Inhibition of PDE4 by Roflumilast ameliorates sleep deprivation-induced cognitive dysfunction in C57BL/6J mice

Abid Bhat[1,2], Muhammed Bishir[1,2], SR. Pandi-Perumal[3], Sulie Chang[4], Saravana B. Chidambaram[1,2,5]

1. Dept. of Pharmacology, JSS College of Pharmacy, JSS Academy of Higher Education & Research, Mysuru, India
2. Centre for Experimental Pharmacology and Toxicology, Central Animal Facility, JSS Academy of Higher Education & Research, Mysuru, India
3. Somnogen Canada Inc, College Street, Toronto, ON, Canada
4. Department of Biological Sciences and Institute of Neuroimmune Pharmacology, Seton Hall University, 400 South Orange Ave South Orange, NJ 07079, USA
5. Special Interest Group Brain, Behaviour and Cognitive Neurosciences, JSS Academy of Higher Education & Research, Sri Shivarathreeshwara Nagar, Mysuru, Karnataka 570015, India

[*] **Corresponding author**

Dr. C. Saravana Babu, MPharm, PhD, FASc (AW), FIC, FST
Prof & Coordinator Central Animal Facility
Dept of Pharmacology, JSS College of Pharmacy
JSS Academy of Higher Education & Research
S.S. Nagar, Mysuru - 570015
Email: babupublications@gmail.com

Highlights

- Sleep deprivation (SD) impaired recognition memory in mice.
- SD increased PDE4B, amyloid-beta (Aβ), and reduced cAMP, pCREB, BDNF, and synaptic proteins (Synapsin I, SAP 97, PSD 95) expression.
- Treatment with Roflumilast improved memory and decreased Aβ pathology in sleep-deprived mice.
- Increased in cAMP level correlates with improved expression of synaptic proteins and memory

Abstract

Sleep deprivation interferes with long-term memory and cognitive functions by over-activation of phosphodiesterase (PDE) enzymes. PDE4 is a non-redundant regulator of the cyclic nucleotides (cAMP), is densely expressed in the hippocampus, and is involved in learning and memory processes. In the present study, we investigated the effects of Roflumilast (ROF), a PDE4 inhibitor, on sleep deprivation induced cognitive dysfunction in a mouse model. Memory assessment was performed using a novel object recognition task and the cAMP level was estimated by ELISA. The alterations in the expressions of PDE4B, amyloid beta, CREB, BDNF, and synaptic proteins (Synapsin I, SAP 97, PSD 95) were assessed to gain insights on the possible mechanisms of action of ROF using the western blot technique. Results show that ROF reverse SD induced cognitive decline in mice. ROF down-regulated PDE4B and amyloid beta expressions. Additionally, ROF improved cAMP levels and the expressions of synapsin I, SAP 97, and PSD 95 in the hippocampal region of SD mice. Taken together, these results suggest that ROF can suppress the deleterious effects of SD-induced cognitive dysfunction via PDE4-mediated cAMP/CREB/BDNF cascade.

Keywords: Sleep deprivation, PDE4, Roflumilast, Memory, cAMP, Synaptic proteins

Introduction

Sleep plays a regulatory role in maintaining cellular and metabolic homeostasis. Increasing evidence have shown that sleep disturbances affect higher brain functions such as learning and memory, and is also linked to various neurological disorders (1–3). Sleep deprivation (SD) is considered as a public health epidemic and impose a negative impact on social, and economic wellbeing (4,5). Accumulating evidence suggest that SD reduces neurogenesis and transcription factors (CREB, BDNF) expression which are crucial regulators for learning and memory and induce hippocampal atrophy (6–8). Functional magnetic resonance imaging (fMRI) and behavioral studies from 150 picture slides showed that one-night sleep deprivation substantially compromises hippocampal function in humans in turn affects memory (9). Evidence also suggests that sleep disturbance predisposes brain accumulation of amyloid-β (Aβ) (10–12). Positron emission tomography (PET) in humans confirmed that SD and sleep fragmentation are associated with increased deposition of Aβ in the brain (13,14).

A mechanistic research report indicates that SD promotes synthesis (15) and impairs the clearance of Aβ protein (15). Intriguingly, the relationship between sleep disturbance and Aβ is bidirectional because increased Aβ deposition the other way was also shown to impair slow wave sleep (16). Furthermore, Aβ reduces the protein expression of Synapsin I, PSD-95, and SAP-102, which indicates that it eliminates synapses and causes loss of neuronal network (17–19). This synapto-toxic effect of Aβ are linked to the reduced expression of NMDA receptors and decreased cAMP content (20,21). The increased accumulation of Aβ and reduced levels of cAMP impairs the release of transcription factors that regulate brain development and synaptic plasticity (22,23).

Phosphodiesterases (PDE) are a diverse family of enzymes/proteins that play a role in cell functioning by regulating intracellular signaling (24). An increased expression of PDE4 enzymes hydrolyse cAMP into inactive forms which have been consistently observed in brains of Alzheimer's disease (AD) (25), and subjects with cognitive impairment (26) and also in the hippocampal region of sleep-deprived mice (27). Inhibition of PDE4 improved learning and memory in a mouse model of AD via increasing hippocampal cAMP levels (25,28). Furthermore, inhibition of PDE4 has also restored the deficits in synaptic proteins such as synaptophysin, PSD 95 (29,30).

Many reports suggest that PDE4 is a viable target in neurological disorders drug discovery (31). PDE4 inhibition was shown to reverse the cognitive decline induced by muscarinic receptor antagonist (32) and also by modulating NMDA receptors mediated transduction mechanisms in rat models. Albeit, the NMDA does not affect PDE4 expression directly, but

the balance between PDE4 and NMDA mediated adenylyl cyclase plays a pivotal role in the memory process (33). ROF, a cAMP-specific PDE4 inhibitor, is approved by USFDA for use in chronic obstructive pulmonary disease (COPD) (34). ROF promotes hippocampal neuron viability (35) and improve memory in rodents and monkeys at non-emetic doses (36). In a clinical study, it is observed that acute administration of ROF improves learning and memory in healthy individuals (37). These data open the question that whether PDE4 inhibition has any role on the levels of Aβ and associated synaptic dysfunction, particularly in sleep-deprived conditions.

Long-term SD could produce AD-like pathological state, wherein increased neuronal accumulations of Aβ, decreased cAMP and synaptic proteins expressions are well established. On the other hand, a fully blown AD imposes various therapeutic challenges. This spurt interest to investigate whether PDE4 expression has any correlation with Aβ, CREB, BDNF expression in SD brains, and also to study the effect PDE4 inhibition, using ROF, on cognitive function in sleep-deprived mice.

Materials and Methods

Animals.

Male C57BL/6J mice (25-30 g) were obtained from Adita Biosys Private Limited, Tumakuru, Karnataka, and housed in Central Animal Facility, JSS Academy of Higher Education & Research, Mysuru, Karnataka. Animals were housed in groups (5 animals/cage) in polypropylene cages under an ambient temperature of 19-26°C and 40-65% relative humidity, with a 12-h light/dark artificial light cycle. Animals were provided with standard rodent feed and purified water ad libitum. Animals were acclimatized for 7 days to the laboratory conditions prior to initiation of the experiments. Animal experiments were performed in full compliance with the guidelines of "Guide for the Care and Use of Laboratory Animals" (Institute of Laboratory Animal Resources, National Academic Press 1996; NIH publication number nos. 85–23, revised 1996). Institutional Animal Ethics Committee (IAEC), Central Animal Facility, JSS AHER, Mysuru, India approved the study (JSSAHER/CPT/IAEC/014/2020).

Reagents and antibodies

Roflumilast, Cresyl violet and Congo red stains were purchased from Sigma Aldrich (India). cAMP ELISA kit was purchased from Cayman (Ann Arbor, MI, USA). Anti-PSD95 (sc-

32290), Anti-SAP97 (sc-9961), Anti-Synapsin-I (sc-376623), Anti-BDNF (sc-65514), Anti-CREB (sc-377154), Anti β-Amyloid (sc-28365) were procured from Santa Cruz Biotechnology, CA, USA. Anti-PDE4B (NB100-2562) was purchased from Novus Biologicals, United States. All other reagents and chemicals were analytical grade.

Roflumilast treatment

Roflumilast (ROF L: 1 mg/kg and ROF H: 3 mg/kg) freshly prepared in 0.5% CMC was given intraperitoneally once a day for three days. The dosages of Roflumilast were selected based on our earlier reports (38).

Sleep deprivation method

A modified multiple platform method was used for the induction of sleep deprivation (39). Mice were placed in cages (41 cm x 34 cm x 16.5 cm), containing platforms (3 cm in diameter) surrounded by water up to 1 cm beneath the surface. Mice were allowed to freely move inside the cage and jump from one platform to the other but were not in a position to lie down. This method is reported to primarily eliminate REM sleep (40). Non sleep deprived (NSD) animals were kept in their cages in the same room. During sleep deprivation, mice had access to food and water. Water in the cages was changed twice a day. Animals were sleep-deprived for 72 hours. The flow of the experiment is provided in figure 1.

