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Papaverine enhances autophagy, synaptic function, and memory in a mouse model of chronic REM sleep deprivation

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Abstract

Objectives: Cyclic nucleotides play a pivotal role in the establishment of synaptic plasticity which in turn facilitates the memory processes. Dysregulation of cyclic nucleotides due to increased phosphodiesterase (PDE) activity has been implicated in various neurodegenerative diseases. In this study, we investigated the potential effects of Papaverine (PAP), a PDE10A inhibitor, on cognitive dysfunction induced by chronic REM sleep deprivation in a mouse model.
Methods: The modified multiple platform method (MMPM) was used for the induction of chronic REM sleep deprivation. Morris water maze was used to access the cognitive functions, while cAMP level was quantified by ELISA technique. Through Western blot analysis, we evaluated the expression of PDE10A, amyloid beta, CREB, BDNF, NR2A, NR2B, Beclin-1, LC3B, and synaptic proteins.
Results: Administration of PAP ameliorated learning and memory deficits in mice subjected to chronic REM sleep deprivation. PAP increased cAMP and PSD-95, Synapsin, SAP97, pCREB, BDNF levels and decreased NR2A, and NR2B expression, along with the restoration of basal autophagy (Beclin-1, LC3B) in the hippocampal region of chronic REM sleep deprived mice. Increased cAMP and autophagy proteins are probably linked to the decrease in PDE10A and amyloid beta expression, respectively.
Conclusions: This study evidences that papaverine, a non-narcotic opium alkaloid and PDE10A inhibitor, can alleviate learning and memory impairments induced by chronic REM sleep deprivation.

Keywords: phosphodiesterase; cAMP; autophagy; chronic sleep deprivation; memory; papaverine

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Introduction

In mammals, rapid eye movement (REM) sleep and non-rapid eye movement (NREM) sleep are the categories of sleep states that have been distinguished. An increasing body of evidence indicates the protective nature of REM sleep in the learning and memory processes [1, 2]. In humans and experimental animals, REM sleep was shown to enhance the long-term consolidation of tasks involving visual discrimination, as well as spatial and emotional memories [3, 4]. In contrast, REM sleep deprivation following a learning task adversely affects the formation of spatial and environmental fear memories in mice [4]. Memory consolidation is a complex mechanism that transforms transient short-term memories into enduring long-term memories [5]. This process relies on neuronal plasticity, which involves the activation of transcription factors that control the transcription of genes of interest and the translation into proteins. Within this array of transcription factors, cyclic

AMP-responsive element binding protein (CREB) holds particular importance in memory consolidation [6, 7]. It should be noted that the cAMP-PKA-CREB pathway is precisely sensitive to sleep deprivation, indicating the intricate relationship between sleep and memory processes [8].

Rats and monkeys have relatively higher concentrations of phosphodiesterase 10 A (PDE10A), an isoform of the cAMP/cGMP phosphodiesterase family, in the striatum than in the hippocampus [9]. PDE10A hydrolyses both cAMP and cGMP, but the rate at which it hydrolyses cAMP is 20-fold higher than cGMP [10]. Hence, inhibition of PDE10A increases the levels of cAMP more than cGMP. Currently, several PDE10A inhibitors are in clinical trials for treating schizophrenia (clinicaltrial.gov), Huntington's disease (HD), and Parkinson's disease. Papaverine (PAP), a selective PDE10A inhibitor, improves social memory and executive function in the HD mouse model [11, 12] and increases synaptic protein expression in human neurons [13]. In this study, we investigated the effects of PDE10A inhibition via PAP treatment on long-term memory and the associated molecular mechanisms in mice subjected to chronic REM sleep deprivation.

Materials and methods

Reagents and antibodies

Animal husbandry

Adult male C57BL/6J mice (25–30 g) were purchased from a local vendor (M/s. Adita Biosys Private Limited, Tumakuru, Karnataka), and subsequently housed (4–6 per cage) in polypropylene cages in a controlled environment. The animal facility unit was well-ventilated (air cycle: 15–20 per hour), temperature-controlled (22 ± 3 °C and 40–65 % relative humidity), with the ability to regulate the light/dark cycle (12 LL:12 DD). The animals were given free access to food and water *ad libitum*. Post acclimatization, the experimental procedures were initiated.

Prior to initiation of any protocol, formal approval from the Institutional Animal Ethics Committee (IAEC), Central Animal Facility, JSS AHER, Mysuru, India, was obtained (JSSAHER/CPT/IAEC/014/2020). An animal experiment using a mouse model was carried out based on the following guidelines of the Institute of Laboratory Animal Resources (Guide for the Care and Use of Laboratory Animals, National Academic Press 1996; NIH publication number nos. 85–23, revised 1996).

