Cyclic AMP Pathway Modifies Memory through Neural Cell Adhesion Molecule Alterations in the Rat Hippocampus

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Abstract- Neural Cell Adhesion Molecules (NCAMs) are known to influence memory by affecting neural cell-cell and cell-extracellular matrix junctions. This study investigated the possible role of cAMP pathway in the expression of hippocampal NCAM and its polysialylated derivative (PSA-NCAM). The following pharmacological tools were employed for manipulation of cAMP pathway: a) forskolin; the activator of adenylyl cyclase (AC), b) 8-Br-cAMP; a protein kinase A (PKA) agonist, c) 8-pCPT-2′-O-Me-cAMP; a selective enhancer of exchange protein activated by cAMP (Epac) and d) Rp-cAMP; a PKA inhibitor. Memory acquisition was tested by passive avoidance paradigm after injecting the above compounds for three consecutive days into the CA1 region of dorsal hippocampus of rats. Forskolin and 8-Br-cAMP enhanced memory retrieval while Rp-cAMP significantly reduced memory and NCAM levels. 8-pCPT-2′-O-Me-cAMP failed to alter memory performance or NCAM levels as compared to vehicle. We observed no significant changes in PSA-NCAM, however the expression of St8sia4 and St8sia2 (the polysialyltransferase isoforms) were altered. The mRNA levels of St8sia4 was down-regulated by 8-Br-cAMP, Rp-cAMP and 8-pCPT while forskolin led to almost 3 and 5 fold increase in mRNAs of St8sia2 and St8sia4, respectively. The current insight might endorse the predominant role of PKA as compared to Epac in cAMP pathway in expression of NCAM and memory function.

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Introduction

Memory retrieval in murine can be facilitates when accompanied with a same context. This type of memory is dependent on hippocampal function both pre- and post-training. Pharmacological studies have identified the crucial role of cAMP through two effectors; PKA and Epac (exchange proteins directly activated by cAMP) in contextual memory processes. The intra-lateral ventricular and intra-hippocampal injections of PKA antagonist prior to the test have yielded distinct outputs in contextual fear freezing (1) and step-down latency (2), respectively. Also administration of an Epac activator Sp-8-pCPT-2'-O-Me-cAMP into hippocampus thirty minutes before testing, can enhances memory retrieval like Sp-6-Phe-cAMPS, the PKA agonist, in the fear conditioning model (3). In addition, cAMP can influence downstream signaling by binding to PKA or Epac, a guanine exchange factor for Rap. Which can activate p42/44MAPK that alters the expression levels of required proteins through affecting the nuclear targets (4).

On the other hand, NCAM and its poly glycosylated form (alpha 2,8-linked sialic acid) are known to play roles in the memory formation processes such as neural plasticity, neurite outgrowth, synaptic locking, synaptogenesis as well as fasciculation in adult mammalians (5-8). NCAM proteins can stabilize neurite junctions by their homophilic or heterophilic bonds. On the contrary, adding PSA moiety via the two main isoforms ST8SIA2 (STX) and ST8SIA4 (PST) to NCAM, promotes plasticity in adult vertebrates' hippocampus and olfactory bulb (9,10). Furthermore, the pharmacological (11) and genetic (12) modifications of

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NCAM proteins in experimental animals alter LTP in CA1 of hippocampus (13) and amygdala (14) during the acquisition or consolidation of memory in passive avoidance (11) and spatial memory task (15). The eradication of polysialic acid (PSA) by the enzymatic action of endo-neuraminidase also yielded similar results (16,17).

Few studies investigated the signaling cascades that lead to the expression of the CAMs in neuronal cells. In endothelial cells, a rise in cAMP and the activation of PKA or Epac-Rap pathway leads to the expression of cadherin (18), tightening of junctions and adhesion to extracellular matrix (19-21). The previous observations about involvement of cAMP effectors on non-neuronal cells adhesion and junction raises the question that it may have the same effect on neural cells CAMs, especially in NCAM; the abundant CAM in CNS. In order to examine the possible effect of cAMP, we explored the effects of the altered intracellular cAMP signaling pathway on the expression levels of NCAM 120, 140, 180 KDa isoforms and PSA-NCAM in hippocampus. Also we investigated the contribution of two cAMP effectors, PKA and Epac, on expressions and effects of CAMs in memory function of rats.

Materials and Methods

Animals

Adult male wild type, W125 Wistar rats were housed under standard conditions, having free access to food and water. They were exposed to 12 hours light/dark cycle. Experiments were carried out in accordance with the principles of laboratory animal care of Tehran University of Medical Sciences. Maximal efforts were made to minimize the animals' suffering during the tests.