Experimental groups

Animals were randomized based on the bodyweight into 4 groups, i.e. – Non-sleep-deprived (NSD) + Vehicle (Cage control and CMC treated); SD + Vehicle (CMC treated); SD + ROF L (1 mg/kg b.wt); SD + ROF (3 mg/kg b.wt.). Each group contained 10 animals.

Novel object recognition test

Novel object recognition task was performed to access the recognition memory as described previously (41). Mice were habituated to explore the empty apparatus for 10 min (2^{nd} day of treatment). During the acquisition trial (T1), two similar objects were placed inside the apparatus and the mouse was allowed to explore the objects for 3 min. After the acquisition trial, the mouse was transferred to its home cage. Discrimination trial (T2) was done twenty-four hours later (4th day). Two different objects, a familiar object, and a novel object were placed in the exploration area. The time spent by the animal exploring the two objects during

T2 was recorded and the discrimination index (DI) was calculated, as per the following formula. DI=RI/ (Time spent in exploring novel object + Time spent in exploring familiar object). Recognition Index = Time spent in exploring novel object-Time spent in exploring the familiar objects. This test was repeated for all the animals in all the cages one at a time. All behavioral assessments were done between 10 am to 3pm.

Measurement of cAMP content

Hippocampal tissues for each of the animal groups were individually homogenized in 500 µL of 0.1M Hydrochloric acid to purify tissue samples from PDE enzymes. Homogenates were centrifuged for 10 min at 1500× g at 4°C, and the supernatants were stored at 4°C. cAMP levels were determined by cAMP enzyme immunoassay kit following the manufacturer's instructions (Cayman Chemical Co., Ann Arbor, MI, USA)

Western blot

Following all the behavioral assessments, the animals were euthanized to collect the brain and stored at -80^{o}C. Hippocampal regions were isolated, and homogenates were prepared with radioimmunoprecipitation assay buffer (RIPA) buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 5 mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS, 50 nM sodium fluoride, 1 mM sodium vanadate) containing a cocktail of protease inhibitor (Sigma Aldrich, MO, USA). Total protein concentrations of the samples were determined by the Pierce™ bicinchoninic acid (BCA) protein assay (Thermofisher scientific), homogenate samples were aliquoted and stored at -80^{o}C until further use. Sample proteins (20 µg) were separated by using 10% bis-tris -SDS-PAGE (electrophoresis). Resolved proteins in the gels were transferred onto polyvinylidene difluoride (PVDF) membranes (Biorad) and electroblotted. Membranes were blocked overnight with 5% non-fat skimmed milk in Tris-Buffered Saline and Tween 20 (TBST) at 4^{o}C. This was followed by a 4-hour incubation with the primary antibodies (PDE4B (1:1000), CREB (1:1000), BDNF (1:1000), β-Amyloid (1:1000), PSD-95 (1:1000), Synapsin-I (1:1000), SAP 97 (1:1000) at room temperature. The membranes were rinsed with TBST (3 washings for 10 minutes each), followed by incubation with the secondary antibodies (HRP conjugated anti-mouse or anti-rabbit IgG) for 1h at room temperature and washed with TBST (3 washings for 10 minutes each). Bands were detected using SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Scientific). Densitometric measurement of bands was done using ImageJ (NIH software). For Western blot analysis, the signal intensity (integrated density value, IDV) of PDE4B, BDNF, β-

Amyloid, PSD-95, Synapsin-I and SAP 97was normalized against the IDV of internal control β-actin, while pCREB was normalized with total CREB and histogram was plotted.

Histopathology

Whole brain was stored in buffered 10% formalin for 48h. Coronal sections (3-5 µm) of the hippocampus region were cut using a microtome. The hippocampal region of the brain was used for histopathological analysis Sections were mounted on a slide, washed, and dehydrated with 95% ethanol.

Nissl staining

Coronal sections (3-5 µm) of the hippocampus region were washed with xylene flowed by five times washing with water for 5 mins each. The samples were stained with 0.2% cresyl violet dye for 30 mins. The prepared slides were examined under the microscope by a pathologist for histopathological analyses.

Congo red staining

Congo red staining was done to detect amyloid plaques in mice hippocampus. Coronal sections (3-5 µm) of the hippocampus region were stained with 1 % Congo Red stain for 30 mins. Amyloid plaques were observed under a microscope.

Statistical analysis

Data are presented as mean ±SEM. The difference between time spent exploring the novel object versus familiar object during the discrimination trial was calculated for each group and the level of significance was analyzed using a two-sided student's t-test. For other parameters, group means differences were analyzed using a one-way ANOVA test followed by Tukey's multiple comparison test as post hoc. Pearson's correlation analysis was performed using SYSTAT 11 (SPSS Inc, Chicago, IL). Graphs were plotted using GraphPad Prism version 7.04 with $p < 0.05$ considered significant.

RESULTS

PDE4B expression is up-regulated in SD mice brains and ROF down-regulated PDE4B

PDE4B is an important PDE expressed in the hippocampal region. Changes in the PDE4B expression have been associated with cognitive functions (42)**.** We assessed the impact of sleep deprivation on PDE4B expression in mice. 72h continuous SD induced a significant *(p*

222 < 0.05) increase in PDE4B expression when compared with the NSD group. We determined
223 whether ROF administration reduces the SD induced PDE4B expression. Daily dose of ROF
224 down-regulated PDE4B expression when compared with vehicle-treated SD group. A
225 significant ($p < 0.01$) decrease in PDE4B expression was found at a dose of 3 mg/kg of ROF**.**
226 Correlation analysis revealed that PDE4B possesses a strong positive correlation with β-
227 amyloid (r = 0.8167) **(Fig 2A)**.
228
229 **Sleep deprivation increased β-amyloid deposition in mice brains and roflumilast down-**
230 **regulated its expression**
231 Neuronal damage is the outcome of excessive deposition of β-amyloid in brain (43)**.**
232 Impaired sleep has been associated with AD as sleep plays a role in clearing the metabolic
233 waste from the brain (11). We performed Western blotting to study the impact of sleep
234 deprivation on β-amyloid expression in mice hippocampal region. 72 h of SD significantly
235 ($p< 0.01$) increased β-amyloid expression in vehicle-treated mice when compared to the non-
236 sleep deprived mice. Inhibition of PDE4B by ROF significantly ($p< 0.01$) reduced the
237 expression of β-amyloid in mice when compared with the SD control group. Correlation
238 analysis revealed that β-amyloid possesses a strong negative correlation with Synapsin (r = -
239 0.9733 [p < <0.0001]), SAP 97 (r = -0.5594 [p < <0.0045]) and PSD 95 (r = -0.4979[p <
240 <0.0133]) in sleep-deprived mice. This data indicates that the PDE4B enzyme has a potential
241 role on β-amyloid expression in the SD state (**Fig 2B**).
242 An increase in β-amyloid deposition was further confirmed by Congo red staining in the
243 hippocampal region of SD mice. We found that vehicle-treated SD mice showed multifocal
244 and moderate increased deposition of amyloid at CA1 and DG region of the hippocampus
245 compared with vehicle-treated NSD group **(Fig.3)**. As shown in Fig. 3 ROF L treated SD
246 mice showed mild deposition of amyloid at hippocampus CA1 and DG regions.
247
248 **Roflumilast improves hippocampal cAMP levels in sleep-deprived mice**
249 cAMP mediates fundamental brain functions relevant to learning and memory. Decline of
250 cAMP levels in hippocampus impairs the memory consolidation (42)**.** To determine the
251 impact of sleep deprivation on cAMP levels in the hippocampus region of mice. We
252 performed an ELISA assay and found that SD significantly ($p < 0.01$) reduced cAMP levels
253 in vehicle-treated mice when compared with vehicle-treated NSD group. Administration of
254 ROF in sleep-deprived mice showed a significant ($p < 0.001$) increase in the levels of cAMP

when compared with vehicle-treated SD group. This indicates that ROF administration rescues sleep deprivation induced decrease in cAMP levels in mice. Correlation analysis revealed a strong negative correlation with β-amyloid (r = -0.8162) and a strong positive correlation with CREB (r = 0.927) and BDNF (r = 0.886) respectively **(Fig. 4).**

Roflumilast improved CREB and BDNF expression in the hippocampus of SD mice

Subsequently, we assessed the impact of sleep deprivation on the hippocampal expression of transcription factors CREB and BDNF. We found that 72-hour sleep deprivation produced a significant ($p < 0.01$) decrease in hippocampal pCREB expression as compared to the NSD control group. Administration of ROF restored the levels of pCREB when compared with the SD control group. A significant ($p < 0.001$) increase in pCREB was observed when compared with vehicle-treated SD mice **(Fig. 5A).** CREB influences the expression of BDNF which is essential in memory consolidation and synaptic function (6), we performed western blot analysis to detect BDNF expression in the hippocampus region of mice. As shown in **Fig. 5B,** SD significantly ($p < 0.001$) decreased the expression of BDNF when compared with NSD mice. ROF administration in sleep-deprived mice significantly ($p< 0.001$) increased BDNF expression when compared to vehicle-treated SD mice. These data suggest that ROF administration improves the expression of transcription factors in SD mice, which might be due to the decreased Aβ toxicity.