Papaverine Sigma Aldrich (India), cAMP ELISA kit (Ann Arbor, MI, USA), Anti-PSD95 (sc-32290), Anti-Synapsin-I (sc-376623), Anti-SAP97 (sc-9961), Anti-BDNF (sc-65514), Anti-CREB (sc-377154), Anti-β-Amyloid (Aβ; sc-28365), Anti-PDE10A (sc-515023) were procured from Santa Cruz Biotechnology, CA, USA. Anti-Beclin (NB500-249), Anti LC3B (NB100-2220). Anti-NR2A (PPS012) and Anti-NR2B (PPS013) were procured from Novus Biologicals, USA. All other reagents and chemicals used were of analytical grade.

Induction of chronic REM sleep deprivation

As described elsewhere, a modified multi-platform method (MMPM) was used for chronic REM sleep deprivation in mice [8]. Animals were subjected to chronic REMD for 6 h per day (from 9:00 to 15:00 h) for a total duration of 21 days [14]. In this experimental method, the individual mouse was placed on a cylindrical platform (3 cm in diameter, 5 cm high), and the surface of the platform was kept 1 cm above the water surface. This method has been known to effectively eliminate REM sleep with a minimum reduction in NREM sleep [15]. All non-sleep-deprived (NSD) animals were housed in polypropylene cages in the same room. A schematic presentation of the experimental design is shown in Figure 1.

Groups and treatment

Mice were randomized (based on stratified body weight) to four groups viz–Non-sleep-deprived (NSD)+Vehicle 0.5 % carboxymethyl cellulose [CMC] treated; chronic REM sleep-deprived+Vehicle (chronic REMD+CMC); cREMD+PAP- Low dose (chronic REMD+2.5 mg/kg b.wt); chronic REMD+PAP–High dose (chronic REMD+10 mg/kg). Each group consisted of 10–12 animals, and either papaverine or CMC was administered daily via the intraperitoneal (i.p.) route for a duration of 21 days.

Morris water maze (MWM)

The MWM test was accomplished as described elsewhere [16], with minor modifications. Training was conducted for 5 days (experimental days 16–21) in a circular water tank, which has four equally divided quadrants. The hidden (escape) platform was positioned in the NW quadrant, 1 cm below the water. During the trial phase mouse was left in the drum from the SE quadrant near the edge of the tank and permitted to search the hidden platform. In case the mouse was incapable of tracing the platform within 90 s, it was directed towards the hidden platform. A probe test was conducted on the 6th day, escape platform to test the retention memory of the animal. The individual mouse swam in

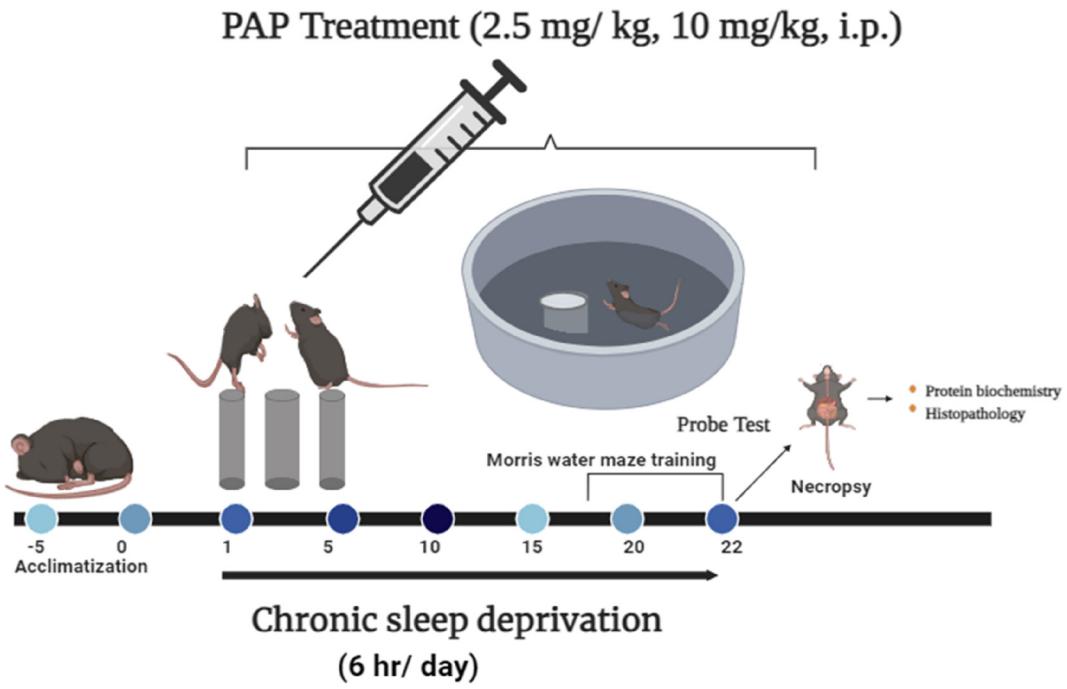

Figure 1: Schematic presentation of animal experiment.

the tank for 60 s; the assessment was video recorded and analysed through ANY-maze software (Version 7.1).