Stereotaxic surgery and treatments

The surgical procedures were performed according to the well described method for stereotaxic operation in rats (22). Animals were anesthetized by intraperitoneal injection of ketamine (75 mg/kg) and xylazine (10 mg/kg) cocktail. The obtained coordination from Paxinos and Watson's atlas of rat brain (23) for the CA1 region of dorsal hippocampus (DH) was AP: -3 mm from Bregma, ML: ± 2 mm and DV: 2.9 mm. Coordinates was measured separately for individual animals according to their Bregma-Lambda distance. Two holes were symmetrically made by dental drill on the surface of skull. The Guide cannulas (22 gauge stainless steel needles) were inserted to the region of interest reaching one millimeter above CA1. Intra-hippocampal injection was performed by 28 gauge needles that were connected to a Hamilton syringe through a polyethylene tube (IntΦ 0.6 mm). Needles were one millimeter longer than the guide cannula. All compounds were dispersed in DMSO 10% and administrated to the animals' hippocampus 24, 48 and 72 h prior to training. A volume of 0.5 µl of the prepared compound was injected into each hippocampus over a 60-second period.

Behavioral test

For evaluation of memory function, the well described step-through testing protocol was employed (24). In brief, the apparatus consisted of a dark and a white chamber, lit by a 100 Watt lamp hanging 20 cm above it. The two chambers were connected through a guillotine door. During the training day, animals were placed in the white chamber and allowed to explore the black one freely. After entering the black chamber, for two second 0.2 or 1 mA electric shock was delivered to their feet. Later on 24 hours, the test was repeated and the latency of entrance to the dark area was considered as an index of memory. The determined cut-off time was 300 seconds (25).

Sample preparation to protein assay

Animals were deeply anesthetized and euthanized after the behavioral test; two hippocampi were extracted and immediately immersed in liquid nitrogen. Samples were stored at -80 ºC for the future experiments. Each hippocampus was homogenized using a 100 µl of the lysis buffer containing 50 mM Tris (pH=8), protease inhibitor cocktail provided from Roche Inc. (one tablet for 10 ml), 1 mM EDTA, 150 mM NaCl, 0.1% triton X10 and 0.25% sodium deoxycholate (all acquired from Merck Inc.).

Protein extraction and immunoblotting

To ensure that equal amounts of protein were loaded into each well, modified method of Lowry's procedure was applied to measure the total amount of each protein sample (26, 27).

The 10% acrylamide/bisacrylamide gel was used for the purpose of protein separation. Protein transferring to the nitrocellulose membrane was performed overnight in a wet system under the 20v constant voltage. Following the transfer, membrane was blocked using the 2% W/V fat-free dry milk in TBST solution (10 mM TrisHCl, 150 mM NaCl, 0.01% V/V Tween 80, PH=7.4). Rabbit anti-rat antibody (Abcam; ab9018), which recognizes all

isoforms of NCAM, Millipore anti-polysialic acid-NCAM antibody (P13591), and GAPDH antibody (Abcam; ab8245) were used for protein blotting. Incubation with primary antibody was performed at 4 ºC overnight. After 4×10 minutes of washing with TBST, membrane was incubated with peroxidase conjugated goat anti-rabbit antibody (Abcam; ab2891) diluted with blocking solution 1:15,000 as a secondary antibody for one hour at room temperature. After 4 times washing, blots were developed with an enhanced chemiluminescence detection kit (Amersham). The blot was then exposed to the x-ray film for an adequate period of time. Area of the bands was outlined and the blots densities were analyzed by the ImageJ software through a semi-quantitative manner. The mean band intensity of the three NCAM isoforms: 120, 140, 180 and the total PSA-NCAM were assessed and normalized to GAPDH protein.

Quantitative real-time PCR

Tissue homogenizing and isolation of total RNA was performed according to the kit manual instruction for lipid tissues provided by Qiagen RNeasy extraction kit (Qiagen, Hilden, Germany). The ratio of rRNA 18s:28s band density confirmed the quality of the extraction of the total RNA. cDNA was synthesized through standard protocol (28). First strand cDNA synthesis from 1000 ng of total RNA was performed using avian myeloblastosis virus reverse transcriptase (Cinnagen) primed by random hexamers (Cinnagen) according to manufacturer's instructions. Quantitative real-time PCR experiments were performed with a Corbett 65H0 instrument (Corbett Research). Primers were purchased from Qiagen company (Gapdh: QT00199633, St8sia2: QT00189385, St8sia4: QT02381687, details are presented in Table 1).