Roflumilast up-regulates the expression of synaptic associated proteins in sleep-deprived mice

Next, we investigated whether improvement in neurotrophic factor expression influences synaptic proteins expression in hippocampus region of SD mice. Synapsin I expression decreased significantly ($p< 0.01$) following SD when compared with the NSD control group. ROF administration significantly ($p< 0.01$) up-regulated the expression of Synapsin I when compared with vehicle-treated SD mice **(Fig 6).** SAP 97 regulates synaptic plasticity by controlling the distribution of glutamate receptors (44). We found a significant decrease in the expression of SAP-97 in SD mice when compared with NSD mice. ROF treatment showed a significant increase in the expression of SAP-97 when compared with vehicle-treated SD mice **(Fig 6).**

Next, we investigated the impact of sleep deprivation on the expression of post-synaptic protein PSD95, we found that 72 h of sleep deprivation caused a significant ($p< 0.01$)

decrease in the expression of PSD 95 when compared with NSD mice. Administration of ROF significantly up-regulated ($p< 0.01$) the expression of PSD95 in SD mice when compared with vehicle SD mice **(Fig 6)**. These results imply PDE4 inhibition may improve synaptic functions, which might be due to its restoration of neurotrophic factors, at least partly, in SD mice

Roflumilast restores sleep-deprived induced cognitive dysfunction in mice

Recognition memory was assessed by a novel object recognition test. Sleep-deprived mice did not show a significant difference in the time spent between familiar and novel objects when compared with NSD mice. SD mice administered with ROF showed significantly (1mg/kg; $p < 0.05$, 3mg/kg; $p < 0.01$) increased time spent in novel object than the familiar object. **(Fig. 7a)**. SD significantly ($p < 0.01$) decreased the discrimination index in mice, whereas ROF significantly (1mg/kg; $p < 0.05$, 3mg/kg; $p < 0.01$) increased the discrimination index in SD mice **(Fig. 7b)**. These results indicate that ROF can restore recognition memory in SD mice.

Roflumilast prevented the morphological changes of hippocampal neurons in sleep-deprived mice

SD induced multifocal moderate neuronal degeneration in the CA1 and dentate gyrus regions of the hippocampus. As shown in fig.6, neurons in CA1 and DG regions in vehicle-treated SD mice showed a decrease in purple Nissl granules and pyknotic nucleus in the perikarya when compared with NSD mice. ROF treated mice showed reduced morphological changes and had regularly shaped cell bodies in CA1 and DG regions when compared with SD mice **(Fig 8).**

Discussion

The present study demonstrates the molecular pathogenetic mechanism behind SD-induced cognitive dysfunctions and the protective effects of PDE4 inhibition using Roflumilast. Our findings reveal that alleviation of Aβ pathology, cAMP signaling, and synaptic proteins expression by Roflumilast via PDE4 inhibition as a crucial mechanism in cognitive restoration in SD mice.

SD aggravates Aβ plaque levels in AD transgenic mouse models (45). A recent study has shown that chronic sleep restriction (3 h per day, 5 days per week, for 4 weeks) increases the

accumulation of hippocampal Aβ in mice, which was corroborated to cognitive decline (46). Aβ deposition in cortical and hippocampal regions initiates inflammatory responses, synaptic dysfunctions (47) and neuronal apoptosis (48). Aβ also compromises the cAMP-response element-binding protein signaling in neurons suggesting that multiple factors contribute to neuronal damage in SD (49).

On the other hand, increased expression of PDE4 is reported to associate with Aβ plaque pathology and memory loss (50). Studies using PDE4 knockout mice showed an improved memory and reduced neuroinflammation and β-amyloidosis (51–53). In the present study, we found that 72 h SD increased the protein expression and deposition of Aβ in hippocampal neurons mice. Earlier, in vivo studies and clinical trials have shown that alleviating cAMP signaling through PDE4 inhibition improved cognitive functions (54,55) and by reducing the Aβ expression in mice (56). The present study reports an increased PDE4 and Aβ expression in SD mice brains with a significant decrease in cAMP levels. Treatment with ROF reversed the changes including Aβ pathology that indicates that PDE4 improves cognition in SD mice probably via alleviating Aβ synthesis or clearance.

Synapse dysfunction is an early consequence of Aβ deposition (57). PSD-95 is an essential regulator of synaptic strength and plasticity. SAP-97 facilitates synaptic plasticity, synaptic vesicles biogenesis, and neurotransmitters release (58). Synapsin I involves in the modulation of neurotransmitters release and its down-regulation is shown to impair neurogenesis and synaptic plasticity (59,60). SD causes synaptic damage in the hippocampus by reducing the expression of presynaptic and postsynaptic proteins (61–63). Alterations in PSD95 and SAP-97 levels adversely affect synaptic connectivity and neural regeneration (64). Recently, we have shown that ROF improves synaptic proteins expression in human neurons exposed to quinolinic acid-induced neurotoxicity (29). The present study shows that SD down-regulated synaptic proteins (SAP-97, Synapsin-I, and PSD-95) in the hippocampal region of mice. Nevertheless, administration of ROF increased SAP-97, Synapsin-I, and PSD-95 expression, which indicates that PDE4 enzymes play a role in synaptic proteins expression.

Cyclic nucleotides play a vital role in memory consolidation (65). Inhibition of cAMP signaling in the hippocampal region is reported to impair consolidation of long-term memory in mice (66). SD reduces the phosphorylation of CREB in the hippocampus and affects the protein expression of neurotrophic factor BDNF (6). Particularly, long-term memory is critically dependent on CREB mediated expression of neurotrophic factors such as BDNF (61). In the present study, ROF administration restores the cAMP/CREB/BDNF signaling

cascade in SD mice. These results are in agreement with earlier studies that report that inhibition of PDE4 improves memory consolidation in rodent models via cAMP/CREB/BDNF cascade, which reveals that PDE4 is connected with this signaling cascade (32,67).

Histopathology examination using Congo red staining showed higher deposition of Aβ aggregates in CA1 and DG region of the hippocampal region in SD mice. Further Cresyl violet staining showed cell death in the hippocampus region of SD mice indicating apoptosis. Recent studies have shown that SD increases cytokine production, microglial activation and initiates neuronal apoptosis, causing lesions in the hippocampus of mice (68,69). Interestingly PDE4 expression is regulated by inflammation and microglial reactivity (70). Transient increase in cAMP stimulates cAMP signal transduction inhibiting inflammatory mediators and early apoptotic factors (71). Nissl staining showed that ROF administration prevented neuronal damage in SD mice. Earlier studies have reported that inhibition of PDE4 reduces the levels of pro-inflammatory and early apoptosis factors (72).

Conclusion

From the current investigation we provided molecular, behavioral and histopathological evidence that Roflumilast rescues SD induced cognitive dysfunction in mice. Roflumilast administration improves recognition memory via PDE4 inhibition mediated cAMP/CREB/BDNF signaling and downregulation of Aβ pathology in SD mice. Additionally, further studies to understand the effect of Roflumilast on NMDA activity and autophagy in chronic sleep restriction are still being investigated.

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Conflict of Interest

All the authors in this study declare no potential conflicts of interest in research and publishing this article.

Availability of data and material

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval

All animal experiments were performed in accordance with the Institutional Animal Ethics Committee (IAEC), Central Animal Facility, JSS AHER, Mysuru, India approved the study (JSSAHER/CPT/IAEC/014/2020).

Author contributions statement

AB: Methodology, Investigation, Data curation, Formal analysis, Writing – original draft, **MB**: Methodology, Investigation. **SBC**: Conceptualization, Supervision, Project administration, Data curation, Writing – review & editing. The manuscript has been read and approved by all authors.

References

1. Bhat A, Pires AS, Tan V, Babu Chidambaram S, Guillemin GJ. (2020) Effects of Sleep Deprivation on the Tryptophan Metabolism. Int J Tryptophan Res.13.

2. Bishir M, Bhat A, Essa MM, Ekpo O, Ihunwo AO, Veeraraghavan VP, et al. (2020) Sleep Deprivation and Neurological Disorders [Internet]. Vol. 2020, BioMed Research International. Hindawi; e5764017.

3. You JC, Jones E, Cross DE, Lyon AC, Kang H, Newberg AB, et al. (2019) Association of β-Amyloid Burden With Sleep Dysfunction and Cognitive Impairment in Elderly Individuals With Cognitive Disorders. JAMA Network Open. e1913383.

4. Chattu VK, Manzar MdD, Kumary S, Burman D, Spence DW, Pandi-Perumal SR. (2018) The Global Problem of Insufficient Sleep and Its Serious Public Health Implications. Healthcare (Basel). 20;7(1):1.

5. Wang W, Yang L, Liu T, Ma Y, Huang S, He M, et al. (2020) Corilagin ameliorates sleep deprivation-induced memory impairments by inhibiting NOX2 and activating Nrf2. Brain Research Bulletin. 160:141–9.