Immunoblotting

After conducting the behavioral assessment, the animals were euthanized, and their brains were carefully extracted and stored at a temperature of −80 °C. The hippocampal regions were isolated from the brain tissue and homogenized with radio immunoprecipitation assay (RIPA) buffer. The resulting homogenate samples were divided into aliquots and stored at −80 °C. The total protein content of these samples was quantified using the Pierce™ bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific, USA). Twenty five µg of protein was separated using a 12 % bis-tris protein gel through electrophoresis. To transfer the proteins from the gel to a PVDF membrane (Biorad, USA). The membranes were then blocked for an hour using 5 % skimmed milk. Later, the membranes were incubated overnight at 4 °C with primary antibodies targeting specific proteins, including PDE10A (1:1,000), Aβ (1:1,000), CREB (1:1,000), BDNF (1:1,000), PSD-95 (1:1,000), Synapsin (1:1,000), SAP 97 (1:1,000), Beclin (1:1,000), LC3B (1:1,000), NR2A (1:1,000), and NR2B (1:1,000). Afterward, the blots subjected to three washes of 10 min each with Tris-buffered saline with Tween (TBST) solution. Membranes were exposed to secondary antibodies (HRP-conjugated anti-mouse or anti-rabbit IgG) for a hour, the membranes were again washed three times with TBST for 10 min. The protein bands were visualized using the SuperSignal West Pico PLUS Chemiluminescent Substrate by Thermo Scientific, USA, and the semi-quantification of these bands was performed using the ImageJ software (NIH, USA; Research Resource Identifier: RRID: SCR_003070) [17].

Hippocampal cAMP estimation

Hippocampal cAMP levels were quantified through ELISA kit protocol (Cayman, MI, USA) [8].

Data analysis

Mean ± Standard error of the mean (SEM) was used to express the data values. GraphPad Prism 7.0 was utilized for statistical comparisons, which involved one-way analysis of variance and Tukey's multiple comparison tests. The threshold for statistical significance was set at $p \leq 0.05$.

Results

Papaverine protected the hippocampal-dependent spatial memory in chronic REM sleep-deprived mice

We used the MWM test to evaluate how chronic REMD affected hippocampus-dependent spatial memory. In contrast to vehicle-treated non-sleep deprived (NSD) mice, the chronic REMD mice had a higher delay to locate the hidden platform during the probing test (n=12, $p<0.01$), as illustrated in Figure 2. Additionally, compared to NSD mice, these mice travelled less distance ($p<0.01$), made fewer entries ($p<0.01$), and spent significantly less time ($p<0.05$) in the target quadrant. These results indicate that chronic REMD mice suffer from memory impairment. Administration of PAP in chronic REMD mice reduced the latency time (n=12, 2.5 mg/kg; $p<0.01$) to find the hidden platform, and increased the number of entries in the target quadrant