Table 1. Primers specifications

Gene Symbol	Entrez gene ID	Gene name	Qiagen Cat. #	Target transcript	Amplified exons	Amplicon length (bp)
St8sia2	117523	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 2	QT00189385	NM_057156	1/2	76
St8sia4	116696	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 4	QT02381687	NM_053914	1/2	120
Gapdh	24383	glyceraldehyde-3-phosphate dehydrogenase	QT00199633	NM_017008	1/3	149

The QuantiFast SYBR Green PCR Kit (Qiagen) was used for the detection purpose. The specificity of PCR products was confirmed by both melting curve analysis and agarose gel electrophoresis. Gapdh was chosen as a housekeeping gene. The acquired data were normalized to the expression level of Gapdh. Changes in the expression of St8sia2 and St8sia4 of each treatment were calculated using Corbett instrument software. The fold change values were calculated using Pfaffl method (29). The expression ratios analyses were performed using Relative Expression Software Tool (REST) 2009 program (29) (n=4 for all groups).

Statistical analysis

ANOVA followed by a post-hoc Tukey test was used to compare all groups' results in the behavioral tasks. The image densities were analyzed using the Imagej an image processing tool. The analyses of the behavioral tests were performed using SPSS software version 20. The evaluation of the expression ratios was done through a randomization test described by REST software guide (29). QRT-PCR and immunoblotting related graphs were drawn by GraphPad Prism software.

Experimental Design

Totally 42 rats were divided into six groups were operated with the described stereotactic technique. The animals received saline, forskolin (0.5 µg/side), 8-Br-cAMP (1.25 µg/side), Rp-cAMP (0.02 µg/side) or 8-pCPT-2′-O-ME-cAMP (5 µg/side) at time 0, 24 and 48 (three consecutive days).The additional seven rats served as sham-operated group. The administration timing procedure was chosen because of the expression period of NCAM and PSA-NCAM after manipulating cAMP pathway in the rat hippocampus (30). The above doses of the compounds were selected according to the related literatures (31).

Result

Passive avoidance test

One-way ANOVA revealed a significant difference in the latency of entrance to the dark zone among the treated groups [0.2 mA: $F(5, 36) = 189$, $P<0.0001$; 1

mA: F (5, 36) =, $P<0.0001$ Fig. A]. Tukey's HSD results showed a significant variation in memory retrieval among forskolin (5 µg/0.5 µl)/8-Br-cAMP (1.25 µg/0.5 µl) groups as compared to other groups which trained by 0.2 mA shock (both, $P<0.001$). However, animals which received 8-Br-cAMP (the activator of PKA), demonstrated no significant difference in the latency of entrance as compared to forskolin-treated rats (0.2 mA: $P=0.069$, 1 mA: $P=0.74$). Similar to the 8-pCPT-2′-O-Me-cAMP (5 µg/0.5 µl), there was no change in the latency of entrance of Rp-cAMP (0.02 µg/0.5 µl) in 0.2 mA shock received groups as compared to control/sham groups (for all, $P>0.05$). But the 1 mA current shock revealed a significant difference between Rp-cAMP and other treated groups [F (5, 36) =, $P<0.0001$]. Lack of difference ($P=0.505$ for 0.2 mA and $P=0.957$ for 1mA) in the duration of latency of entrance between the control and sham-operated groups demonstrates that the injection has no effect on rats' memory function (Figure A).