6. Alhaider IA, Aleisa AM, Tran TT, Alkadhi KA. (2011) Sleep deprivation prevents stimulation-induced increases of levels of P-CREB and BDNF: Protection by caffeine. Molecular and Cellular Neuroscience.46(4):742–51.

7. Cao Y, Li Q, Liu L, Wu H, Huang F, Wang C, et al. (2019) Modafinil protects hippocampal neurons by suppressing excessive autophagy and apoptosis in mice with sleep deprivation. British Journal of Pharmacology.176(9):1282–97.

8. Havekes R, Park AJ, Tudor JC, Luczak VG, Hansen RT, Ferri SL, et al. (2016) Sleep deprivation causes memory deficits by negatively impacting neuronal connectivity in hippocampal area CA1. Takahashi JS, editor. eLife. 5:e13424.

9. Yoo S-S, Hu PT, Gujar N, Jolesz FA, Walker MP. (2007) A deficit in the ability to form new human memories without sleep. Nature Neuroscience. 10(3):385–92.

10. Ooms S, Overeem S, Besse K, Rikkert MO, Verbeek M, Claassen JAHR. (2014) Effect of 1 night of total sleep deprivation on cerebrospinal fluid β-amyloid 42 in healthy middle-aged men: a randomized clinical trial. JAMA Neurol. 71(8):971–7.

11. Shokri-Kojori E, Wang G-J, Wiers CE, Demiral SB, Guo M, Kim SW, et al. (2018) β-Amyloid accumulation in the human brain after one night of sleep deprivation. PNAS. ;115(17):4483–8.

12. Winer JR, Mander BA, Kumar S, Reed M, Baker SL, Jagust WJ, et al. (2020) Sleep Disturbance Forecasts β-Amyloid Accumulation across Subsequent Years. Curr Biol. ;30(21):4291-4298.e3.

13. Sharma RA, Varga AW, Bubu OM, Pirraglia E, Kam K, Parekh A, et al. (2018) Obstructive Sleep Apnea Severity Affects Amyloid Burden in Cognitively Normal Elderly. A Longitudinal Study. Am J Respir Crit Care Med.197(7):933–43.

14. Sprecher KE, Koscik RL, Carlsson CM, Zetterberg H, Blennow K, Okonkwo OC, et al. (2017) Poor sleep is associated with CSF biomarkers of amyloid pathology in cognitively normal adults. Neurology. 89(5):445–53.

15. Lucey BP, Mawuenyega KG, Patterson BW, Elbert DL, Ovod V, Kasten T, et al. (2017) Associations Between β-Amyloid Kinetics and the β-Amyloid Diurnal Pattern in the Central Nervous System. JAMA Neurol. 74(2):207–15.

16. Mander BA, Marks SM, Vogel JW, Rao V, Lu B, Saletin JM, et al. (2015) β-amyloid disrupts human NREM slow waves and related hippocampus-dependent memory consolidation. Nature Neuroscience. 18(7):1051–7.

17. Marsh J, Bagol SH, Williams RSB, Dickson G, Alifragis P. (2017) Synapsin I phosphorylation is dysregulated by beta-amyloid oligomers and restored by valproic acid. Neurobiol Dis. 106:63–75.

18. Proctor DT, Coulson EJ, Dodd PR. (2010) Reduction in post-synaptic scaffolding PSD-95 and SAP-102 protein levels in the Alzheimer inferior temporal cortex is correlated with disease pathology. J Alzheimers Dis. 21(3):795–811.

19. Walsh DM, Selkoe DJ. (2007) Aβ Oligomers – a decade of discovery. Journal of Neurochemistry.101(5):1172–84.

20. Chen Y, Huang X, Zhang Y, Rockenstein E, Bu G, Golde TE, et al. (2012) Alzheimer's β-Secretase (BACE1) Regulates the cAMP/PKA/CREB Pathway Independently of β-Amyloid. J Neurosci. ;32(33):11390–5.

21. Parihar MS, Brewer GJ. (2010) Amyloid Beta as a Modulator of Synaptic Plasticity. J Alzheimers Dis. 22(3):741–63.

22. Campbell IG, Guinan MJ, Horowitz JM. (2002) Sleep Deprivation Impairs Long-Term Potentiation in Rat Hippocampal Slices. Journal of Neurophysiology. Aug 1;88(2):1073–6.

23. Ravassard P, Pachoud B, Comte J-C, Mejia-Perez C, Scoté-Blachon C, Gay N, et al. (2009) Paradoxical (REM) Sleep Deprivation Causes a Large and Rapidly Reversible Decrease in Long-Term Potentiation, Synaptic Transmission, Glutamate Receptor Protein Levels, and ERK/MAPK Activation in the Dorsal Hippocampus. Sleep. 32(2):227–40.

24. Bhat A, Ray B, Mahalakshmi AM, Tuladhar S, Nandakumar D, Srinivasan M, et al. (2020) Phosphodiesterase-4 enzyme as a therapeutic target in neurological disorders. Pharmacological Research.105078.

25. Zhang H, Wang H, Zhang F, Li B, Zhou Y, Yu H, et al. (2019) Inhibition of phosphodiesterase-4D reverses memory deficits and depression-like effects via cAMP signaling in mouse models of Alzheimer's disease. The FASEB Journal.33(S1):806.1-806.1.

26. Perez-Gonzalez R, Pascual C, Antequera D, Bolos M, Redondo M, Perez DI, et al. (2013) Phosphodiesterase 7 inhibitor reduced cognitive impairment and pathological hallmarks in a mouse model of Alzheimer's disease. Neurobiology of Aging.34(9):2133–45.

27. Havekes R, Park AJ, Tolentino RE, Bruinenberg VM, Tudor JC, Lee Y, et al. (2016) Compartmentalized PDE4A5 Signaling Impairs Hippocampal Synaptic Plasticity and Long-Term Memory. J Neurosci. 36(34):8936–46.

28. Xu Y, Zhu N, Xu W, Ye H, Liu K, Wu F, et al. (2018) Inhibition of Phosphodiesterase-4 Reverses Aβ-Induced Memory Impairment by Regulation of HPA Axis Related cAMP Signaling. Front Aging Neurosci.;10.

29. Bhat A, Tan V, Heng B, Lovejoy DB, Sakharkar MK, Essa MM, et al. (2020) Roflumilast, a cAMP-Specific Phosphodiesterase-4 Inhibitor, Reduces Oxidative Stress and Improves Synapse Functions in Human Cortical Neurons Exposed to the Excitotoxin Quinolinic Acid. ACS Chem Neurosci. DOI:10.1021/acschemneuro.0c00636

30. Cui S-Y, Yang M-X, Zhang Y-H, Zheng V, Zhang H-T, Gurney ME, et al. (2019) Protection from Amyloid β Peptide–Induced Memory, Biochemical, and Morphological Deficits by a Phosphodiesterase-4D Allosteric Inhibitor. J Pharmacol Exp Ther. 371(2):250–9.

31. Bollen E, Prickaerts J. (2012) Phosphodiesterases in neurodegenerative disorders. IUBMB Life. 64(12):965–70.

32. Zhang H-T, O'Donnell JM. (2000) Effects of rolipram on scopolamine-induced impairment of working and reference memory in the radial-arm maze tests in rats. Psychopharmacology 150(3):311–6.

33. Zhang H-T, Crissman AM, Dorairaj NR, Chandler LJ, O'Donnell JM. (2000) Inhibition of Cyclic AMP Phosphodiesterase (PDE4) Reverses Memory Deficits Associated with NMDA Receptor Antagonism. Neuropsychopharmacology. 23(2):198–204.

34. Rabe KF, Bateman ED, O'Donnell D, Witte S, Bredenbröker D, Bethke TD. (2005) Roflumilast--an oral anti-inflammatory treatment for chronic obstructive pulmonary disease: a randomised controlled trial. Lancet. 366(9485):563–71.

35. Peng S, Yan H-Z, Liu P-R, Shi X-W, Liu C-L, Liu Q, et al. (2018) Phosphodiesterase 4 Inhibitor Roflumilast Protects Rat Hippocampal Neurons from Sevoflurane Induced Injury via Modulation of MEK/ERK Signaling Pathway. Cell Physiol Biochem.;45(6):2329–37.

506 36. Vanmierlo T, Creemers P, Akkerman S, van Duinen M, Sambeth A, De Vry J, et al.
507 (2016) The PDE4 inhibitor roflumilast improves memory in rodents at non-emetic
508 doses. Behav Brain Res. 303:26–33.

509 37. Blokland A, Van Duinen MA, Sambeth A, Heckman PRA, Tsai M, Lahu G, et al.
510 (2019) Acute treatment with the PDE4 inhibitor roflumilast improves verbal word
511 memory in healthy old individuals: a double-blind placebo-controlled study.
512 Neurobiology of Aging. ;77:37–43.