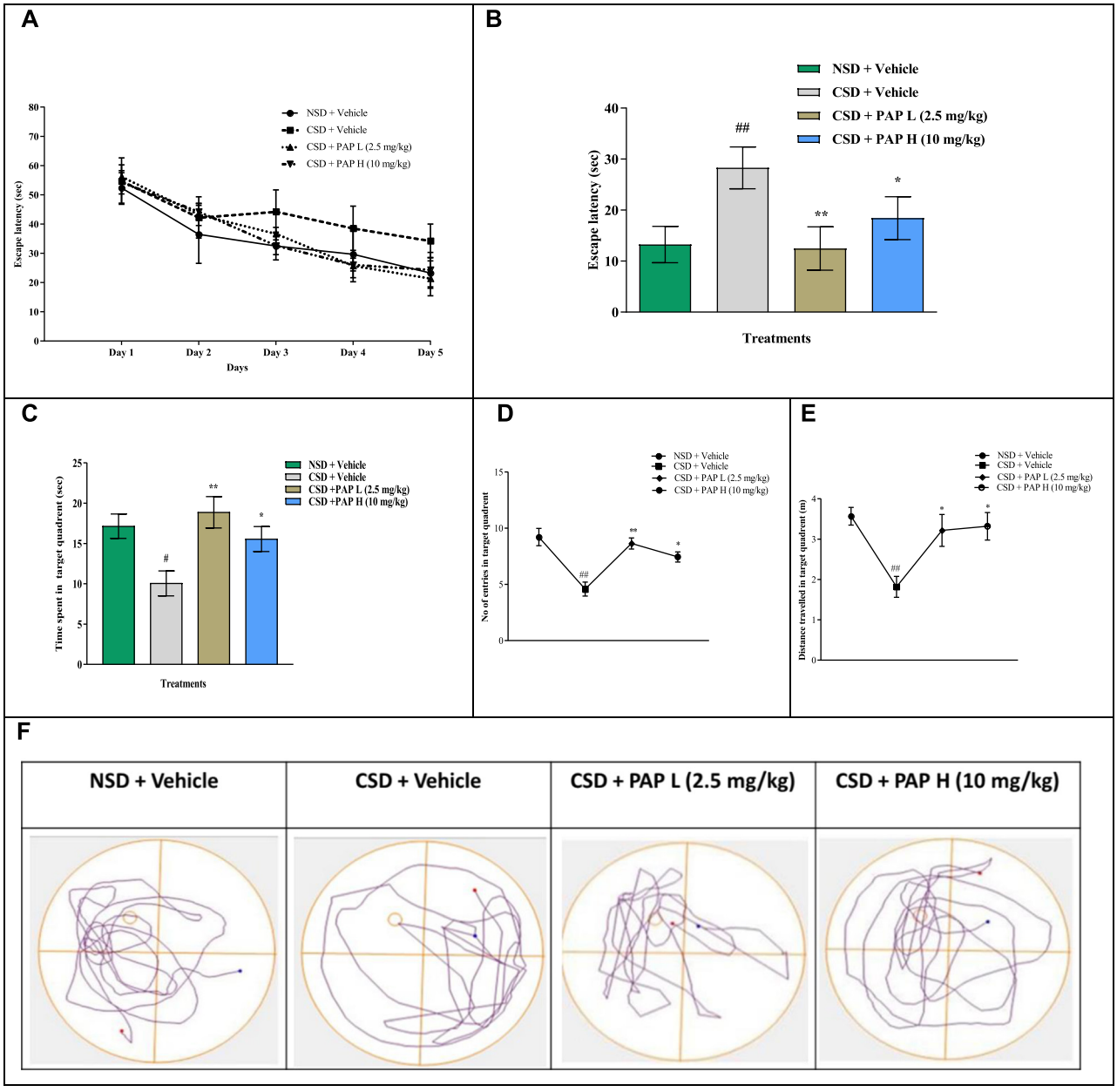

Figure 2: Papaverine administration restored hippocampal dependent spatial memory in cREMD mice (A) escape latency during training trials (B) escape latency in probe test (C) time spent in target quadrant in the probe test. (D) number of entries in target quadrant in probe test (E) distance travelled in target quadrant in probe test (F) swimming pattern track plot. Data are presented as mean ± SEM; # denotes $p<0.05$, ## denotes $p<0.01$ versus the vehicle-treated NSD group, * and ** denote $p<0.05$ and <0.01, respectively, versus the vehicle-treated cREMD group.

(2.5 mg/kg; p<0.01). PAP increased the time spent (2.5 mg/kg; p<0.01) and covered further distance in the target quadrant PAP (p<0.05) compared to the vehicle-treated chronic REMD group. Representative MWM track plots of the swimming across the platform in the probe trial are presented in Figure 2F.

Papaverine improved hippocampal cAMP levels in chronic REM sleep-deprived mice

Vehicle-treated chronic REMD mice exhibited a significant (*n*=8, p<0.01) reduction in cAMP levels compared with NSD mice. PAP significantly (10 mg/kg: p<0.001) increased the levels of cAMP in the hippocampus region of chronic REMD mice when compared with NSD mice (Figure 3).

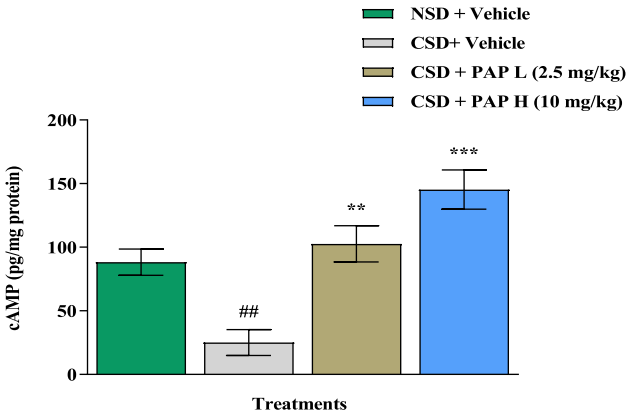

Figure 3: Papaverine treatment increased cAMP level in cREMD mice. Data are presented as mean ± SEM; ## denotes p<0.01 versus NSD mice, ** and *** denotes p<0.01 and 0.001, respectively, versus the vehicle-treated cREMD group.

