the animals were sacrificed after 24 h of the behavioural analysis, and the brain was isolated. The hippocampus, somatosensory cortex, and prefrontal cortex regions were selected as these areas are engaged in

the cognitive process and are primarily affected during behaviour disorders. The brain of four animals selected randomly from each group was used for histopathological analysis and immunohistochemistry, while the remaining brains were dissected to isolate all the three regions for gene expression and western blotting studies. The uterus was also isolated from all the animals to calculate adjusted weight. A schematic representation of the experimental protocol is shown in Fig. 1.

Assessment of cognitive functions

The Morris water maze test (MWMT) test was conducted for subsequent 5 days following 10 weeks' treatment to animals. The procedure involved learning phase of 4 days to record escape latency (time to trace a submerged platform). On the next day of the last learning phase, the extent of memory retention was recorded in terms of exploration in a target quadrant. After MWMT, recognition memory in rats was accessed using a novel object recognition test (NORT). In the test, ability of an animals to distinguish a familiar object from a novel object was noted as discrimination ratio and preference index. The details of cognitive tests are included in Supplementary Information (Supplement 1).

Behavioural assessment

The anxiety-like behaviour and locomotor function of rats were studied using an open field test (OFT). Forced swim test (FST) was carried out 1 h after OFT to assess depression-like behaviour in experimental animals, and details are described in Supplementary Information (Supplement 1).

Quantitative real-time PCR analysis (qRT-PCR)

The isolated brain parts were homogenized in Trizol reagent (Sigma-Aldrich, USA) and processed to pellet down the RNA [28]. The RNA was quantified and reverse transcribed into the cDNA following RNAse treatment. A complete qRT-PCR method with details of primers used in the study is described in Supplementary Information (Supplement 1), Table S1. A relative expression of each gene with respect to *sham-N* was calculated using 2^ΔΔCT method.

Western blotting

The crude protein was extracted from the isolated brain parts using Trizol method as described earlier [28]. Total protein was denatured in 1X sample loading buffer, separated on SDS–polyacrylamide gel electrophoresis, transferred to

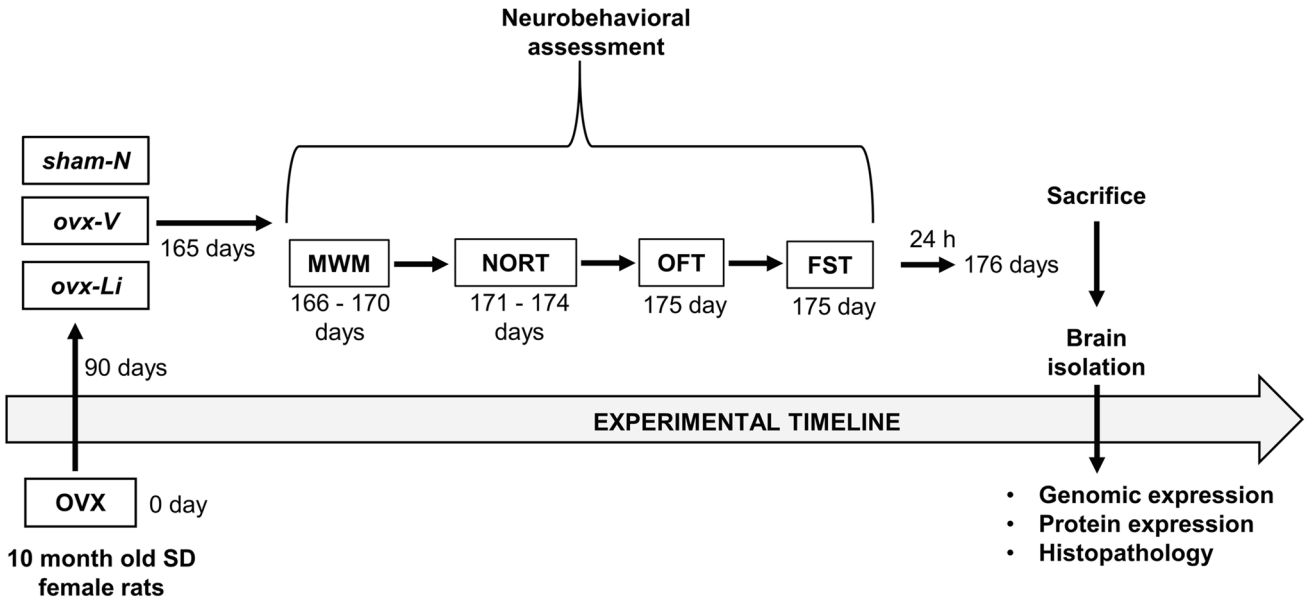

Fig. 1 Schematic representation of experimental timeline. 10-month-old SD female rats underwent the ovariectomization (*ovx*) procedure and were kept for 90-day post-surgical procedure to develop post- menopause-associated neurobehavioural impairment. After 90 days, the rats were randomly divided into three groups named as *sham-N*, *ovx-V,* and *ovx-LI,* respectively. *sham-N* and *ovx-V* were continuing on the normal chow diet, while *ovx-LI* groups received lithium containing chow diet for further 75 days. Animals were sacrificed following the 24 h of last behaviour parameter, and brain was isolated to perform the mRNA expression, protein expression, and histopathological analysis. ***ovx:*** ovariectomy; **MWM:** Morris water maze; **NORT:** Novel object recognition test; **OFT:** Open field test; **FST:** Forced swim test; ***sham-N***: Group subject to sham surgery with normal chow diet; ***ovx-V***: Group subject to ovariectomy with normal chow diet; ***ovx-LI***: Group subject to ovariectomy with lithium chow diet; and **h**: Hours

a PVDF membrane and incubated with primary antibodies [Gsk-3β, p-Gsk-3β(Ser^{9}), β-catenin, mTOR, p-mTOR (Ser^{2448}), BDNF, CREB, and p-CREB (Ser^{133})]. The protein expression was quantified using ImageJ software following normalization with β-actin. A detailed methodology is included in Supplementary Information (Supplement 1).

Histopathological analysis

The isolated brain was washed in freshly prepared PBS. The brain was sagittal separated into two equal halves. A half was fixed in the formalin for the histopathological and immunohistochemistry analysis, while the remaining part was used for the Golgi-Cox staining.

Golgi-Cox (GC) staining

GC staining was performed to understand the structural synaptic plasticity. The left hemisphere of brain was dissected to separate the prefrontal cortex and somatosensory region. Both the dissected regions were processed for GC staining [29]. The details of staining procedure, and section analysis for dendritic morphology and spine density [30] are described in Supplementary Information (Supplement 1). For the quantitative analysis of pyramidal morphology, only those neurons which fulfil the following criteria were selected: (1) well-impregnated pyramidal neuron characterized by triangular soma shape; (2) cell body within the middle of specified subregions of the hippocampus (CA1 region), layer V of the somatosensory cortex as well as II/III layer of the prefrontal cortex; (3) neuron relatively isolated from the neighbouring neuron; (4) the presence of at least two primary basal dendritic shafts; and (5) apical dendrite without truncated branches.

Immunohistochemistry

The right hemisphere was kept in formalin and finally embedded in paraffin wax. The sections were processed [described in Supplementary Information (Supplement 1)] to study localized expression of glial fibrillary acidic protein (GFAP) [31] and neuronal nuclear protein (NeuN).

Nissl staining

Nissl staining was performed to understand the morphology and pathology of the neuron. Briefly, the 5 µM section of paraffin-embedded brain tissue was taken on the slide and processed for Nissl staining using a 1% solution of cresyl violet [32], as is further described in Supplementary Information (Supplement 1). The sections on slides were analysed under a bright-field microscope (Olympus BX53F) to count the number of dark-stained dead neurons. In addition, to reduce the ambiguity in results, the average number of the dark neurons/field from the *sham-N* group was deducted from the rest of the experimental groups [32].

Statistical analysis

The results are represented as a mean ± standard error. Distribution of the data was assessed by Shapiro–Wilk test. One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was performed for normally distributed data, whereas the parameters with skewed data were analysed by Kruskal–Wallis test and Mann–Whitney U post hoc. Student's t test was applied to find the statistic significant difference between the groups in Nissl staining. Repeated measure of ANOVA was done to assess the escape latency in MWM and difference in number of intersections by Sholl method. Mauchly's test of sphericity was used with the repeated measure of ANOVA, and degree of freedom was corrected by using epsilon Greenhouse–Geisser to more conservative value for the factor in which sphericity assumption was violated. Further effective size was also calculated by applying a formula, $r = Z/\sqrt{N}$, for Mann–Whitney U test (where N = total samples number, r = correlation coefficient, and Z = standardized value for the U value). Epsilon-squared formula was applied for Kruskal–Wallis test, as $E_R^2 = \frac{H}{(n2-1)/(n+1)}$ [where n = the total number of observations, H = value earned in the Kruskal–Wallis test (the Kruskal–Wallis H test statistic), and E_R^2 = coefficient, which assumes the value from 0 (no relationship) to 1 (a perfect relationship). Cohen's d was used to calculate the effective size during pair-wise comparison, while Eta square (η2) is done for one-way ANOVA. A value of $P < 0.05$ for the studied parameters was considered as significant.

Results

The detailed results including variation between the sample mean/difference within the sample, exact P value among different groups, and the effective size are given in the Supplementary Information (Supplement 1).