Fig. A

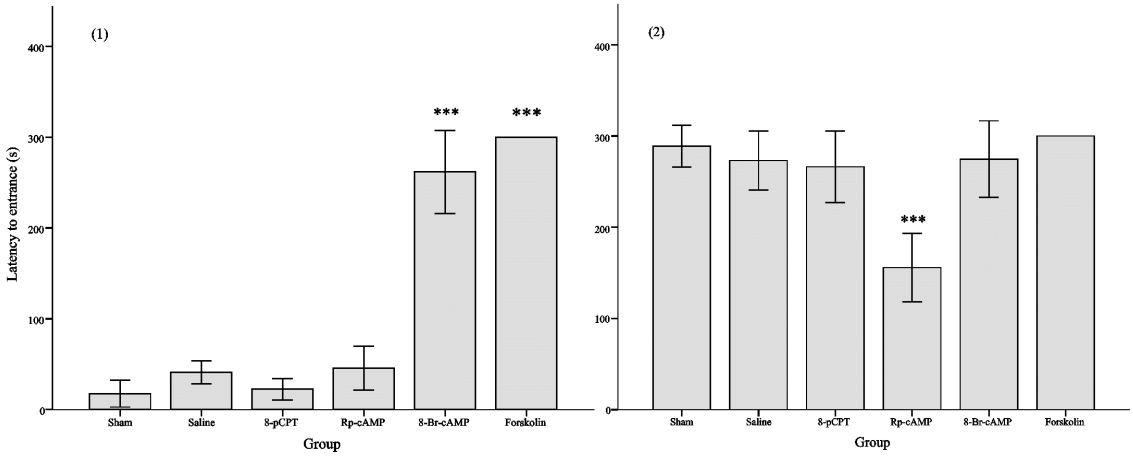


Figure A. Latency to entrance to the dark zone in step-through passive avoidance test; Animals received 0.2 mA **(1)** or 1 mA **(2)** shock 24 hours prior to the test. Bars demonstrate mean and standard error of mean, considered confidence limit is 95%. Tukey's post hoc was applied to compare results in between groups. Forskolin (0.5 µg/side) and 8-Br-cAMP (1.25 µg/side) groups in the low shock and Rp-cAMP (0.02 µg/side) in the high shock experiment showed significant differences as compared to control and sham groups (n=7, *** $P<0.0001$)

Immunoblotting

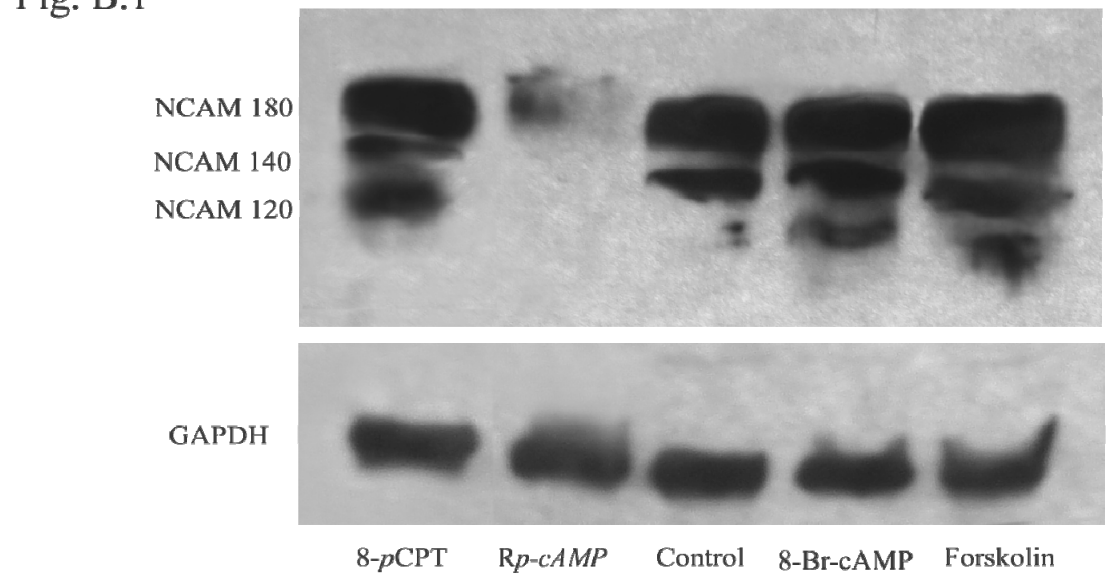
Because of the effect of high shock intensity in these cell adhesion molecules levels all molecular studies were performed on the low shock received animals. Immunoblotting images demonstrated a significant difference in the expression of hippocampal NCAM 120 [F (4, 15)=7.18, $P=0.0019$], 140 [F (4, 15)=6.01, $P=0.004$], 180 [F (4, 15)=5.5, $P=0.0065$] and total NCAM [F (4, 15)=8.5, $P=0.0009$] expression of the groups. Post-hoc test showed the reduction of NCAM expression in the Rp-cAMP injected tissues as compared to the control group ($P<0.05$). However PKA and adenylyl cyclase-activated hippocampi expressed equivalent levels of all isoforms of NCAM proteins compared to the control group ($P>0.05$). The reduction of NCAM content was remarkable in NCAM 140 and 180 kDa isoforms in Rp-cAMP group, whereas the blots showed a rise in the expression of 120 kDa isoform of forskolin and 8-Br-cAMP groups compared to the group which received vehicle. The 8-pCPT, in comparison with forkolin and 8-Br-cAMP, reduced NCAM. This reduction was most notable in 120 and 140 kDa isoforms ($P<0.001$). However, such alterations were not detectable in the 8-pCPT- treated hippocampi as compared to vehicle treated tissues (Figure B. 1).