Papaverine reduced PDE10A and Aβ expression in chronic REM sleep-deprived mice

Next, we investigated the influence of chronic REMD on PDE10A and Aβ expression in the hippocampus of experimental mice. The hippocampus of vehicle-treated chronic REMD mice exhibited a significant (p<0.05) increase in PDE10A expression compared to NSD mice. Administration of PAP in chronic REMD mice reduced (p<0.01) PDE10A expression compared with vehicle-treated chronic REMD mice (Figure 4A).

In addition, we also observed a significant (p<0.05) increase in Aβ expression in chronic REMD mice in contrast to NSD mice. Administration of PAP (10 mg/kg: p<0.01) reduced Aβ expression in chronic REMD mice in contrast to vehicle-treated chronic REMD mice (Figure 4B).

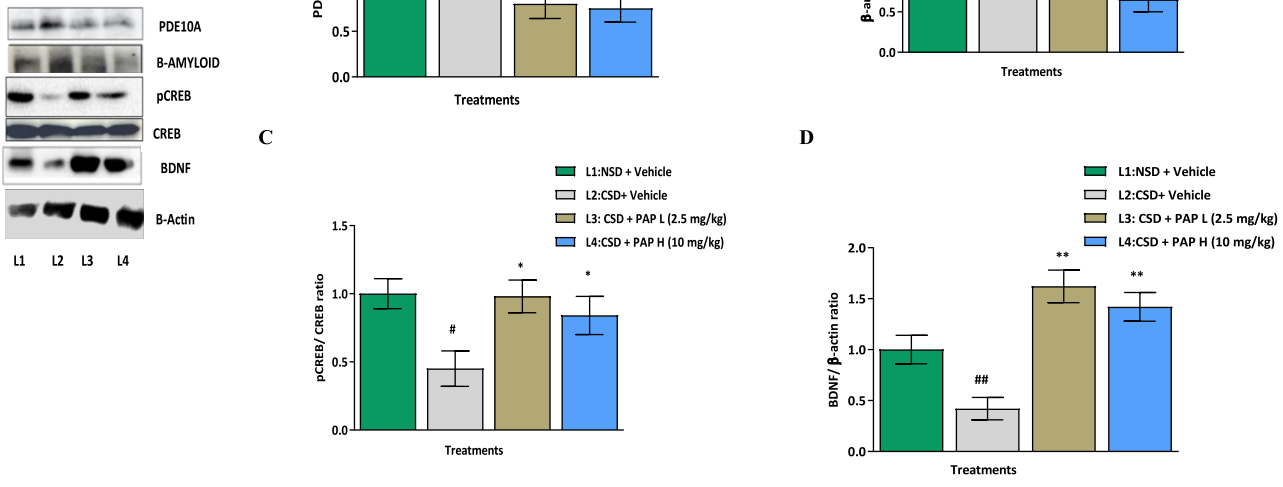

Figure 4: Papaverine administration reduced PDE10A, Aβ and improved BDNF and CREB expression in cREMD mice (A) PDE10A/β-actin expression (B) Aβ/β-actin expression (C) pCREB/TCREB expression (D)BDNF/β-actin expression. Data are presented as mean ± SEM, # denotes p<0.05, ## denotes p<0.01 versus vehicle-treated NSD group, * and ** denotes p<0.05, and p<0.01, respectively, versus vehicle-treated cREMD group.