Ovariectomy reduced uterine weight

Ovariectomization significantly ($P < 0.05$) reduced the uterine/body weight in the *ovx-V* group (0.162 ± 0.0186) compared to *sham-N* group. Interestingly, no change ($P = 0.93$) in uterine weight was observed in *ovx-LI* group (0.147 ± 0.01) as that of *ovx-V*; however, it remained significantly ($P < 0.05$) reduced in comparison with *sham-N* group (0.442 ± 0.045).

Lithium treatment improved ovariectomy-induced neurobehavioural impairments

The escape latency, a measure of consolidation process of spatial memory in MWMT, was significantly ($P < 0.05$) decreased day-wise within the experimental groups. However, insignificant change in the escape latency was observed between the experimental groups (Fig. 2A). The *ovx-LI* group and *sham-N* group spent significantly ($P < 0.05$) more time in the target quadrant during probe trial on day 5 in comparison with the *ovx-V* group, thus suggesting that lithium rescued the reconsolidation process of spatial memory (Fig. 2B). In NORT, episodic memory was also altered in *ovx-V* group, indicated by a significant ($P < 0.05$) decrease in the discrimination ratio (Fig. 2C) and preference index (Fig. 2D) as compared to *sham-N* group. However, lithium treatment significantly increased ($P < 0.05$) both the parameters when compared with the *ovx-V* group.

Further, OFT revealed insignificant change in total number of virtual square entities among different groups, indicating no effect on total locomotion (Fig. 2E). There was induction of anxiety-like behaviour in *ovx-V* group evidenced by a significant ($P < 0.05$) decrease in the central square entries in comparison with the *sham-N* group. Interestingly, lithium treatment in *ovx-LI* group do not overcome the anxiety-like behaviour when compared with *ovx-V* group, as insignificant change in central square entries was observed among both the groups (Fig. 2F). Furthermore, *ovx-V* group also showed a significant ($P < 0.05$) increase in immobility period in FST as compared to the *sham-N* group (Fig. 2G). Thus, suggested

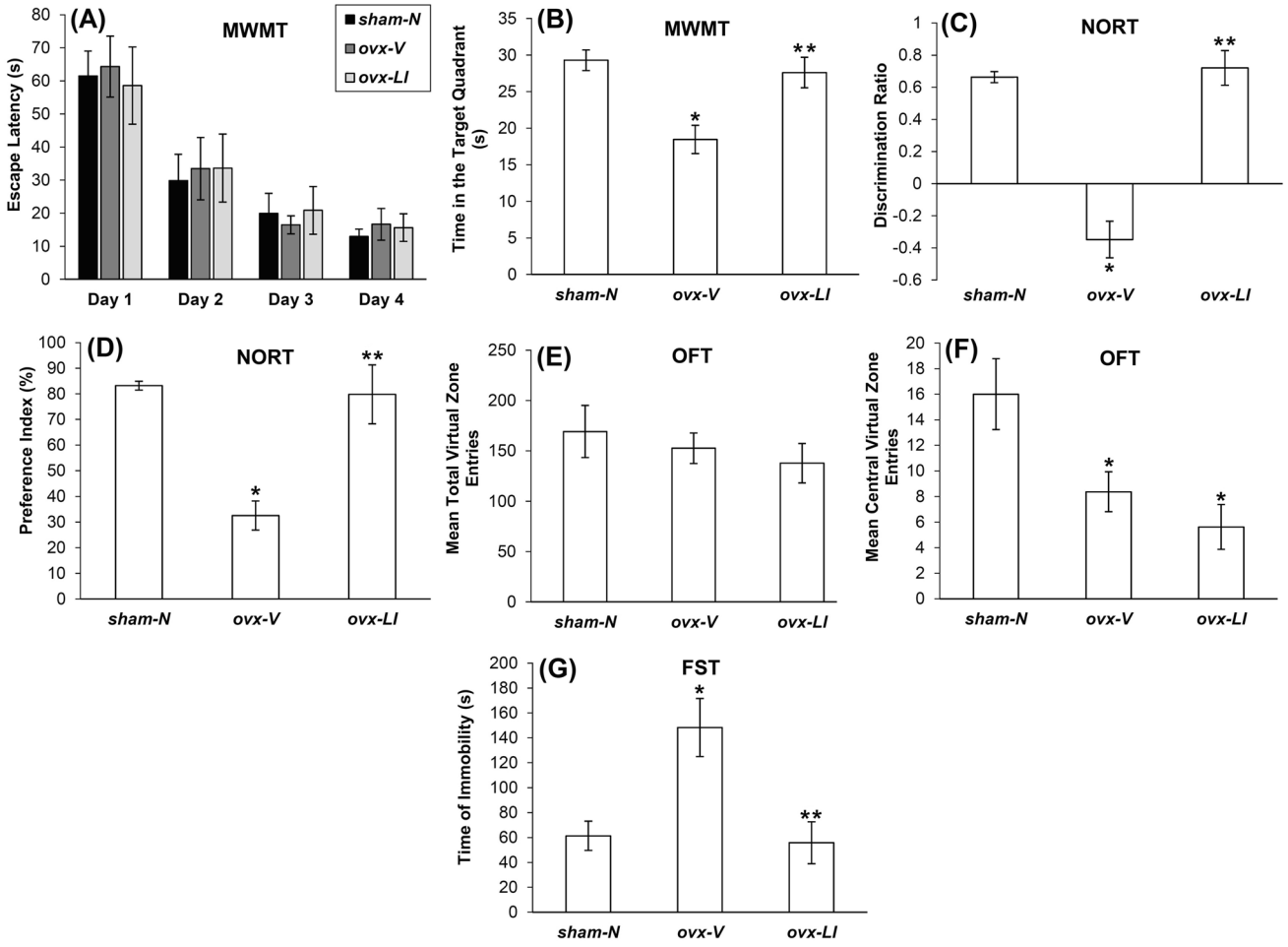

Fig. 2 Effect of lithium treatment on neurobehaviour impairment in *ovx* rat. (**A**): Average escape latency from day 1 to day 4 in MWMT; (**B**): Probe trial on day 5 to assess the time spent in the target quadrant in MWMT; (**C**) and (**D**): Recognition memory analysis by calculating the discrimination ration and percentage of preference index by NORT; (**E**): Locomotion analysis (Mean total virtual zone entries) in OFT; (**F**): Anxiety-like behaviour analysis (Mean central square entries) in OFT; and (**G**): Depression-like behaviour by recording immobility time in FST. *$P < 0.05$ as compared to *sham-N*; **$P < 0.05$ as compared to *ovx-V*. ***sham-N***: Group subject to sham surgery with normal chow diet; ***ovx-V***: Group subject to ovariectomy with normal chow diet; ***ovx-LI***: Group subject to ovariectomy with lithium chow diet; **MWMT**: Morris water maze test; **NORT**: Novel object recognition test; **OFT**: Open field test; **FST**: Forced swimming test; and **s**: Seconds

appearance of depression-like behaviour in vehicle control group following *ovx*. However, lithium treatment ameliorated depression-like behaviour, as a significant decrease ($P < 0.05$) in the immobility period was observed in *ovx-LI* group in comparison with *ovx-V* group. The results of cognitive-behaviour tests suggested that *ovx* leads to the alteration in the neurobehaviour function, while lithium treatment rescued the rats from these abnormalities.

Lithium improved neuronal population and maintained pyramidal neuron architecture

Nissl staining was performed to evaluate the neuronal morphology in all the selected regions of the brain. The somatosensory cortex and CA1 region of the hippocampus in *ovx-V* group showed darker neuron population when studied using Nissl staining. A significant ($P < 0.05$) reduction in dark neuron density was observed in the regions following lithium treatment in the *ovx-LI* group compared to *ovx-V* group. However, the hippocampal CA3 region did not show any significant change in the dark neuron population among all the experimental groups. The prefrontal cortex of the *ovx-V* group also showed a higher density of Nissl-stained neurons, which significantly ($P < 0.05$) reduced post-lithium treatment in the *ovx-LI* group (Fig. 3A).