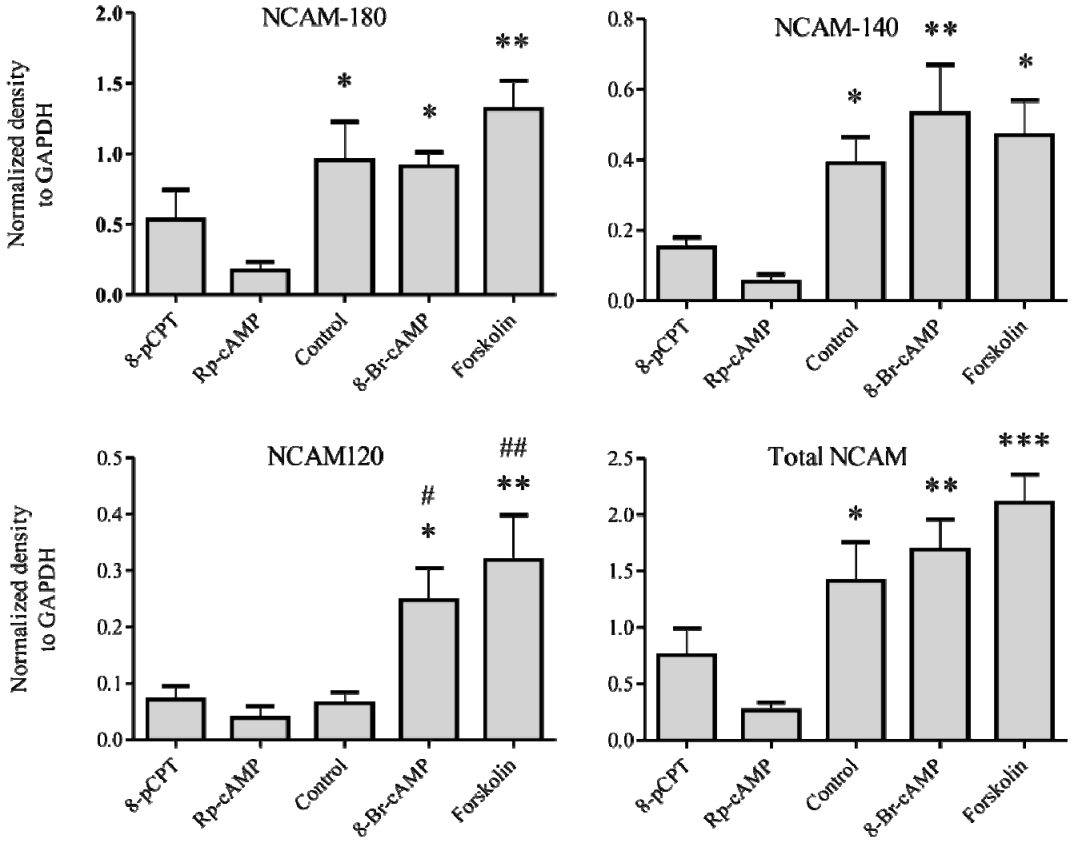
In terms of PSA-NCAM blotting data (Fig. B. 2), there were no significant difference in the level of polysialylated form of NCAM in the treated groups. The PSA-NCAM amount of all groups remained the same as the control group [F (4, 20) =1.81, $P=0.59$].

Real-time PCR

To explore the role of modulating agents on cAMP pathway in the polysialylation of the adhesion molecules, we examined hippocampal mRNA levels of St8sia2 (Polysialyltransferase-1) and St8sia4 (Polysialyltransferase-2) of groups of animal which received 0.2 mA. In spite of the level of polysialylated NCAM, mRNA expression of St8sia2 after a 3-day injection of cAMP pathway modifying agents was altered in the rat hippocampus.