513 38. Jabaris SSL, Sumathy H, Girish R, Narayanan S, Sugumar M, Saravana Babu C, et al.
514 (2015) Phosphodiesterase-4 inhibitors ameliorates cognitive deficits in
515 deoxycorticosterone acetate induced hypertensive rats via cAMP/CREB signaling
516 system. Brain Research.1622:279–91.

517 39. Patti CL, Zanin KA, Sanday L, Kameda SR, Fernandes-Santos L, Fernandes HA, et al.
518 (2010) Effects of Sleep Deprivation on Memory in Mice: Role of State-Dependent
519 Learning. Sleep. 33(12):1669–79.

520 40. Grahnstedt S, Ursin R. (1985) Platform sleep deprivation affects deep slow wave sleep
521 in addition to REM sleep. Behav Brain Res.18(3):233–9.

522 41. Prickaerts J, van Staveren WCG, Şik A, Markerink-van Ittersum M, Niewöhner U, van
523 der Staay FJ, et al. (2002) Effects of two selective phosphodiesterase type 5 inhibitors,
524 sildenafil and vardenafil, on object recognition memory and hippocampal cyclic GMP
525 levels in the rat. Neuroscience. 113(2):351–61.

526 42. McGirr A, Lipina TV, Mun H-S, Georgiou J, Al-Amri AH, Ng E, et al. (2016) Specific
527 Inhibition of Phosphodiesterase-4B Results in Anxiolysis and Facilitates Memory
528 Acquisition. Neuropsychopharmacology. 41(4):1080–92.

529 43. Gupta A, Goyal R. (2016) Amyloid beta plaque: a culprit for neurodegeneration. Acta
530 Neurol Belg.116(4):445–50.

531 44. Li D, Specht CG, Waites CL, Butler-Munro C, Leal-Ortiz S, Foote JW, et al. (2011)
532 SAP97 directs NMDA receptor spine targeting and synaptic plasticity. J Physiol. 589(Pt
533 18):4491–510.

534 45. Kang J-E, Lim MM, Bateman RJ, Lee JJ, Smyth LP, Cirrito JR, et al. (2009) Amyloid-
535 beta dynamics are regulated by orexin and the sleep-wake cycle. Science.
536 326(5955):1005–7.

537 46. Brice KN, Hagen CW, Peterman JL, Figg JW, Braden PN, Chumley MJ, et al. (2020)
538 Chronic sleep restriction increases soluble hippocampal Aβ-42 and impairs cognitive
539 performance. Physiol Behav. 226:113128.

540 47. Palop JJ, Mucke L. (2010) Amyloid-β Induced Neuronal Dysfunction in Alzheimer's
541 Disease: From Synapses toward Neural Networks. Nat Neurosci. 13(7):812–8.

542 48. Wang L, Xiaokaiti Y, Wang G, Xu X, Chen L, Huang X, et al. (2017) Inhibition of
543 PDE2 reverses beta amyloid induced memory impairment through regulation of
544 PKA/PKG-dependent neuro-inflammatory and apoptotic pathways. Scientific Reports.
545 7(1):12044.

546 49. Tong L, Thornton PL, Balazs R, Cotman CW. (2001) Beta -amyloid-(1-42) impairs
547 activity-dependent cAMP-response element-binding protein signaling in neurons at
548 concentrations in which cell survival Is not compromised. J Biol Chem. 276(20):17301–
549 6.

550 50. Paes D, Lardenoije R, Carollo RM, Roubroeks JAY, Schepers M, Coleman P, et al.
551 (2021) Increased isoform-specific phosphodiesterase 4D expression is associated with
552 pathology and cognitive impairment in Alzheimer's disease. Neurobiol Aging. 97:56–
553 64.

554 51. Aizawa T, Wei H, Miano JM, Abe J, Berk BC, Yan C. (2003) Role of
555 phosphodiesterase 3 in NO/cGMP-mediated antiinflammatory effects in vascular
556 smooth muscle cells. Circ Res. 93(5):406–13.

557 52. Chong YH, Shin SA, Lee HJ, Kang JHL, Suh Y-H. (2002) Molecular mechanisms
558 underlying cyclic AMP inhibition of macrophage dependent TNF-alpha production and
559 neurotoxicity in response to amyloidogenic C-terminal fragment of Alzheimer's
560 amyloid precursor protein. J Neuroimmunol. 133(1–2):160–74.

561 53. Tibbo AJ, Tejeda GS, Baillie GS. (2019) Understanding PDE4's function in
562 Alzheimer's disease; a target for novel therapeutic approaches. Biochemical Society
563 Transactions. 47(5):1557–65.

564 54. Feng H, Wang C, He W, Wu X, Li S, Zeng Z, et al. (2019) Roflumilast ameliorates
565 cognitive impairment in APP/PS1 mice via cAMP/CREB/BDNF signaling and anti-
566 neuroinflammatory effects. Metab Brain Dis. 34(2):583–91.

567 55. Prickaerts J, Heckman PRA, Blokland A. (2017) Investigational phosphodiesterase
568 inhibitors in phase I and phase II clinical trials for Alzheimer's disease. Expert Opin
569 Investig Drugs. 26(9):1033–48.

570 56. Ashour NH, El-Tanbouly DM, El Sayed NS, Khattab MM. (2021) Roflumilast
571 ameliorates cognitive deficits in a mouse model of amyloidogenesis and tauopathy:
572 Involvement of nitric oxide status, Aβ extrusion transporter ABCB1, and reversal by
573 PKA inhibitor H89. Progress in Neuro-Psychopharmacology and Biological Psychiatry.
574 111:110366.

575 57. Lacor PN, Buniel MC, Furlow PW, Clemente AS, Velasco PT, Wood M, et al. (2007)
576 Aβ Oligomer-Induced Aberrations in Synapse Composition, Shape, and Density Provide
577 a Molecular Basis for Loss of Connectivity in Alzheimer's Disease. J Neurosci.
578 27(4):796–807.

579 58. Chen F, He Y, Wang P, Wei P, Feng H, Rao Y, et al. (2018) Curcumin can influence
580 synaptic dysfunction in APPswe/PS1dE9 mice. Journal of Traditional Chinese Medical
581 Sciences. 5(2):168–76.

582 59. Barbieri R, Contestabile A, Ciardo MG, Forte N, Marte A, Baldelli P, et al. (2018)
583 Synapsin I and Synapsin II regulate neurogenesis in the dentate gyrus of adult mice.
584 Oncotarget.9(27):18760–74.

585 60. Corradi A, Zanardi A, Giacomini C, Onofri F, Valtorta F, Zoli M, et al. (2008)
586 Synapsin-I- and synapsin-II-null mice display an increased age-dependent cognitive
587 impairment. J Cell Sci.121(18):3042–51.

588 61. Alhaider IA, Aleisa AM, Tran TT, Alzoubi KH, Alkadhi KA. (2010) Chronic Caffeine
589 Treatment Prevents Sleep Deprivation-Induced Impairment of Cognitive Function and
590 Synaptic Plasticity. Sleep.33(4):437–44.

591 62. Vecsey CG, Huang T, Abel T. (2018) Sleep deprivation impairs synaptic tagging in
592 mouse hippocampal slices. Neurobiol Learn Mem. 154:136–40.

593 63. Wadhwa M, Sahu S, Kumari P, Kauser H, Ray K, Panjwani U. (2015) Caffeine and
594 modafinil given during 48 h sleep deprivation modulate object recognition memory and
595 synaptic proteins in the hippocampus of the rat. Behav Brain Res. 294:95–101.

596 64. Ansari MA, Roberts KN, Scheff SW. (2008) Oxidative stress and modification of
597 synaptic proteins in hippocampus after traumatic brain injury. Free Radic Biol Med.
598 45(4):443–52.

599 65. Havekes R, Bruinenberg VM, Tudor JC, Ferri SL, Baumann A, Meerlo P, et al. (2014)
600 Transiently Increasing cAMP Levels Selectively in Hippocampal Excitatory Neurons
601 during Sleep Deprivation Prevents Memory Deficits Caused by Sleep Loss. J Neurosci.
602 34(47):15715–21.

603 66. Bollen E, Puzzo D, Rutten K, Privitera L, De Vry J, Vanmierlo T, et al. (2014)
604 Improved Long-Term Memory via Enhancing cGMP-PKG Signaling Requires cAMP-
605 PKA Signaling. Neuropsychopharmacology.39(11):2497–505.

606 67. Wang C, Yang X-M, Zhuo Y-Y, Zhou H, Lin H-B, Cheng Y-F, et al. (2012) The
607 phosphodiesterase-4 inhibitor rolipram reverses Aβ-induced cognitive impairment and
608 neuroinflammatory and apoptotic responses in rats. International Journal of
609 Neuropsychopharmacology.15(6):749–66.

610 68. Smith C, Trageser KJ, Wu H, Herman FJ, Iqbal UH, Sebastian-Valverde M, et al.
611 (2021) Anxiolytic effects of NLRP3 inflammasome inhibition in a model of chronic
612 sleep deprivation. Transl Psychiatry. 11(1):1–15.