NeuN is a protein marker used to define mature neuron population. The expression studies of NeuN validated the results of Nissl staining in our study. There was a significant decrease ($P < 0.05$) in NeuN positive cells reactivity in the somatosensory cortex, CA1 region of the hippocampus, and the prefrontal cortex of *ovx-V* group compared to *sham-N* group. The group treated with lithium showed a significant increase ($P < 0.05$) in NeuN reactivity compared to *ovx-V* group (Fig. 3B). However, insignificant change in NeuN

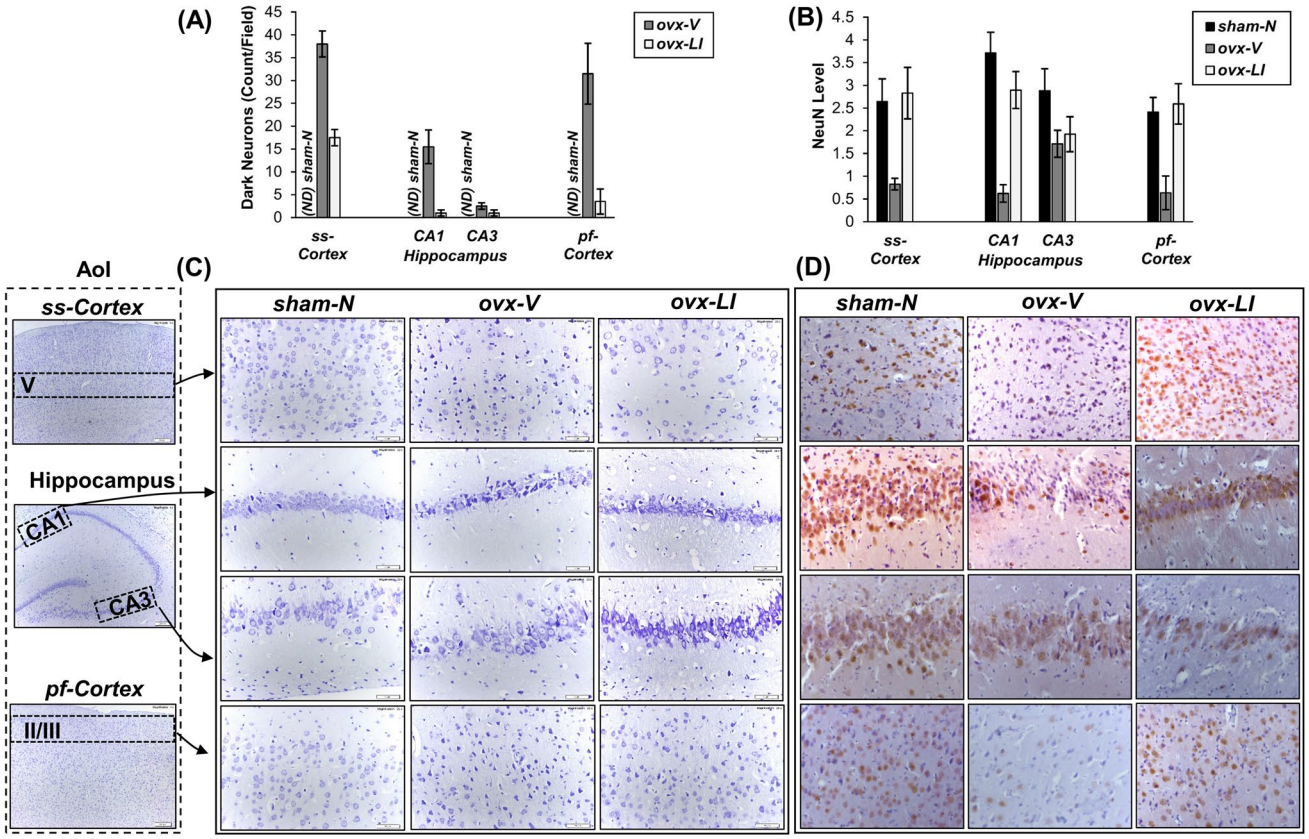

Fig. 3 Histopathological analysis to assess the effect of lithium treatment on neuronal population. (**A**): Analysis of Nissl-stained dark neurons in the *ss-cortex*, *hippocampus* (*CA1* and *CA3* regions) and *pf-cortex*; (**B**): Analysis of NeuN protein expression as DAB-stained area (%) in the *ss-cortex*, *hippocampus* (*CA1* and *CA3* regions) and *pf-cortex*; (**C**): Representative image of Nissl-stained dark neurons in the *ss-cortex*, *hippocampus* (*CA1* and *CA3* regions) and *pf-cortex* (Scale bar 50 µm); and (**D**): Representative image of NeuN protein expression in the *ss-cortex*, *hippocampus* (*CA1* and *CA3* regions) and *pf-cortex* (Scale bar 100 µm). *$P < 0.05$ as compared to *sham-N*; **$P < 0.05$ as compared to *ovx-V*. ***sham-N***: Group subject to sham surgery with normal chow diet; ***ovx-V***: Group subject to ovariectomy with normal chow diet; ***ovx-LI***: Group subject to ovariectomy with lithium chow diet; ***ND***: No dark neuron; ***ss-Cortex***: Somatosensory cortex; ***CA1***: *Cornu Ammonis 1; CA3*: *Cornu Ammonis* 3; ***pf-cortex***: Prefrontal cortex; and **AoI:** Area of interest. The average number of dark neurons/ fields observed in the *sham-N* group subtracted from rest of other section to avoid the artefacts, hence represented as *ND*

expression was observed in the CA3 region among all the studied groups.

Morphological analysis of pyramidal neurons was also carried out to understand the effect of lithium on dendritic arborization. The effect was studied on the both apical and basal dendrite of the pyramidal neurons. There was a significant decrease ($P < 0.05$) in total apical and basal dendritic length in all the three regions of the brain in *ovx-V* group compared to *sham-N* group. Lithium treatment significantly increased the dendritic length in *ovx-LI* group in comparison with *ovx-V* group; however, no significant change was observed in the apical dendrite of the somatosensory cortex following the lithium treatment in the *ovx-LI* group (Fig. 4A). Furthermore, a significant ($P < 0.05$) reduction in average dendritic length of apical dendrite was also observed in *ovx-LI* group compared to *ovx-V* group in all the three regions of the brain. However, insignificant change in the average dendritic length of basal dendrite was observed among all the studied groups (Fig. 4B). The number of nodes and branch termination points was also significantly ($P < 0.05$) increased in both the apical and basal dendrite following lithium treatment in *ovx-LI* group in comparison with *ovx-V* group (Fig. 4C and D).

In this study, dendritic bifurcation and spine density of pyramidal neuron were also quantified, as decrease in the both the processes interferes with neuronal signalling that results in development of neurobehavioural impairment. There was a significant ($P < 0.05$) decrease in dendritic bifurcation of apical, as well as basal dendrite in all the studied brain regions in *ovx-V* group compared to *sham-N* group. However, lithium treatment showed a significant increased bifurcation at both the sections ($P < 0.05$) in *ovx-LI* group as compared to *ovx-V* group (Fig. 5B-D). Similarly, to the dendritic bifurcation, the *ovx-V* group also showed a significant ($P < 0.05$) decrease in the spine density of the apical and basal dendrite in all the three regions of brain compared with the *sham-N* group. However, lithium treatment showed a significant ($P < 0.05$) recovery in spine number of apical and basal dendrites in *ovx-LI* group when compared to *ovx-V* group. However, insignificant change in spine density was observed between *ovx-LI* and *ovx-N* groups (Fig. 6). The results suggested that lithium treatment increased dendritic bifurcation and spine number recovery that might be responsible for improvement in neurobehavioural functions.

Lithium downregulated the expression of pro-inflammatory mRNA

A significant ($P < 0.05$) upregulation in the mRNA expression of pro-inflammatory markers in the discrete brain regions was observed in *ovx-V* group in comparison with *sham-N* group. The results revealed that lithium treatment in *ovx-LI* group after a chronic period of *ovx* significantly ($P < 0.05$) downregulated the mRNA expression of *Il2*, *Il6,* and *Il1b* genes in all the three studied regions of the brain in contrast to *ovx-V* group (Fig. 7A).

Lithium improved neuroinflammation via reactive astrogliosis suppression

Gfap is a marker of reactive astrogliosis that is upregulated in several neurodegenerative conditions and triggers a variety of neuroinflammatory events. The results showed a significant ($P < 0.05$) upregulation of *Gfap* expression in all the three regions of the brain in *ovx-V* group, when compared with *sham-N* group (Fig. 7B). However, lithium treatment downregulated its expression in the *ovx-LI* group when compared with *ovx-V* group. On the contrary, lithium treatment in *ovx-LI* group showed a significant ($P < 0.05$) upregulation of *Pparg* expression ($P < 0.05$) in all the three regions of the brain in comparison with *ovx-V* group. The expression of *Pparg* was significantly ($P < 0.05$) reduced in *ovx-V* group as compared to *sham-N* group in all the studied regions (Fig. 7B).

Further to confirm reactive astrogliosis, morphological analysis of the GFAP positive cells was performed. There was a significant increase ($P < 0.05$) in total astrocyte process length, average astrocyte process length, and number of the nodes in *ovx-V* group in all the three studied regions compared to *sham-N* group (Fig. 8(B1-B3, C1-C3, and D1-D3). Interestingly, lithium treatment in *ovx-LI* group significantly ($P < 0.05$) attenuated the observed changes as compared to *ovx-V* group. Increased astrocyte process bifurcation is considered as a characteristic feature of reactive astrogliosis. In this study, reactive astrogliosis was studied by using concentric ring method of Sholl [24]. A significant ($P < 0.05$) increase in the astrocyte process bifurcation was observed in all the three brain regions of *ovx-V* group compared to *sham-N* group (Fig. 8(B 4, C 4, and D 4). The astrocyte process bifurcation was normalized following the lithium treatment in *ovx-LI* group as a significant ($P < 0.05$) decrease is observed in comparison with *ovx-V* group. The results suggested lithium to be a potential agent to overcome reactive astrogliosis and to maintain homeostasis in the brain following *ovx*.