Fig. B.1


NCAM 180
NCAM 140
NCAM 120
GAPDH
8-pCPT
Rp-cAMP
Control
8-Br-cAMP
Forskolin


NCAM-180
NCAM-140
NCAM120
Total NCAM
Normalized density to GAPDH
8-pCPT
Rp-cAMP
Control
8-Br-cAMP
Forskolin

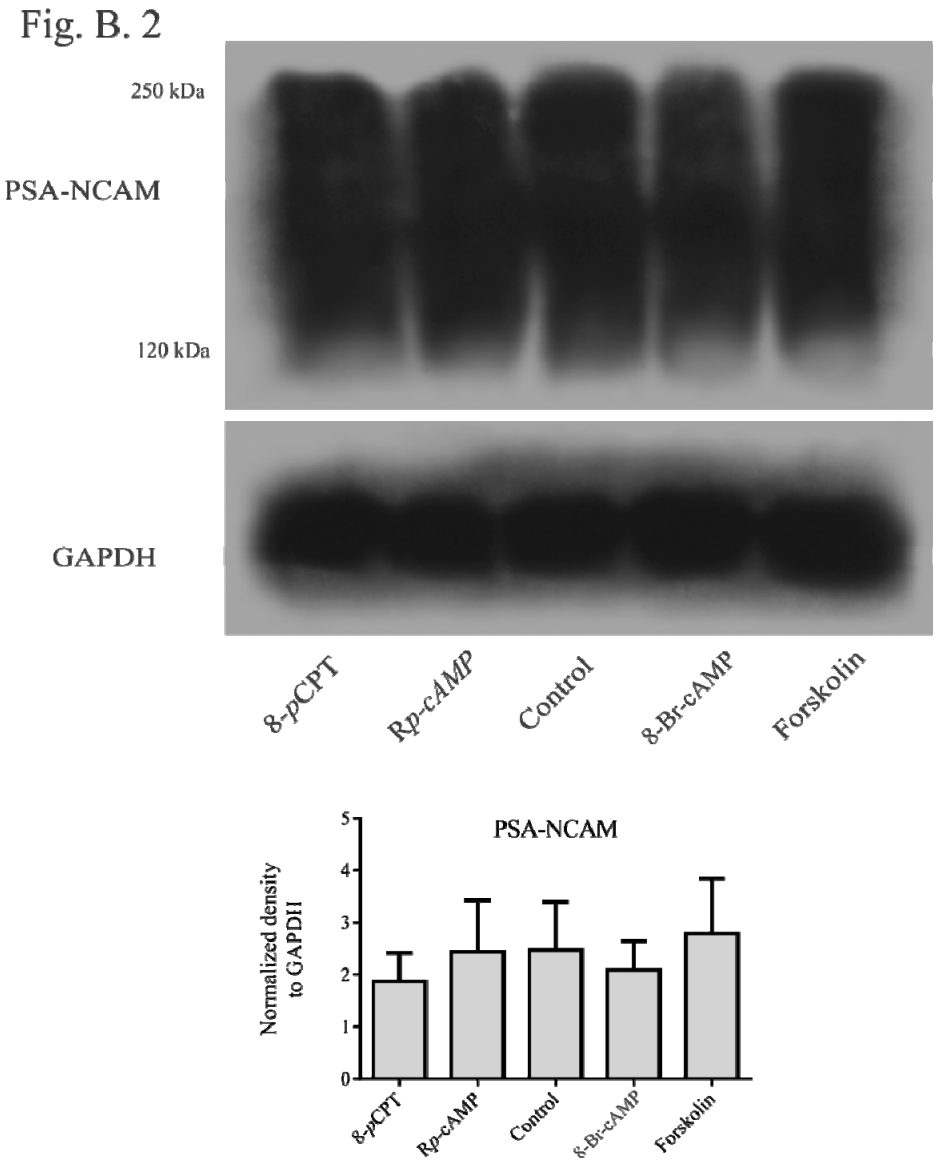


Figure B. Representative immunoblotting of the effect of 0.5 µl of DMSO 10% as control, Forskolin (0.5 µg), 8-Br-cAMP (1.25 µg), 8-*p*CPT-2′-O-ME-cAMP (5 µg) and Rp-cAMP (0.02 µg) on hippocampus for 3 days in expression of the hippocampal NCAM 120,140 and 180 **(B-1)** and PSA-NCAM **(B-2)** in male rats (n=4, * $P<0.05$, ** $P<0.01$, *** $P<0.001$ different from Rp-cAMP group and # $P<0.05$, ## $P<0.01$ different from control group

Real-time PCR

To explore the role of modulating agents on cAMP pathway in the polysialylation of the adhesion molecules, we examined hippocampal mRNA levels of St8sia2 (Polysialyltransferase-1) and St8sia4 (Polysialyltransferase-2) of groups of animal which received 0.2 mA. In spite of the level of polysialylated NCAM, mRNA expression of St8sia2 after a 3-day injection of cAMP pathway modifying agents was altered in the rat hippocampus. The randomization test p-values less than the α-level (0.05) were considered significant for all comparisons. qRT-PCR data revealed almost a two folds decrease in mRNA levels of St8sia2 when treated with 8-Br-cAMP (P=0.032) and Rp-cAMP (P=0.018) whereas AC and Epac activators up regulated the mRNA levels by approximately three folds as compared to the control hippocampi (both, P=0.015). Changes in the St8sia4, another isozyme mRNA levels, were the same as polysialyltransferase-1 isozyme in forskolin, 8-Br-cAMP and Rp-cAMP groups [5