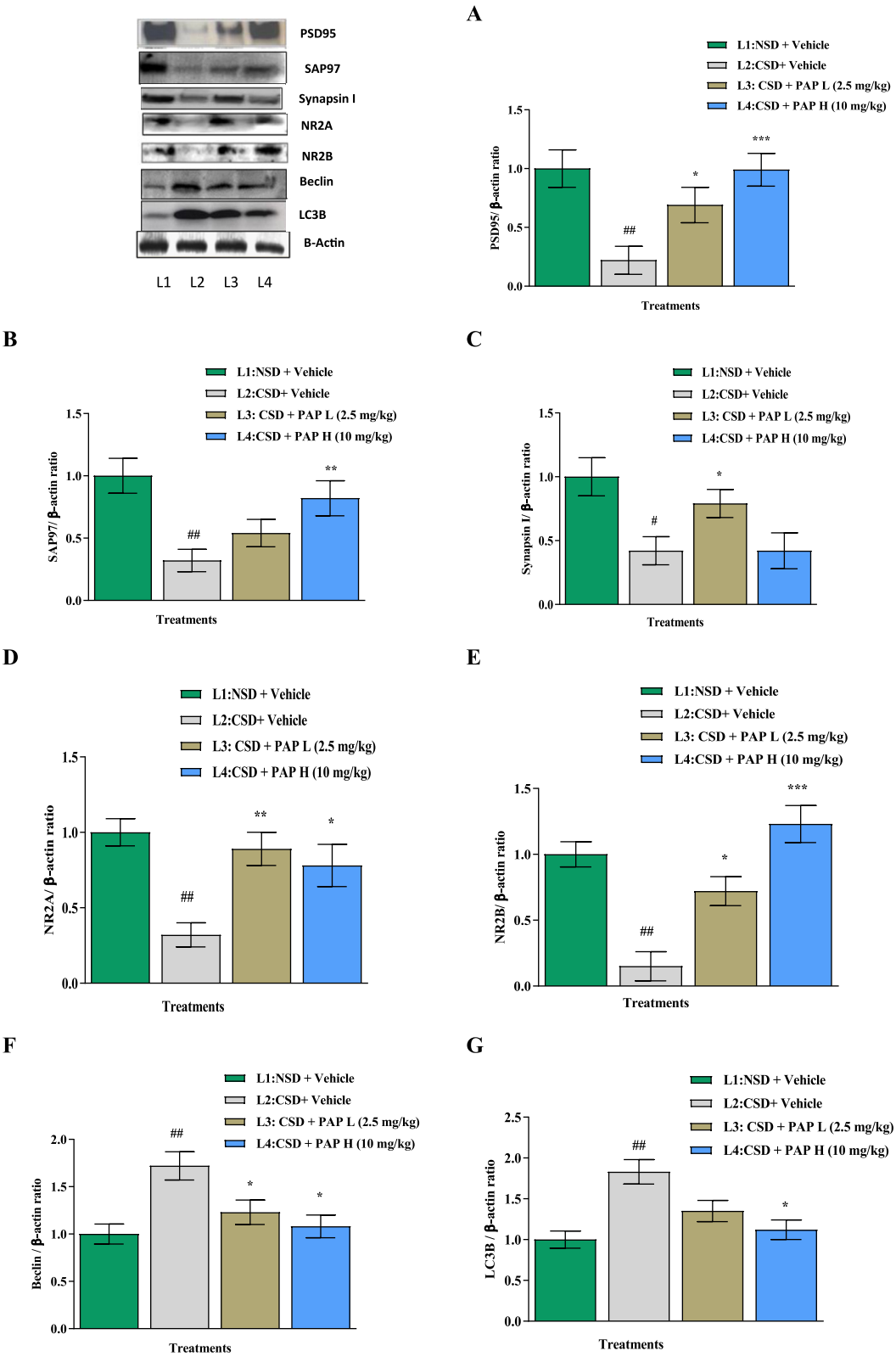

Figure 5: Inhibition of PDE10A by papaverine administration increased synaptic, NMDA receptor proteins expression and restored basal autophagy in cREMD mice (A) PSD95/β-actin expression (B) SAP97/β-actin expression (C) synapsin I/β-actin expression (D)NR2A/β-actin expression (E) NR2B/β-actin expression (F) beclin/β-actin expression (G) LC3B/β-actin expression. Data are presented as mean ± SEM, $^{\#}$ & $^{\#\#}$ denotes $p<0.05$, and <0.01, respectively, versus vehicle-treated NSD group, *, ** and *** denotes $p<0.05$, <0.01 and <0.001, respectively, versus vehicle-treated cREMD group.

Papaverine increased hippocampal CREB and BDNF expression in chronic REM sleep-deprived mice

In our study, Western blot analysis revealed a significant reduction in hippocampal pCREB ($p<0.05$) and BDNF ($p<0.01$) expression in chronic REMD mice compared to NSD mice. 21-day administration of PAP in chronic REMD mice improved the hippocampal expression of pCREB ($p<0.05$) and BDNF ($p<0.01$) in contrast to vehicle-treated chronic REMD mice (Figure 4C and D, respectively).

Papaverine upregulated synaptic proteins in the hippocampus region of chronic REM sleep-deprived mice

The results demonstrated that vehicle-treated chronic REMD mice exhibited a significant decrease in the expression of key synaptic proteins, namely PSD95 ($p<0.01$), SAP-97 ($p<0.01$), and Synapsin-I ($p<0.05$) (Figure 5A–C) when compared to the NSD group. The reduction in synaptic protein expression indicates synaptic dysfunction in the mice subjected to chronic REMD. Administration of PAP restored the expression of PSD95 ($p<0.001$), SAP97 ($p<0.01$), and Synapsin I ($p<0.05$) in the chronic REMD mice (Figure 5A–C).

Papaverine improved NMDA receptors (NR2A and NR2B) expression in the hippocampus region of chronic REM sleep-deprived mice.