Lithium maintained the BBB integrity by upregulation of *Ocln* and *Tjp1* expression

Increase in the BBB permeability is an indicator of neurodegenerative events in various neurological conditions. The BBB strength depends on the expression of junction proteins, which has been reported to decline following oestrogen deprivation [33]. In the present study, a significant ($P < 0.05$) downregulation in the mRNA expression of *Ocln* and *Tjp1* was observed in all the studied regions in *ovx-V*

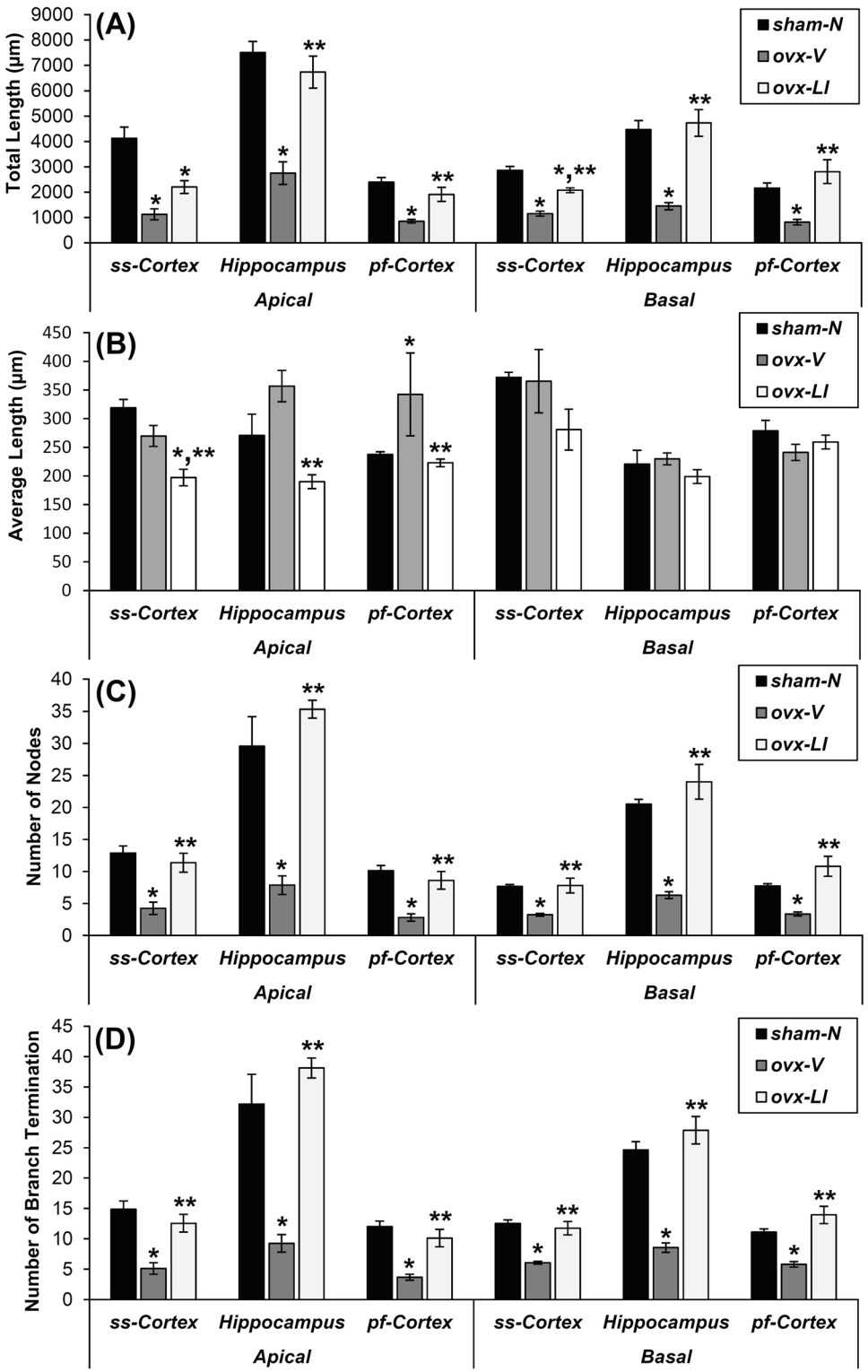

Fig. 4 Effect of lithium treatment on pyramidal neuron morphology. (**A**): Quantification of total dendritic length of apical and basal pyramidal neuron in the *ss-cortex*, *hippocampus,* and *pf-cortex*; (**B**): Quantification of average dendritic length of apical and basal pyramidal neuron in the *ss-cortex*, *hippocampus,* and *pf-cortex;* (**C**): Quantification of number of nodes in the apical and basal dendrite of pyramidal neurons with consecutive 20 µm spaced concentric ring in the *ss-cortex*, *hippocampus,* and *pf-cortex;* and (**D**): Quantification of number of branch termination in the apical and basal dendrite of pyramidal neurons with consecutive 20 µm spaced concentric ring in the *ss-cortex*, *hippocampus,* and *pf-cortex*

group compared with *sham-N* group (Fig. 7C). However, lithium treatment significantly upregulated the expression of both the genes in the hippocampus, somatosensory cortex, and prefrontal cortex of *ovx-LI* group compared to *ovx-V* and *sham-N* groups.

Lithium treatment attenuated ovariectomy-induced alteration in proteins expression

Gsk-3β has been well-reported to be involved in numerous of neurobehavioural conditions [20]. The results revealed

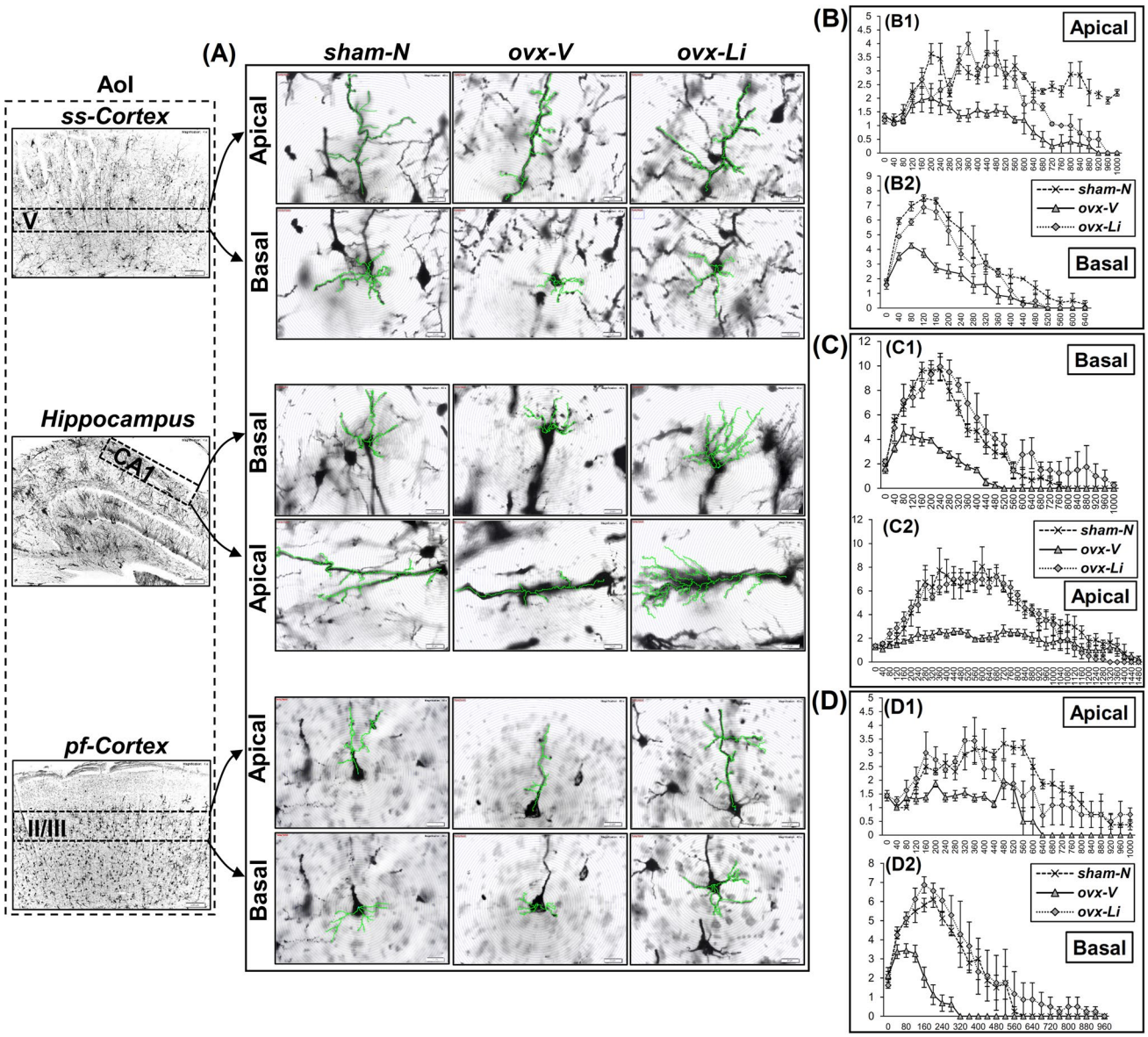

Fig. 5 Effect of the lithium treatment on the dendritic arborization. (**A**): Representative image of apical and basal traced neurite over Golgi-Cox-stained neurons micrograph of *ss-cortex*, *hippocampus* (CA1 regions), and *pf-cortex* under 40X magnification; Scale bar 20 µm; (**B, C, and D**): Quantification of apical and basal dendritic bifurcation of pyramidal neurons in the *ss-cortex*, *hippocampus* (*CA3* regions), and *pf-cortex*; (**B1, C2, and D1**): Number of apical dendritic intersection of pyramidal neurons with consecutive 20 µm spaced concentric ring; (**B2, C1, and D2**): Number of basal dendritic intersection of pyramidal neurons with consecutive 20 µm spaced concentric ring. *$P < 0.05$ as compared to *sham-N*; **$P < 0.05$ as compared to *ovx-V*. ***sham-N***: Group subject to sham surgery with normal chow diet; ***ovx-V***: Group subject to ovariectomy with normal chow diet; ***ovx-LI***: Group subject to ovariectomy with lithium chow diet; ***ss-Cortex***: Somatosensory cortex; ***pf-cortex***: Prefrontal cortex; and **AoI:** Area of interest. X-axis in the Fig. 5(B1, B2, C1, C2, D1, and D2) represent distance from the nucleus (µm), whereas Y-axis represents number of intersections