(*P*=0.01), 0.1 (*P*=0.013), and 0.3 (*P*=0.032) respectively]. Significant difference was observed in the 8-pCPT administered hippocampi between Polysialyltransferase-1 and -2 mRNA quantities. The Epac activator could upturn the first isoform gene expression, whereas the alterations of the second isozyme mRNA levels were not significant (*P*=0.3; Figure C).

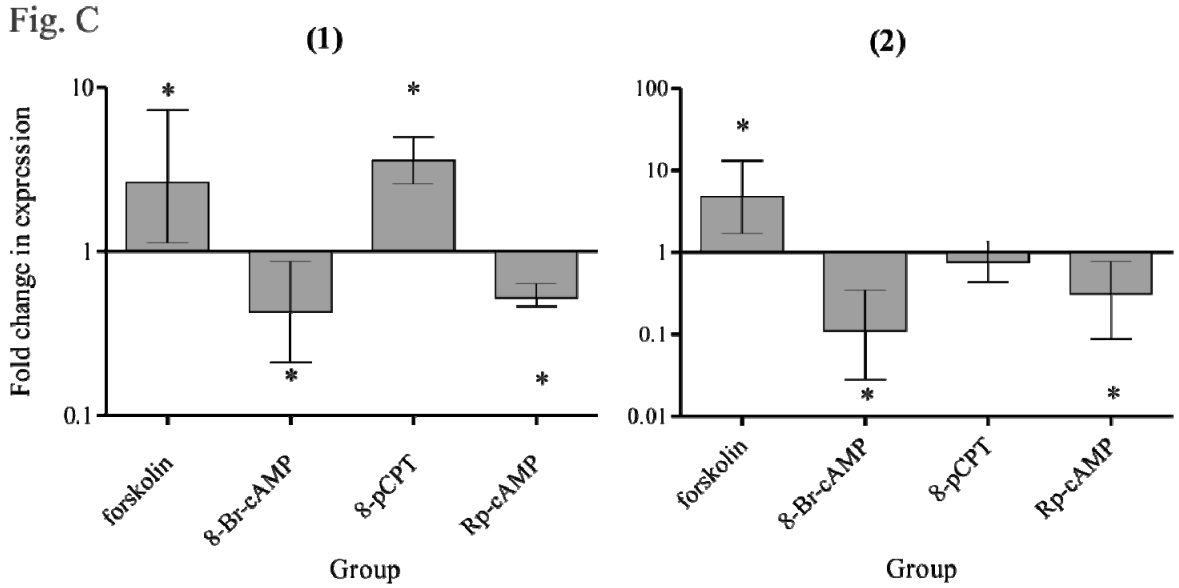


Figure C. St8sia2 (**1**) and St8sia4 (**2**) mRNA expression of forskolin (0.5 µg), 8-Br-cAMP (1.25 µg), Rp-cAMP (0.02 µg) and 8-*p*CPT-2′-O-ME-cAMP (5 µg) in three days treated hippocampi; Y axis on a logarithmic scale demonstrates mRNA expression ratio level relative to the control group. mRNA level of each sample were normalized to its own Gapdh mRNA level. Data are shown as Mean ± SE (n=4, randomization test, * $P<0.05$)

Discussion

In the present work we reported new data regarding the modification of hippocampal NCAM by cAMP and its downstream effectors. The obtained data suggest new mechanisms of action of Gs-AC-PKA/Epac in memory modification.

Several reports pointed out the effects of cAMP signaling pathway in learning and memory (32,33). This idea is originated from the positive impact of norepinephrine (NE) a G-protein coupled receptor agonist on memory as well as the propensity of beta-1 blockers to impair memory. Consistent with the above findings, antagonist of PKA or Gs-coupled receptor can disturb memory retrieval. However, studies on Dbh-/- mice showed that NE deficiency failed to impair the hippocampus-associated memory formation (34). Moreover, hippocampal NCAM up-regulation is documented for memory consolidation in rats (35). Thus, we investigated the effects cAMP pathway modifying agents on the possible alterations of synaptic plasticity process within DH after three consecutive day's treatment. The expression of the NCAM, PSA-NCAM and polysialylation-involved enzymes, the essential effectors of hippocampal plasticity were measured.