Vehicle-treated chronic REMD mice showed a significant decrease in NR2A ($p<0.01$) and NR2B ($p<0.01$) expression compared to NSD mice. Chronic REMD mice treated with PAP significantly ($p<0.01$) showed increased NR2A expression in contrast with vehicle-treated chronic REMD mice. Administration of PAP in chronic REMD mice also showed a significant ($p<0.001$) increase in the expression of NR2B compared to vehicle-treated chronic REMD mice (Figure 5D and E).

Papaverine restored the autophagy process in the hippocampus of chronic REM sleep-deprived mice

Autophagy plays a pivotal role in synaptic plasticity and memory formation [18]. To explore the impact of chronic REMD on autophagy, we assessed the expression of beclin-1 and LC3B in the hippocampus region of the brain. chronic REMD mice exhibited a significant increase in beclin-1 ($p<0.01$) and LC3B ($p<0.01$) expression in the hippocampal region when compared with the NSD mice. PAP administration in chronic REMD mice was shown to reduce the expression of beclin-1 ($p<0.05$) and LC3B ($p<0.05$) when compared with vehicle-treated chronic REMD mice (Figure 5F and G).

Discussion

Increasing evidence indicates that chronic REMD is one of the major risk factors for neurodegenerative diseases [18–20]. Chronic REMD negatively impacts neural circuits, leading to disruptions in hippocampal-dependent learning and memory [22, 23]. However, the precise molecular changes triggered by chronic REMD that directly contribute to synaptic dysfunction and memory impairment remain unclear. In this study, we observed that 21 days of chronic REMD caused spatial memory deficits in mice. This memory decline was accompanied by increased PDE10A and Aβ expression, along with perturbed cAMP signalling, increased autophagy, and NMDA receptor expression in the hippocampal region of chronic REMD mice. Our findings showed that PDE10A inhibition improved cAMP, basal autophagy, NMDA receptors, and synaptic proteins expression in the hippocampal region of chronic REMD mice.

The hippocampus is regarded as the central structure for learning and memory processes. Chronic sleep deprivation disrupts the hippocampus structure, functions, and reduces blood flow, which results in neurocognitive deficits [24]. The chronic REMD studies in rodents have consistently shown that sleep loss disrupts the long-term storage of episodic and spatial memory [25, 26]. Chronic REMD has the biggest impact on memory consolidation as well as sleep deprivation for just 3 h, which commenced 1 h after the training session, impairs hippocampal long-term potentiation and disrupts neuronal connectivity [27]. Consistent with the previous reports, we also observed that chronic REMD impairs cognitive function in experimental animals, as evidenced by the increased latency time to find the escape platform and the reduced number of platforms crossed during the platform-free period, indicating difficulties in retaining the learned spatial information in MWM. The role of cAMP in molecular mechanisms of memory acquisition, consolidation, retrieval, and extinction is well documented. An increase in the expression of PDE enzymes depletes cAMP levels in the hippocampus in SD [8, 28]. In this study, we observed that 21 days of cREMD increased hippocampal expression of PDE10A, which could be a possible reason for the decrease in cAMP hydrolysis. Simultaneously, pharmacological inhibition of PDE10A during chronic REMD in mice preserved cAMP, CREB, and BDNF expression, which could be attributed to the improved memory.

Dysfunction of the synapse is implicated in a range of neurological disorders. Cyclic adenosine monophosphate response element binding (CREB) protein plays an essential role in memory formation [29, 30]. CREB phosphorylation occurs in CA1 and DG regions of the hippocampus in aversively motivated learning in rats [31]. It is the main regulator of BDNF, which is also associated with learning and memory [32], and enhances long-term potentiation in the cortex and hippocampus regions of mice [33]. Our results support the previous findings and suggest that activation of the cAMP, in turn, CREB, and BDNF cascade could be associated with improved spatial memory in the MWM test.

Higher dementia risk is associated with a lack of sleep in midlife [34, 35] and linked to AD-like pathology [36]. A buildup of Aβ and tau is linked to impaired cognitive functions in AD. These clumps of Aβ interfere with cAMP signaling, reduce the expression of NMDA receptors, and impair synapse communications. Sleep plays a vital role in clearing excessive Aβ deposits from the brain [37]. cREMD has been shown to increase Aβ production in human and rodent brains [38]. On the other hand, increased expression of PDE isoenzymes was recorded in AD patients [39]. Our study showed that chronic REMD simultaneously increased the expression of both Aβ and PDE10A, which could have exerted unfavourable effects on spatial memory formation as evidenced in the MWM test. The improved memory in chronic REMD mice could be corroborated by the decreased expression of Aβ and PDE10A in PAP-treated mice. Post-mortem brains of AD patients were shown to have fewer synapses with a predominant accumulation of Aβ [40]. Synapse proteins such as SAP-97, Synapsin, PSD-95, etc., are involved in regulating pre- and postsynaptic neurogenesis, neuronal outgrowth, and synaptic plasticity [41, 42]. Alterations in synapse-associated proteins have an adverse impact on synaptic transmission and neural regeneration. Several studies have shown that acute and chronic SD impair the expression of SYP, SYN-1, and PSD-95 in the hippocampus, resulting in synaptic damage [43, 44]. Our data showed that PAP administration increased the expression of SAP-97, Synapsin I, and PSD-95 in chronic REMD mice. Further, PAP also decreased Aβ expression, which indicates that PDE10A inhibition would have facilitated Aβ clearance and this spurts to check the status of autophagy in chronic REMD mice brains.