613 69. Xie G, Huang X, Li H, Wang P, Huang P. (2021) Caffeine-related effects on cognitive
614 performance: Roles of apoptosis in rat hippocampus following sleep deprivation.
615 Biochemical and Biophysical Research Communications. 534:632-638

70. Pearse DD, Hughes ZA. (2016) PDE4B as a microglia target to reduce neuroinflammation. Glia. 64(10):1698–709.

71. Zuo G, Zhang T, Huang L, Araujo C, Peng J, Travis Z, et al. (2019) Activation of TGR5 with INT-777 attenuates oxidative stress and neuronal apoptosis via cAMP/PKCε/ALDH2 pathway after subarachnoid hemorrhage in rats. Free Radical Biology and Medicine. 143:441–53.

72. Wang H, Zhang F, Xu Y, Fu H, Wang X, Wang L, et al. (2020) The Phosphodiesterase-4 Inhibitor Roflumilast, a Potential Treatment for the Comorbidity of Memory Loss and Depression in Alzheimer's Disease: A Preclinical Study in APP/PS1 Transgenic Mice. International Journal of Neuropsychopharmacology. 23(10):700–11.

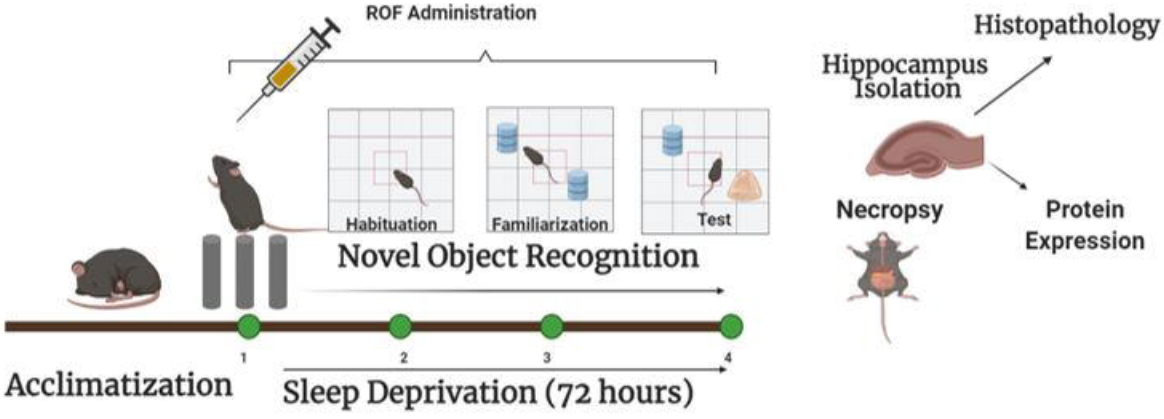


Figure 1: A schematic outline of the study design

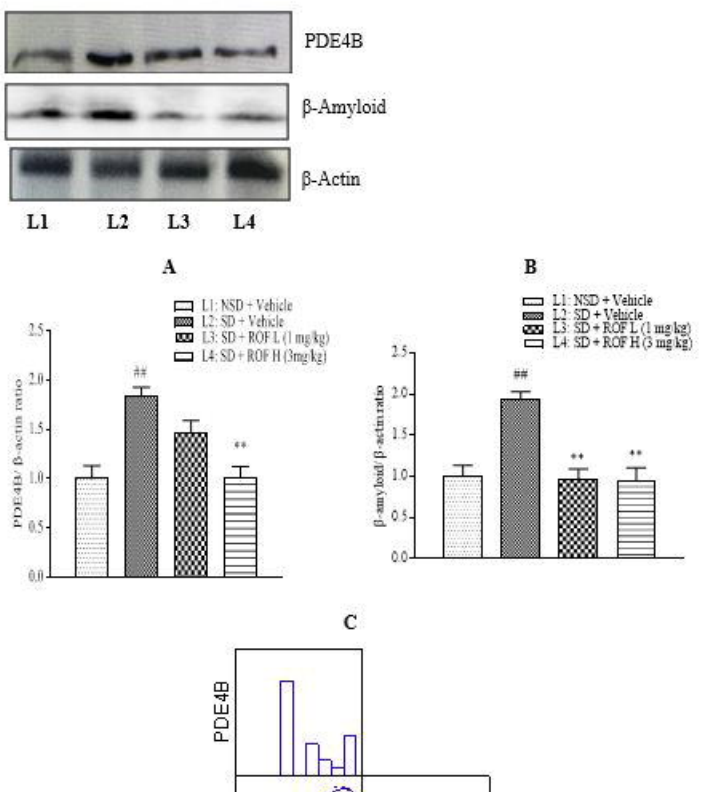


Figure 2: Roflumilast downregulates the expression of PDE4B and β-amyloid in hippocampus of SD mice. (A) Quantification of PDE4B/β-actin (B) Quantification of β-amyloid /β-actin. Data are presented as the mean ± SEM (n=6), ## denotes $p < 0.01$ versus vehicle treated NSD group, ** denotes $p < 0.01$ versus vehicle treated SD group. Correlation analysis was performed using Pearson's correlation test; graphs showing the correlation between PDE4B and Aβ.

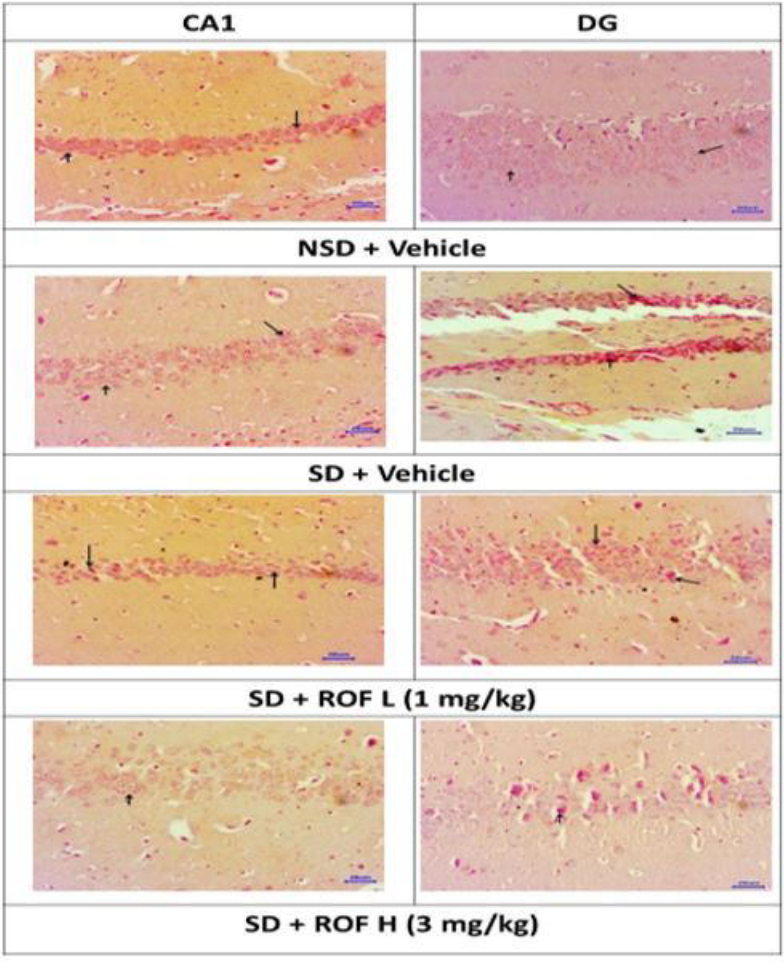


Figure 3: Effect of ROF on amyloid-β plaques in CA1 and DG regions of hippocampus in sleep deprived mice. Congo red staining of CA1 and dentate gyrus region of hippocampus.