an insignificant change in total Gsk-3β level in all the three studied brain regions among different groups. However, a significant ($P < 0.05$) reduction in p-Gsk-3β(Ser^9) was observed in the somatosensory cortical region of *ovx-V* group compared with *sham-N* group, thus suggested activation of Gsk-3β following *ovx*. Lithium treatment in *ovx-LI* group significantly ($P < 0.05$) upregulated the somatosensory cortical expression of p-Gsk-3β(Ser^9) in comparison with *ovx-V* group (Fig. 9A1). On the contrary, the hippocampus and the prefrontal cortex showed a significant upregulation of p-Gsk-3β(Ser^9) in *ovx-V* group ($P < 0.05$) compared to *sham-N* group. Interestingly, there was no significant change in p-Gsk-3β(Ser^9) expression observed in both the regions following the lithium

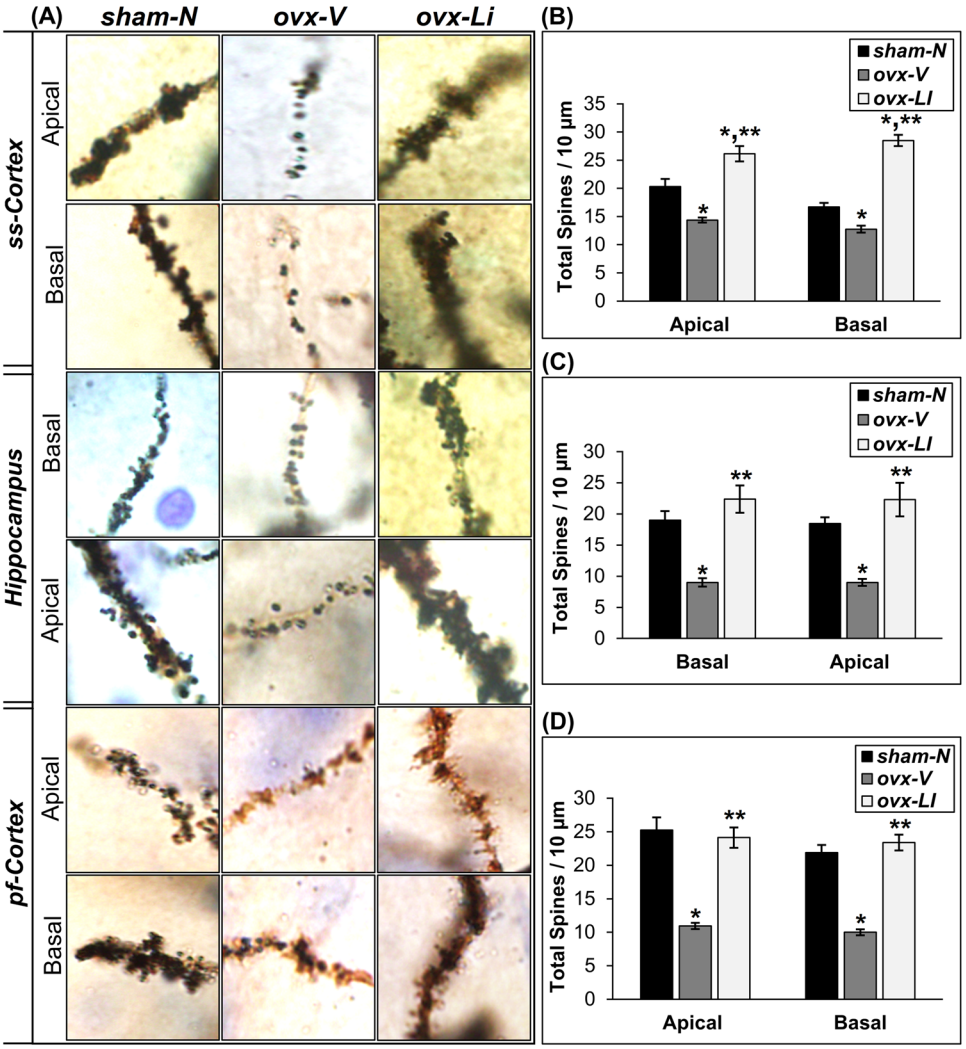

Fig. 6 Effect of lithium treatment on the spine density. (**A**): Representative image of dendritic spine of apical and basal dendrite of pyramidal neuron in the *ss-cortex*, *hippocampus* (CA1 regions), and *pf-cortex* under 100X magnification (oil immersion); Scale bar 10 µm. Dendritic segments of 10 µm length from second order of branching were included in the study. (**B**, **C,** and **D**): Quantification of total spine/10 µm in the apical and basal dendritic of pyramidal neurons from *ss-cortex*, *hippocampus,* and *pf-cortex*, respectively. *P < 0.05 as compared to *sham-N*; **P < 0.05 as compared to *ovx-V*; ***sham-N***: Group subject to sham surgery with normal chow diet; ***ovx-V***: Group subject to ovariectomy with normal chow diet; ***ovx-LI***: Group subject to ovariectomy with lithium chow diet; ***ss-Cortex***: Somatosensory cortex; and ***pf-cortex***: Prefrontal cortex

treatment in *ovx-LI* group compared to *ovx-V* group (Fig. 9B1 and C1).

β-catenin is a major component of Wnt/β-catenin signalling that triggers proteasomal degradation following activation of Gsk-3β. In the present study, the somatosensory cortical region of *ovx-V* group showed a significant (P < 0.05) downregulation of β-catenin expression compared to *sham-N* group. However, lithium treatment significantly (P < 0.05) upregulated β-catenin expression in *ovx-LI* group in comparison with *ovx-V* group (Fig. 9A1). Surprisingly, the hippocampus and the prefrontal cortex did not show variation in the expression of β-catenin in any of the experimental group (Fig. 9B1 and C1).

mTOR is also an indirect downstream target of Gsk-3β. The selected brain regions did not show any significant change in total mTOR protein expression among different experimental groups. Phosphorylation of mTOR on serine^{2448} residue is an essential step for the further signalling cascade. The somatosensory cortical region of *ovx-V* group showed a significant (P < 0.05) downregulation of p-mTOR (Ser^{2448}) compared to *sham-N* group. Lithium treatment in *ovx-LI* group significantly (P < 0.05) upregulated p-mTOR (Ser^{2448}) expression when compared *ovx-V* group (Fig. 9A1). Interestingly, the hippocampus and the prefrontal cortex showed a significant upregulation (P < 0.05) of p-mTOR (Ser^{2448}) in *ovx-V* group as compared to the *sham-N* group. However, lithium treatment did not show any significant change in p-mTOR (Ser^{2448}) protein expression in *ovx-LI* group when compared with *ovx-V* group. There was significant increase for the expression of same observed in *ovx-LI* group as compared to *sham-N* group (Fig. 9B1 and C1).

There was insignificant change observed in the level of total CREB protein in the studied brain regions among different experimental groups. Further, we studied p-CREB (Ser^{133}) protein expression to understand the downstream signalling cascade of CREB signalling pathway. The prefrontal cortex of *ovx-V* group showed a significant (P < 0.05) downregulation of p-CREB (Ser^{133}) expression compared to *sham-N* group. The level was restored to normal following