Basal neuronal autophagy is crucial in the maintenance of functional synaptic vesicles by facilitating the removal of damaged or dysfunctional proteins (proteinopathies) and organelles (mitophagy) [45, 46]. Several reports have demonstrated that excessive autophagy promotes the activation of apoptotic cascades, leading to neurodegeneration [47]. Beclin mediates the recruitment of other autophagy proteins to the pre-autophagosomal membrane and is a part of the type III phosphatidylinositol-3-kinase (PI3K) complex, which is involved in the production of autophagosomes. Beclin is highly expressed in the nervous system and is crucial for neuronal survival [48]. Light chain 3 (LC3), a microtubule-associated protein 1, is essential for autophagosome development. Cytosolic LC3-A is conjugated to phosphatidylethanolamine (PE) on the surface of developing autophagosomes to produce LC3-B, a common marker for autophagosomes [49]. Increased accumulation of LC3-immunoreactive autophagosomes in the hippocampus contributes to memory dysfunction in aged mice [50]. In agreement with previous reports, we observed that chronic REMD increased Beclin-1 and LC3B expression in the hippocampal region of mice. PAP reversed these changes, which may be corroborated by the decreased extracellular Aβ expression and increased cAMP via cyclic AMP (cAMP)/protein kinase A (PKA) cascade [51].

Activation of NMDA receptors influences synaptic transmission by increasing adenylyl cyclase activity and synapse formation [52]. On the other hand, accumulation of oligomeric Aβ decreases NMDA receptors expression at both synaptic and extra-synaptic sites [53, 54]. Previous reports indicate that SD reduces the surface expression of NR1 and NR2A in the hippocampus of rats [55, 56]. In our study, chronic REMD reduced the expression of NR2A and NR2B receptors, which may be corroborated by the overexpression of PDE10A, Aβ, and depletion of cAMP levels. These results indicate increased glutamatergic transmission, i.e., excitotoxicity, in the hippocampus regions of chronic REMD mice. The increased cAMP with PAP could be the possible reason for the improved expression of NR2A and NR2B in chronic REMD mice. Increased expression of PDE10A in PD is responsible for reduced activity of dopamine receptor D1 (D1) mediated striatonigral and corticostriatal neurotransmission [57, 58]. Higher expression of PDE10A has been linked with schizophrenic episodes [59]. Blocking PDE10A mediated hydrolysis of cAMP and/or cGMP results is a promising strategy for neurological disorders connected to neuroinflammation, disturbed neurotransmission of striatal and basal ganglia, including schizophrenia, HD, PD, or stroke. Regional and cellular expression of PDE10A varies between the neurodegenerative diseases, which might be responsible for the variability in response to the PDE10 inhibition efficacy. Increased levels of the intracellular cyclic nucleotides by PDE10A inhibitor are associated with improved cortical regulation of striatal output, improved cognition, and reduced liability for extrapyramidal effects in animals [59–61]. PDE10A inhibition has been reported to

attenuate neuroinflammatory responses [63]. In our study, enhancing the cAMP levels by inhibiting the cREMD induced PDE10A expression prevents the depletion of the synaptic proteins and restores the basal autophagy in the hippocampus of mice, which is responsible for enhanced memory. Therefore, targeting PDE10A with papaverine presents a promising strategy for addressing cognitive dysfunction in neurological disorders.

Research ethics: Not applicable.
Informed consent: Not applicable.
Author contributions: A.R.B. performed experiment, literature collection, original manuscript drafting, M.B. assisted in performing experiment, M.K. manuscript editing and preparation, P.T editing of manuscript, S.L.C. editing of manuscript, S.R.P.P. editing of manuscript, S.B.C conceptualization, designing of Experimentation, editing of manuscript.
Use of Large Language Models, AI and Machine Learning Tools: None declared.
Conflict of interest: All other authors state no conflict of interest.
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Data availability: The data that supports the findings of this study are available at CPT, JSSAHER, Mysuru, India.

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