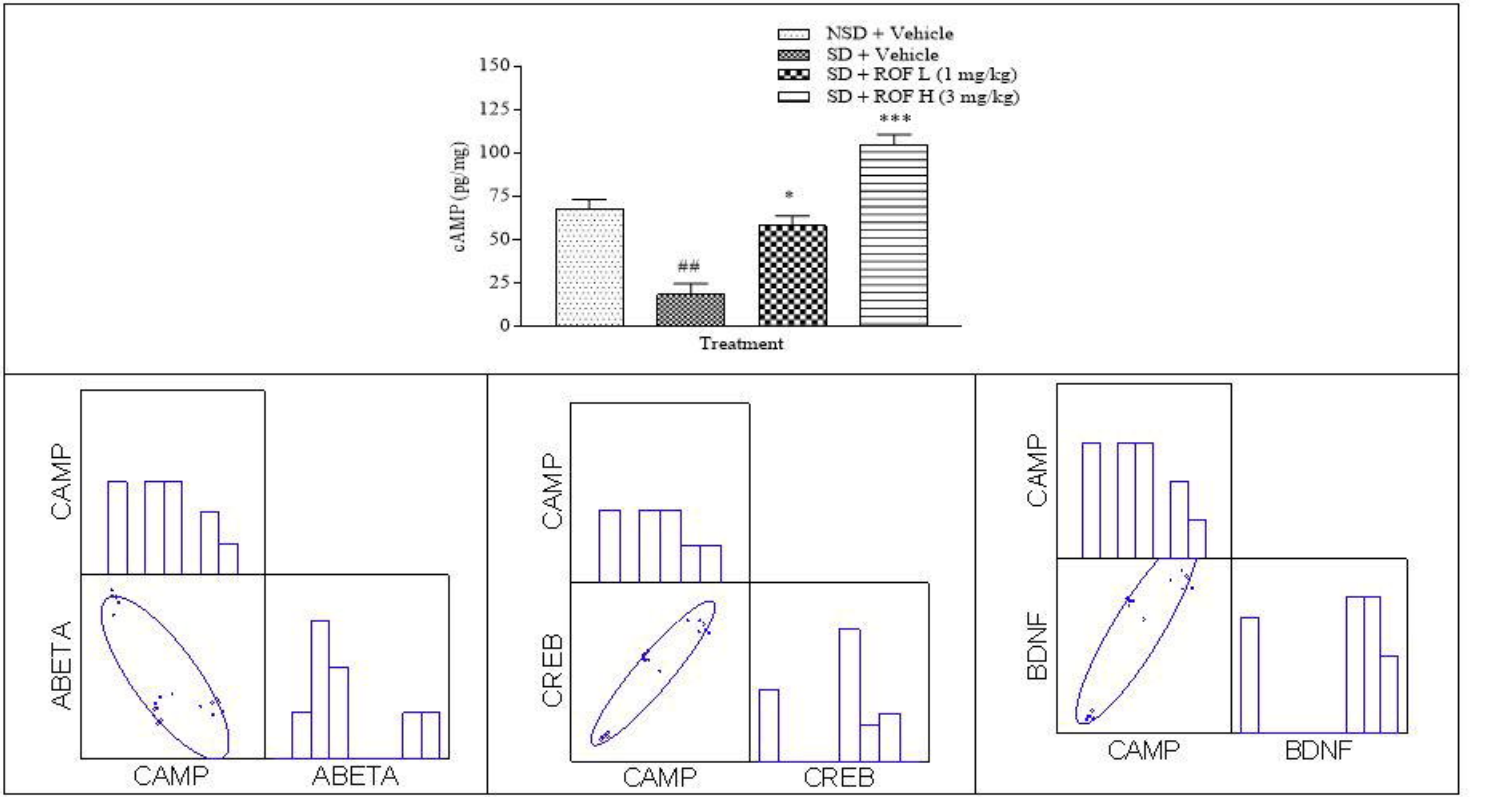


Figure 4: Roflumilast administration restores SD mediated decrease in cAMP level in mice. Data are presented as the mean ± SEM (n = 6). ## denotes $p < 0.01$ versus vehicle treated NSD group, * denotes $p < 0.05$, and ** denotes $p < 0.01$ versus vehicle treated SD group. Correlation analysis was performed using Pearson's correlation test; graphs showing the correlation between cAMP and Aβ, cAMP and CREB, cAMP and BDNF.

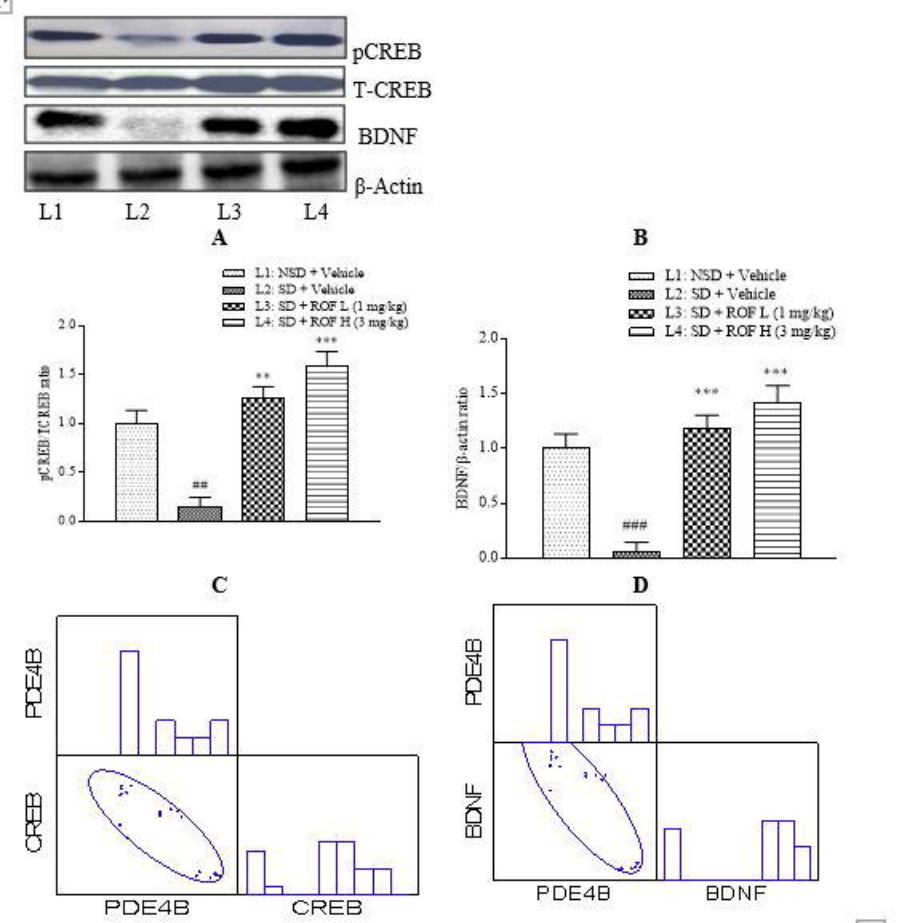


Figure 5: Roflumilast administration increases transcription factors in sleep deprived mice. Quantification of pCREB/TCREB (B) Quantification of BDNF /β-actin. Data are presented as the mean ± SEM (n = 6), ## denotes $p < 0.01$, ### denotes $p < 0.001$ versus vehicle treated NSD group, ** denotes $p < 0.01$, *** denotes $p < 0.001$ versus vehicle treated SD group. Correlation analysis was performed using Pearson's correlation test; graphs showing the correlation between (C) CREB and PDE4B, (D) BDNF and PDE4B.

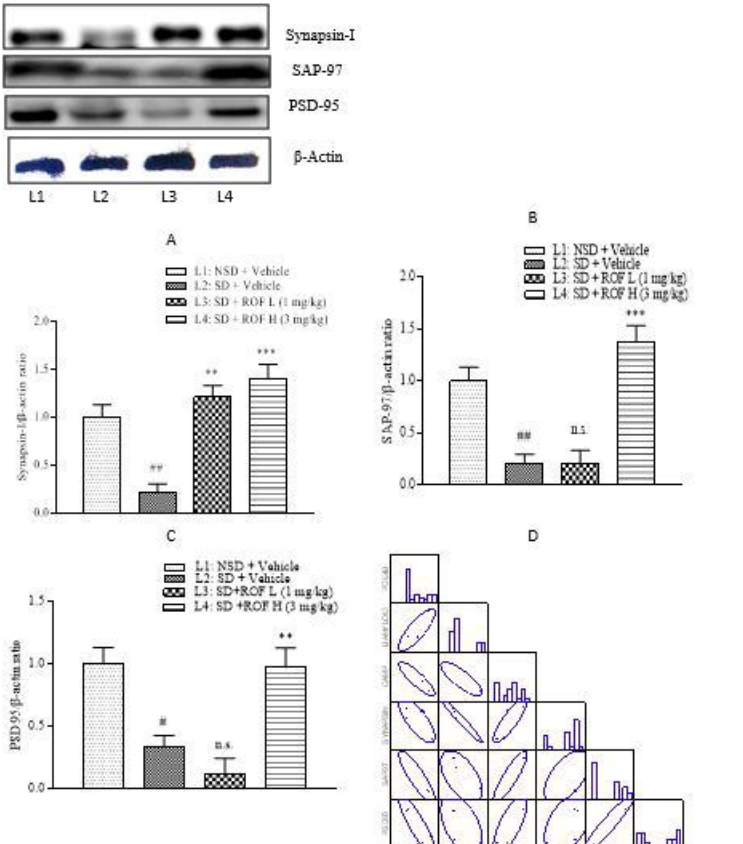


Figure 6: Roflumilast upregulates the expression of synaptic proteins in SD mice. (A) Quantification of Synapsin-I/β-actin. **(B)** Quantification of SAP-97/β-actin. **(C)** Quantification of PSD-95/β-actin. Data are presented as the mean ± SEM (n=6). # denotes $p < 0.05$, ## denotes $p < 0.01$ versus vehicle treated NSD group, ** denotes $p < 0.01$ and *** denotes $p < 0.001$ versus vehicle treated SD group, n.s. denotes non-significant. **(D)** Correlation analysis was performed using Pearson's correlation test; graphs showing the correlation between cAMP, PDE4B, Aβ with SAP-97, Synapsin-I, PSD-95.