As expected, forskolin had the greatest effects on the cAMP pathway upon memory retrieval during the passive avoidance test. A possible explanation could be that the three-day activation of adenylyl cyclase might have prepared the hippocampus for its memory tasks. The activation of PKA by 8-Br-cAMP also improved the memory retrieval in the passive avoidance test. While the high-dose (5 µg) of 8-pCPT-2′-O-Me-cAMP, an Epac activator failed to potentiate it. Consequently among cAMP targets, PKA retains to have a greater impact on memory retrieval over the mentioned period of time. The difference between Rp-cAMP and control groups in the latency of entrance to the dark chamber is dependent on shock intensity. The low foot shock was unable to differentiate the memory impairment response of Rp-cAMP from other groups but the significant difference was observed when 1 mA shock was used. Also in the study it was reported that the blockage of

PKA 10 minutes prior to passive avoidance test impaired the rats memory retrieval previously trained by using 0.4 mA shock (2). During PKA blockade, the PKA independent learning and memory may also be considered. Because the cyclic nucleotide-gated- and hyperpolarization-activated (HCN), as well as the cyclic nucleotide-activated ion channels (CNG) are the two cAMP effectors that may have a role in memory impairment (36).

Studies about the foot shock stress reported the alteration of NCAM expression in rats is due to raise of glucocorticoids levels (37). They showed that the low and intermediate (0.2 and 0.4 mA) foot shock intensity have no effect on rat hippocampal NCAM levels after 24 hours of training but its levels were raised after high current intensity (1 mA). Since, we expect that there is no influence of stress on the expression of NCAM after applying 0.2 mA shock in the passive avoidance test; a statement that is not attributable to the 1 mA shock. Therefore all of the present immunoblotting and qRT-PCR experiments were performed on the groups that received low foot shock.

Since the cAMP signal propagation lead to the activation of cAMP response element-binding protein (CREB), therefore the elevation of some gene transcriptions and proteins synthesis is expected in the treated hippocampus. Quantity/content shift of the CAMs are the prerequisites in the locking and formation of synapses, axonal and dendritic growth which occur during memory formation (38,39). Changes in expression of cell adhesion molecules were investigated under the manipulated cAMP condition in the neuronal cell culture (40). Therefore, some NCAM modifications were anticipated after the manipulation of the hippocampal cAMP pathway. Our results showed that the activation of adenylyl cyclase or PKA possibly makes a remarkable contribution to the NCAM expression changes. Basal PKA or adenylyl cyclase activities are required for the adhesive property of neuronal junctions which is required during the consolidation phase of memory. Inhibition of PKA by its selective inhibitor possibly decline neuronal NCAM levels and rendered synapses non-sticky, hence could be resulted in a relative failure in consolidation of the acquired information.

NCAM 120 is the cell membrane anchored isoform which was predominantly found in glial cells (41). It might be pointed out that hippocampal NCAM over-expression following cAMP up-regulation was more prominent in glial cells. However, due to the low contents of NCAM in glial cells (42) the observed increase could not impact on the total rats' hippocampal NCAMs.

These results are in line with previous studies which shows that no STX/PST mRNA changes occur even until 4 hours after LTP induction in the hippocampus (43). Nevertheless, current results demonstrated an elevation of St8sia2 and St8sia4 by PKA stimulation and of St8sia2 by Epac activation in an extended time course (72 hours). Surprisingly, variation in polysialylated derivative of NCAM was not significant 48 hours after the stimulation/suppression of the cAMP pathway. These findings may imply that hippocampal polysialylation is mainly dependent on enzyme production and activation and not upon the new transcription of the relevant mRNA.

Of the two major polysialylating enzymes, ST8SIA4 is the predominant enzyme in adult hippocampus while ST8SIA2 is active during the embryonic stages and is responsible for neuronal development (44). It is plausible that the increase in the ST8SIA2 expression by forskolin and 8-pCPT-2′-O-Me-cAMP play a major role in PSA-NCAM production in adult rat hippocampus. Although not within the scope of the present study, these findings may support the presence of a link between adenylyl cyclase, Epac and ST8SIA2 during the embryonic brain development (45).

Our results confirmed that the up-regulation of NCAM in course of learning is possibly dependent on PKA but not the Epac function. This was concomitant with a tardive elevation in polysialylating enzymes expression. The present findings may further emphasize on the predominant role of PKA in signal transduction of cAMP, resulting in formation of new proteins in the hippocampus.

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