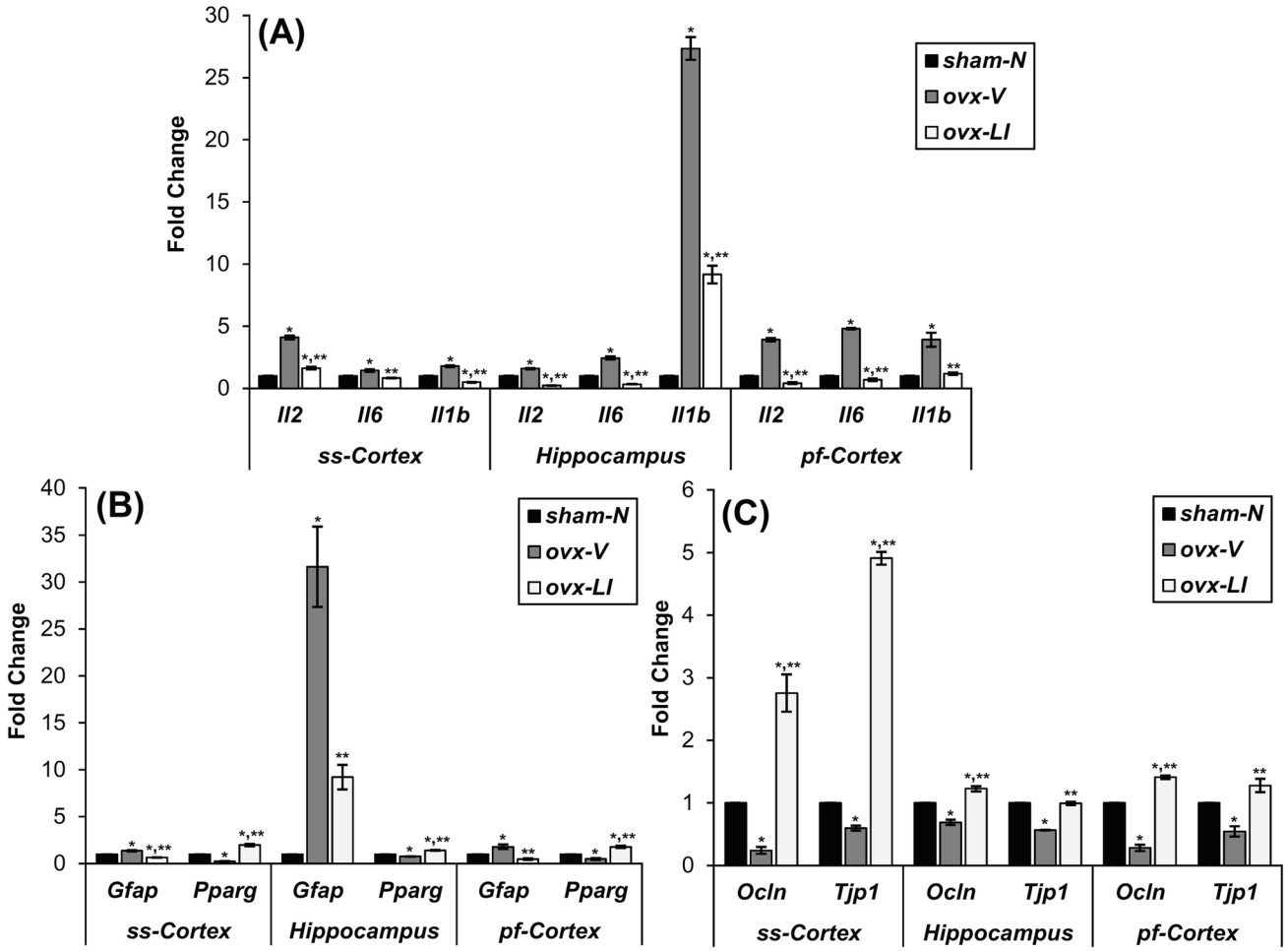

Fig. 7 Effect of lithium treatment on the messenger ribonucleic acid expression of targeted genes. (A): Expression of pro-inflammatory related genes (*ll2*, *ll6,* and *ll1b*) in the *ss-cortex*, *hippocampus,* and *pf-cortex*; (**B**): Expression of reactive astrogliosis-related genes (*Gfap* and *Pparg*) in the *ss-cortex*, *hippocampus,* and *pf-cortex;* and (**C**): Expression of junction protein-related genes (*Ocln* and *Tjp1*) in the *ss-cortex*, *hippocampus,* and *pf-cortex*. *P < 0.05 as compared to *sham-N*; **P < 0.05 as compared to *ovx-V*. ***sham-N***: Group subject to sham surgery with normal chow diet; ***ovx-V***: Group subject to ovariectomy with normal chow diet; ***ovx-LI***: Group subject to ovariectomy with lithium chow diet; ***ll2***: Interleukin 2, ***ll6:*** Interleukin 6; ***ll1b***: Interleukin 1 beta; ***Gfap***: Glial fibrillary acidic protein; ***Pparg***: Peroxisome proliferator-activated receptor gamma; ***Ocln***: Occludin; ***Tjp1***: Tight junction protein 1; ***ss-cortex***: Somatosensory cortex; and ***pf-cortex***: Prefrontal cortex

lithium treatment in *ovx-LI* group and significantly increased in comparison with *ovx-V* group (Fig. 9C1). However, no change in the expression of p-CREB (Ser^{133}) was observed in the somatosensory cortex and the hippocampus regions of brain among different experimental groups (Fig. 9A1 and B1). Further, the expression of total BDNF was also analysed, as its promoter consists of CREB response element. There was a significant downregulation ($P < 0.05$) of total BDNF protein expression in the hippocampus and the prefrontal cortex of *ovx-V* group, when compared with *sham-N* group. However, lithium treatment in *ovx-LI* group significant ($P < 0.05$) increased BDNF level compared to *ovx-V* group (Fig. 9B1 and C1). However, insignificant change in the level of BDNF was observed in the somatosensory cortex of all the experimental groups (Fig. 9A1).

Discussion

Alteration in the neuronal circuit following menopause is associated with a variety of cognitive and behaviour impairments. The present study explored the defensive effect of lithium treatment to overcome neurobehavioural abnormalities in *ovx* rats, a preclinical model that mimics clinical post-menopausal conditions. The results showed that chronic lithium treatment following *ovx* sustained the structural synaptic plasticity via maintaining the normal polarization state of glial cells, reducing neuroinflammation, and preventing BBB permeability.

Due to increased average life expectancy, one-third age of the majority of women is impacted with menopause. The two ways of seizing the menstrual cycle are either by

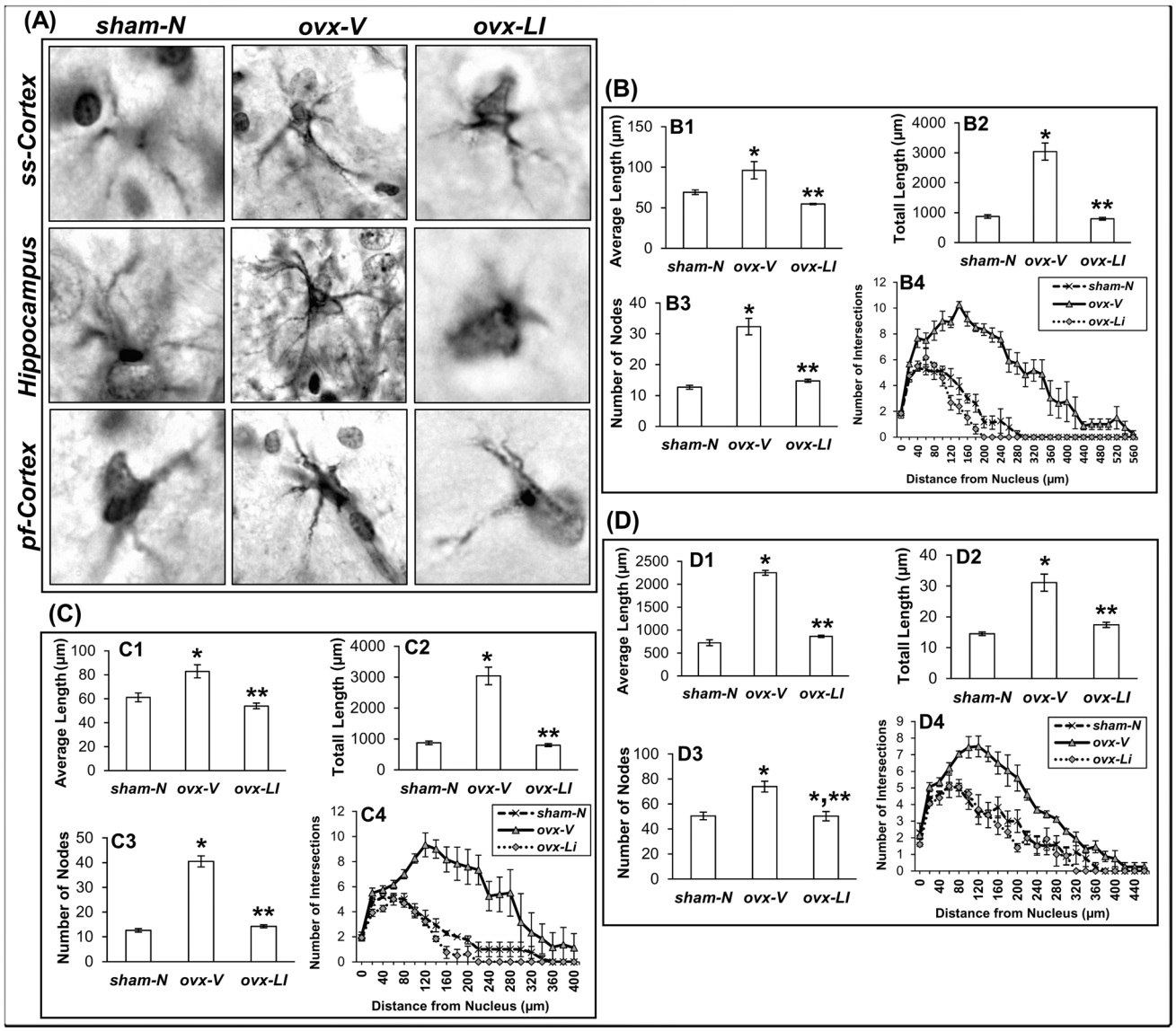

Fig. 8 Effect of lithium treatment on the reactive astrogliosis. (**A**): Representative image of GFAP- DAB-stained micrograph of *ss-cortex*, *hippocampus* (*CA1* regions), and *pf-cortex* under 100X magnification, Scale bar 10 µm; (**B-D**): Quantification of reactive astrogliosis in the *ss-cortex*, CA1 region of the *hippocampus,* and *pf-cortex*; (**B1, C1, and D1**): Average astrocytic process length in (µm); (**B2, C2, and D2**): Total astrocytic process length in (µm); (**B3, C3, and D3**): Numbers of nodes by concentric ring method; and (**B4, C4, and D4**): Number of intersections of astrocytic process with consecutive 4 µm spaced concentric ring. *P < 0.05 as compared to *sham-N*; **P < 0.05 as compared to *ovx-V*. ***sham-N***: Group subject to sham surgery with normal chow diet; ***ovx-V***: Group subject to ovariectomy with normal chow diet; ***ovx-LI***: Group subject to ovariectomy with lithium chow diet; **GFAP**: Glial fibrillary acidic protein; ***ss-Cortex***: Somatosensory cortex; and ***pf-cortex***: Prefrontal cortex