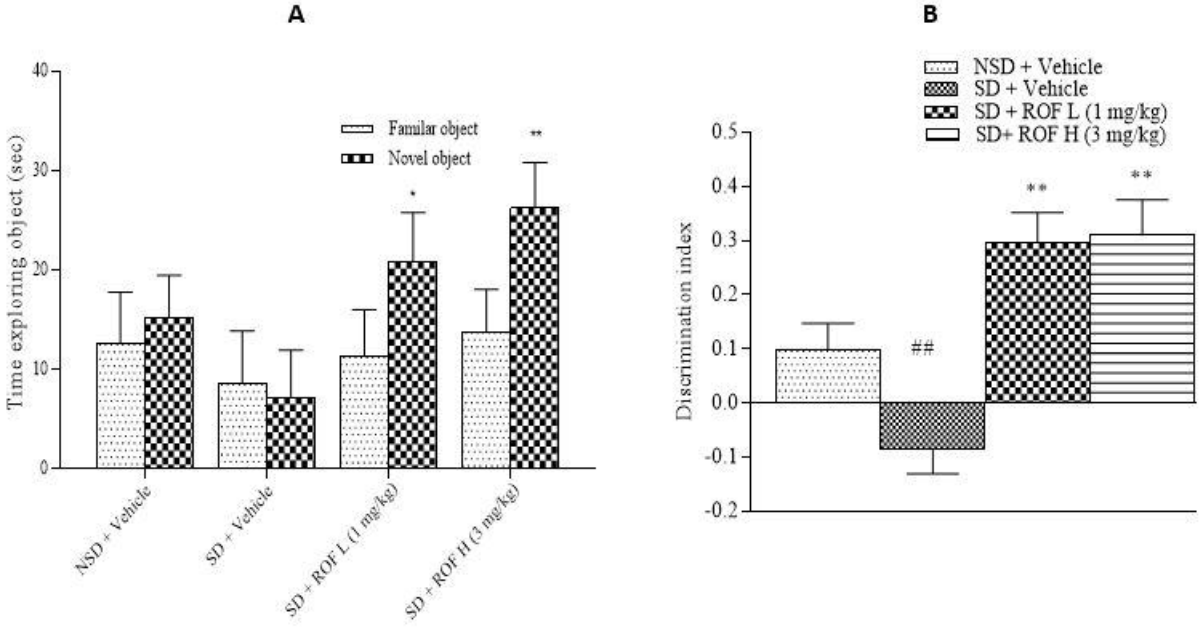


Figure 7: Effect of Roflumilast on recognition memory evaluated by NORT in sleep deprived mice. (A) time exploring the familiar and novel object (B) Discrimination index. Data are presented as the mean ± SEM (n = 10). ## denotes $p < 0.01$ versus NSD group and * denotes $p < 0.05$, ** denotes $p < 0.01$, versus SD group.

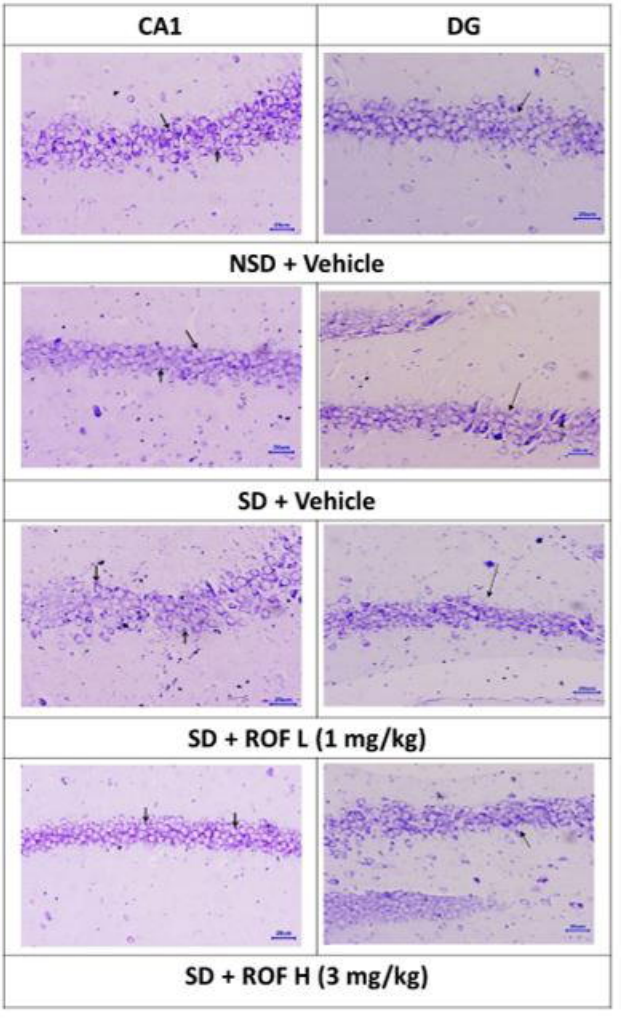
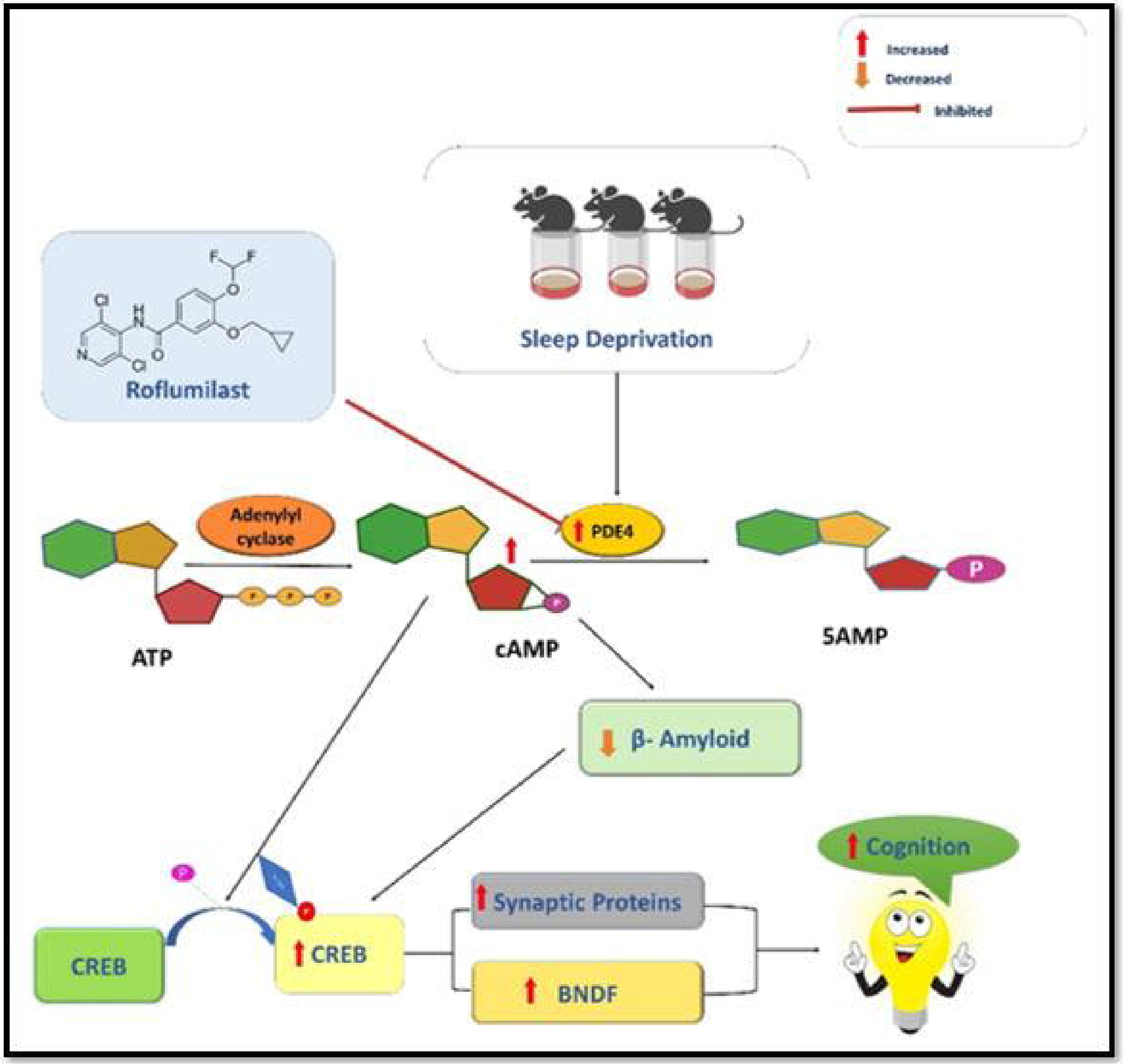


Figure 8: Effect of ROF on hippocampal neuronal morphology of mice sleep deprived for 72 h. Nissl staining of CA1 and dentate gyrus region of hippocampus.


Increased
Decreased
Inhibited
Roflumilast
Sleep Deprivation
Adenylyl cyclase
PDE4
ATP
cAMP
SAMP
β- Amyloid
Cognition
Synaptic Proteins
CREB
CREB
BNDF