surgical removal of ovaries in women to treat the reproductive organ-associated complications or through the natural biological process. Deficiency of ovarian hormone following menopause is reported to interfere with normal cognition and behavioural processes [34]. Depression and anxiety are the major psychiatric conditions observed in women following menopause that negatively impact the inflicted person and their quality of life. Deficiency in the sexual hormone creates instability in the monoaminergic neurotransmitter, exposing the female to psychiatric conditions [35]. FST and OFT were performed in the present study to study the *ovx*-associated depression-like and anxiety-like behaviour, respectively. In line with the previous findings, *ovx* increased immobility time in FST and decreased central square entries in OFT, indicating induction of behavioural alterations [3, 36] in the *ovx-V* group. Chronic activation or hyperactivation of the hypothalamic–pituitary–adrenal (HPA) axis is reported in depressed patients [37], which disrupts the

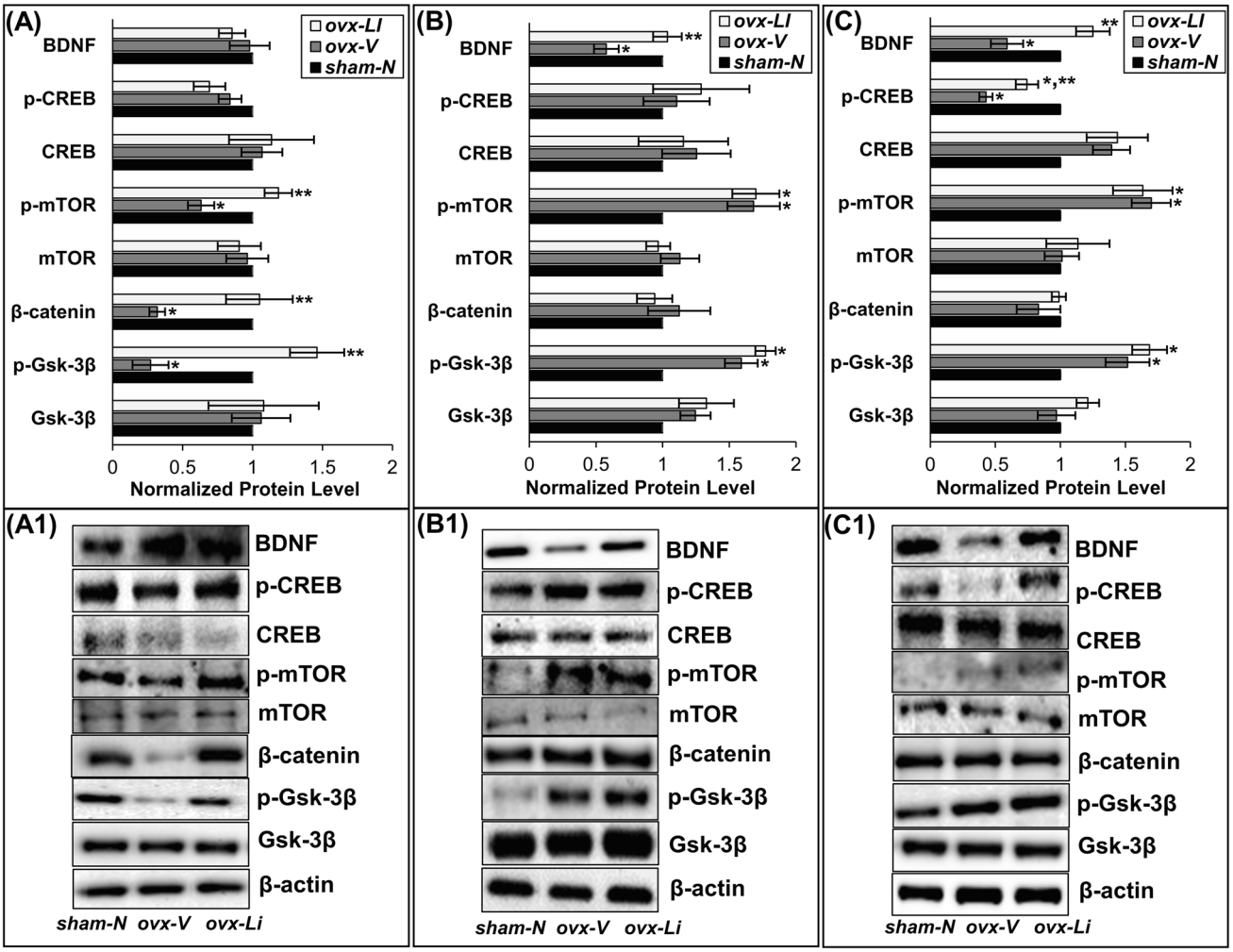

Fig. 9 Effect of lithium treatment on the protein expression. (**A, B,** and **C**): Graphic representation of relative fold change of protein expression relative to *sham-N* in the *ss-cortex*, *hippocampus,* and *pf-cortex.* The phosphorylated forms of p-CREB, p-mTOR, and p-Gsk-3β were normalized with their native, *i.e.* CREB, mTOR, and Gsk-3β, respectively, whereas all the native forms of the protein BDNF, CREB, mTOR, β-catenin, and Gsk-3 β were normalized with β-actin. (**A1, B1,** and **C1**): representative Western blotting image of BDNF, p-CREB, CREB, p-mTOR, m-TOR, β-catenin, p-Gsk-3β, Gsk-3β, and β-actin in the *ss-cortex*, *hippocampus,* and *pf-cortex*. *P < 0.05 as compared to *sham-N*; **P < 0.05 as compared to *ovx-V*. ***sham-N***: Group subject to sham surgery with normal chow diet; ***ovx-V***: Group subject to ovariectomy with normal chow diet; ***ovx-LI***: Group subject to ovariectomy with lithium chow diet; **BDNF**: Brain-derived neurotrophic factor; **p-CREB**: Phosphorylated cAMP-response element binding protein; **CREB:** cAMP-response element binding protein; **p-mTOR**: Phosphorylated mammalian target of rapamycin; **mTOR**: mammalian target of rapamycin; **β-catenin**: Beta catenin; **p-Gsk-3β**: Phosphorated glycogen synthase kinase-3 beta, **Gsk-3β:** Glycogen synthase kinase-3 beta**; β-actin**: Beta actin; ***ss-Cortex***: Somatosensory cortex; and ***pf-cortex***: Prefrontal cortex

synaptic plasticity via elevating the cortisol level [38]. Sexual hormones regulate the HPA axis via negative feedback, and during menopause, alterations in these hormones cause an increase in cortisol levels [39]. The hippocampus and the somatosensory cortical regions play a crucial role in the pathogenesis of depression. The dendritic atrophy with decreased spine count in the pyramidal neuron of the hippocampus and the prefrontal cortex is usually observed in depressive disorders [7, 40].

Spines are the major functional unit of neurons involved in the formation and maintenance of memory [40], and alteration in their number is associated with cognitive impairment. Thus, in the present study, the spatial memory functions of the animals were evaluated using MWMT to record consolidation and reconsolidation processes. Following the earlier findings, we did not observe any change in the consolidation process of spatial memory. Still, impairment in the reconsolidation process was recorded following *ovx* [3, 41] in *ovx-V* group. The pyramidal neurons in the CA3 region of the hippocampus participate in the consolidation of the spatial memory, whereas neurons of CA1 areas are involved in the reconsolidation process

[42]. Likewise, in the present study, we did not find any degenerative changes in the histopathological analysis of the CA3 region of the hippocampus. The observation could be a reason for the normal consolidation process of memory in the *ovx-V* group. However, degenerative neurons, alteration in the dendritic arborization, and spine number were observed in the CA1 region, which might be responsible for the impaired reconsolidation process in the *ovx-V* group. The degeneration and alteration of pyramidal neurons were also observed in the neocortical areas linked with non-spatial memory (recognition memory) impairments [43, 44]. The degenerative neurons, alteration in the dendritic arborization, and spine number in all the three studied regions in the *ovx-V* group further supported behavioural impairments. The results of the present study and literature findings supported the crucial role of the sexual hormone in maintaining the neuronal circuit, which seems to be disrupted following menopause.

Previous studies have proposed the elevated risk of neurobehaviour impairment, breast cancer, endometrium cancer, cerebral stroke, and cardiovascular disease in women after exogenous oestrogen therapy [14, 15, 45]. Hence, as an alternative therapeutic intervention over hormone replacement therapy and managing menopause-associated neurological conditions, lithium carbonate was selected in the present study, as it directly or indirectly regulates signalling cascades that are under the regulation of oestrogen [46]. Lithium salt is not only used to treat bipolar disorder, but it also showed promising effects in major depressive disorder, including treatment-resistant depression [44]. A preclinical study in a mouse model of chronic unpredicted stress supported an antidepressant effect of lithium salt and suggested its ability to instigate the firing rate of neurons in the brain's frontal cortex [44]. Lithium is often considered a multitargeted molecule that maintains neurotransmitters levels, elevates neurotrophic factors, controls apoptosis, and the second messenger system to overcome clinical depression [44, 47]. In line with the previous reports, we also observed the antidepressant activity of lithium in the *ovx* rats. However, no protection against anxiety-like behaviour in *ovx* rats was observed following treatment with lithium.

The pharmacological effect of lithium is not only limited to mood disorders, but it is also known to rescue the hippocampal-dependent memory formation via improving synaptic plasticity and adult neurogenesis [23]. Valdes et al. [46] showed that lithium treatment in *ovx* mice improved episodic memory through maintaining the normal mRNA expression of the genes related to synaptic plasticity such as *BDNF, BCL-2,* and *NR1* [46]. Similarly, lithium treatment in our study increased dendritic length, maintained dendritic arborization, and increased spine number, resulting in improved cognitive processes in the *ovx-LI* group. These shreds of evidence suggest that lithium therapy helps to keep the normal structural synaptic plasticity to overcome neurobehaviour impairments following *ovx*.

Neuroinflammation plays a central role in the pathogenesis of various neurological conditions, which eventually disrupts the neuronal circuit and triggers neurodegeneration cascades in the later stages of life [48]. Ding et al. [49] found that there is a metabolic transition in the brain from glucose to fatty acid following *ovx* to sustain the normal pool of ATP [49]. This transition is a type of adaption, but it also generates free radicals, leading to neuroinflammation [50]. Glucose deprivation alters the metabolic function of astrocytes, increases cell volume, and initiates transcription of genes that triggers the transformation of normal astrocytes to reactive astrocytes [51]. Activated astrocytes lose their normal functions and release neurotoxic substances that induce neuronal and oligodendrocytes death [52] via triggering the pro-inflammatory cytokine IL-6, IL-1β, and TNF-α expression in the cortex and the hippocampus following *ovx* [53]. The elevated pro-inflammatory cytokines stimulate the HPA axis [54] and result in the elevation of glucocorticoid level, which finally triggers neurodegenerative events. GFAP, an astrocyte intermediate filament, is reported to be upregulated during reactive astrogliosis in patients affected with bipolar disorder and is reverted to a normal level following lithium treatment [55]. Alteration in the physiological functioning of astrocytes also affects the normal synaptic signalling in bipolar disorder patients that is normalized after lithium therapy [56].

Apart from GFAP, peroxisome proliferator-activated receptor gamma (*Pparg*) is a nuclear receptor subfamily that controls the transformation of reactive astrogliosis and neuroinflammation [57]. Lithium treatment was reported to be associated with increased expression of *Pparg* and reactive astrogliosis perturbations via suppressing the activity of lysyl oxidase [58]. Further lithium treatment also helped to overcome the neuroinflammation via downregulating the expression of the pro-inflammatory marker in the different models of neurodegenerative disease [59, 60]. Similarly, in the present study, lithium treatment upregulated the *Pparg* expression. At the same time, it downregulated the expression of *Gfap*, *Il2*, *Il6*, and *Il1b,* reduced total astrocytic process length, and the number of nodes in the *ovx-LI* group, thus supporting the anti-inflammatory activity of lithium.

Deprivation of sexual hormones and neuroinflammatory events following menopause also enhances BBB permeability by altering tight junction proteins' expression [33]. These changes result in diapedeses of blood-derived inflammatory molecules towards the brain parenchyma that further participate in inflammation cascades [61]. Lithium treatment has been reported to improve the BBB permeability by maintaining normal expression of the junction proteins leading to antidepressant effect in a rat model [62]. Similarly, in

the present study, we also observed upregulation in mRNA expression of *Ocln* and *Tjp1*, improving neurobehaviour impairment in *ovx* rats following lithium treatment. Results of the current study and literature support the ability of lithium to improve and maintain the integrity of BBB after menopause. Hence, lithium treatment may decrease the risk of neurodegenerative disease in women following menopause.

The role of Gsk-3β has been well-explored in the induction of neurobehavioural impairments in the *ovx* rat model [3]. Furthermore, Gsk-3β instigation in the brain of *Dixdc1* KO mice induced depression-like behaviour and disrupted arborization of pyramidal neuronal in the cortex, which was improved after lithium administration [63]. Further, β-catenin downregulation in the *ovx-V* group confirmed the stimulation of Gsk-3β protein. Being a dual-nature protein, β-catenin appears to function as a cell adhesion molecule to maintain synaptic strength between neurons [64] and in gene transcription control associated with cell proliferation, maturation, and adult neurogenesis pathways [10]. A key component of the Wnt signalling pathway, β-catenin, is degraded after Gsk-3β activation [64], and decreased expression of the total β-catenin protein is reported in the brain of depressed patients [65]. However, lithium treatment maintained a normal protein level and relieved the depression-like behaviour [66], thus suggesting Gsk-3β inactivation.

Another important transcription regulator, a serine-threonine kinases mTOR, is primarily involved in the protein translation cascade, synaptic plasticity, axon sprouting, and myelination, also get inhibited following Gsk-3β activation [67, 68]. Chronic unpredicted stress-induced depression in mouse impaired mTOR signalling that was reverted to normal level following lithium therapy [69]. Alteration in mTOR activity interferes with the normal translation mechanism of synaptic proteins such as postsynaptic density (PSD95), synapsin 1, and GluR1, resulting in abnormal neuronal arborization that further leads to neurobehaviour impairments [70]. Surprisingly, in our study, Gsk-3β was found to be inactive in the hippocampus and the prefrontal cortex of the *ovx-V* group and remained at the same level following the lithium treatment. Activation of Gsk-3β is not only linked with neurodegeneration, but also contributes towards the long-term potentiation (LTP) and long-term depression (LTD) during memory processing [71]. An impaired reconsolidation process of spatial memory was observed following the inhibition of Gsk-3β in a mouse model, suggesting its role in memory reactivation [41]. In our study, lithium treatment rescued the reconsolidation process without activating Gsk-3β in the *ovx-LI* group animals.

Memory generation, maintenance, and storage are complex phenomena requiring synchronized involvement of various brain regions and complex interaction of signalling cascades [72, 73]. BDNF is a neurotrophic factor known for its involvement in learning and memory processes via maintaining the structural synaptic plasticity in the brain [73]. Depression-like behaviour following chronic-unpredicted stress in a mouse model showed downregulation in the BDNF signalling [44], upregulated following lithium treatment. Downregulation of BDNF signalling is also linked with structural synaptic plasticity distortion, which further impedes the hippocampus and the prefrontal cortex-dependent spatial and recognition memory reconsolidation process [74–76]. BDNF is a multifunctional protein that involves the memory reconsolidation process and regulates the mTOR-dependent translational cascade [77]. As previously reported, downregulation of BDNF expression in the hippocampus impairs the reconsolidation process, which was recovered after exogenous administration of the same protein [77]. Similarly, we also observed normalization of BDNF protein level and cognitive and behaviour process recovery following the lithium treatment.

Lithium is a drug of choice in psychiatric medicine, despite of some associated side effects that usually appear with improper monitoring. Chronic lithium treatment is linked with the development of nephrogenic diabetes insipidus [78]. Nausea, diarrhoea, tremor, and weight gain are common side effects associated with lithium therapy [79]. It has a narrow therapeutic index, and a slight rise in serum lithium level beyond therapeutic concentration (0.5 to 1.2 mM) leads to adverse effects [80]. Such consequences need to be taken into consideration while scheduling lithium therapy.

Conclusion

The present study revealed that lithium treatment prevented neurobehavioural impairments and improved structural synaptic plasticity following *ovx*. Neuroinflammatory events were also obscured following lithium treatment due to suppression of reactive gliosis and maintenance of the BBB strength. Lithium also regulated Gsk-3β activity, maintained a normal level of BDNF that ultimately helped to overcome neuroinflammation, and maintained the structural synaptic plasticity. Overall, the results supported the efficacy of lithium therapy to overcome the post-menopause-associated neurobehavioural complications after the critical therapeutic window period. However, in-depth research is still required to transfer the lithium from bench to bed in place of the hormone replacement therapy.

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Authors' contribution AKR performed the animal surgery, behaviour experiments, protein expression, histopathology, and wrote the manuscript. SS carried out the gene expression studies. VP analysed the histopathology data. DS conceptualized the idea, analysed the data, wrote, and edited the manuscript.

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Data availability The data generated during the course of study is included in the manuscript in the form of figures, images, and Supplementary Information (Supplement 1).

Declarations

Ethics approval The study protocol was duly approved by the Institutional Animal Ethics Committee (IAEC) of the CSIR-IHBT established by the CPCSEA, Ministry of Fisheries, Animal Husbandry and Dairying, Government of India.

Consent for publication The authors have provided the consent for publication in the journal Molecular Neurobiology.

Competing interests None declared.

Consent to participate Not applicable.

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