

CREB Phosphorylation Coincides with Transient Synapse Formation in the Rat Hippocampal Dentate Gyrus Following Avoidance Learning

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SUMMARY

Spine density change in the hippocampal dentate gyrus accompanies memory consolidation and coincides with the increased expression of ribosome-rich, hyperchromatic granule cells. Although this suggests increased protein synthesis to be required for synaptic growth in the 5 to 7 h post-training period, little temporal mapping of the associated molecular mechanisms has been done. Here, we demonstrate a similar frequency of hyperchromatic cells in naïve animals and in those sacrificed 6 h post-training, suggesting a transient repression of protein synthesis in the early post-training period. Immunoblot analysis of CREB phosphorylation in the dentate gyrus supported this view, with downregulation from basal levels observed at 2 to 3 h and at 12 h post-training. Protein synthesis reactivation appears to be specific for *de novo* spine production as no change in spine frequency accompanies the immediate post-training period of depressed protein synthesis. These findings support the view that CREB-mediated gene transcription is a requirement for long-term memory consolidation and may be directly implicated in the process of synaptic growth.

KEYWORDS

passive avoidance paradigm, dentate gyrus, axospinous synapses, CREB, NCAM

INTRODUCTION

The acquisition and consolidation of long-term memory may be divided into discrete temporal phases, as has been demonstrated using amnesic agents like protein synthesis inhibitors (Ng & Gibbs, 1991; Davis & Squire, 1984). These interventive studies suggest that the triggering of molecular mechanisms during memory consolidation has consequences that outlast the initiating stimulus by 'many hours' (McGaugh, 1966; 2000; Doyle et al., 1992; Mondadori et al., 1991, 1994). One consequence of learning, observed across species and paradigms, is an alteration in synaptic number and/or structure (Bailey & Kandel, 1993; Doubell & Stewart, 1993; Hunter & Stewart, 1993; Weiler et al., 1995; O'Malley et al., 1998, 2000; Toni et al. 1999). This synaptic plasticity must occur in parallel with alterations in gene transcription and protein synthesis.

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Molecular biological studies imply most models of memory formation to invoke common gene transduction pathways that are conserved across species (Bailey et al., 1996; Mayford & Kandel, 1999). Most notably, cyclic AMP response element binding protein (CREB)-mediated gene transcription is required for memory consolidation, and CREB homologues are detected in species as diverse as *Aplysia*, *Drosophila*, rodents, and humans (Frank & Greenberg, 1994; Silva et al., 1998). Although mutation analyses have confirmed the conserved role of CREB in long-term memory and LTP, several aspects of its function remain unclear (Bourtchuladze et al., 1994; Yin et al., 1994). For example, in mice, long-term memory deficits induced by CREB mutation may be overcome by increasing training sessions and/or the inter-trial interval (Kogan et al., 1996). This suggests the timing of CREB activation to be of importance, an aspect supported by antisense-mediated CREB disruption studies in which amnesia for a spatial learning paradigm is dependent on times of administration and recall (Guzowski et al., 1997). CREB-mediated gene transcription is regulated at several levels, but most prominently by phosphorylation of serine 133 that is required for the nuclear translocation and transcription of genes containing the cAMP response element (CRE). Nevertheless, temporal activation of CREB-mediated transcription and the nature of the transcribed proteins associated with memory formation remain to be elucidated.

In previous studies, we have mapped the temporal regulation of protein synthetic events that are involved in learning-associated synaptic growth in the hippocampus (O'Connell et al., 1997; O'Malley et al., 1998, 2000). Specifically, we have described the existence of a population of ribosome-rich granule cells, termed hyperchromatic (HC) cells, within the rat hippocampal dentate gyrus that stain darkly with the basophilic dye toluidine blue (O'Connell et al., 1997). These HC cells were

found to undergo a learning-specific increase in their frequency in the 5 to 7 h period following avoidance training, suggesting that protein synthesis in the dentate becomes activated in this post-training period. Furthermore, dendrites emanating from HC cells were found to be microtubule-rich and specifically elaborated synapses during memory consolidation (O'Connell et al., 1997; O'Malley et al., 1998). We have now investigated how change in synaptic complement and/or protein synthesis correlates with CREB phosphorylation in the hippocampal dentate gyrus during consolidation of a passive avoidance task. In addition, we have extended our temporal mapping of HC cell frequency change and spine density analysis in the same hippocampal region.

EXPERIMENTAL PROCEDURES

Materials

Agar 100 epoxy resin and coated slot grids were purchased from Agar Scientific (UK). The preformed mold used for sectioning was from Zivac Millar (USA). Antibodies to phosphoCREB were purchased from Upstate Biotechnology (USA) and New England Biolabs (NEB; USA). The enhanced chemiluminescence substrate was purchased from Pierce, UK. ImageQuant software for densitometric analysis was from Molecular Dynamics (USA). Sodium pentobarbitone was from Rhone-Merrieux (France). All other laboratory chemicals, including secondary antibodies, were from Sigma Chemical Co. (UK).

Passive avoidance training

Male Wistar rats (postnatal day 80, Biomedical Facility, University College Dublin) were individually caged in a 12-h light/dark cycle with *ad libitum* access to food and water. The procedure

was identical to that described previously (Fox et al., 1995). Briefly, animals were trained in a one-trial, step-through, light-dark passive avoidance paradigm. The smaller, illuminated compartment was separated from a larger, dark compartment by a shutter, which contained a small entrance. The floor of the training apparatus consisted of a grid of stainless steel bars that could deliver a remotely controlled, scrambled shock (0.75 mA every 0.5 msec) of 5 sec duration when the animal entered the dark chamber. Before training, rats were handled, weighed, and their locomotor activity assessed in an open field apparatus over a period of 3 d. The animals were tested for recall of this inhibitory stimulus at the appropriate time post-training by placing them into the light compartment and noting their latency to enter the dark compartment within a criterion period of 300 sec. The animals were then terminally anaesthetized with sodium pentobarbitone (60 mg/kg, i.p.). Latency values significantly different from the control were determined using the Mann-Whitney *U*-test for nonparametric data and *P* values <0.05 were considered significant.

Naïve animals used in the HC cell study received the same handling procedure that was used for the animals to be trained. In the spine density study, naïve rats were sacrificed immediately upon removal from a large housing cage, thus receiving no exposure to the handling procedure, open field apparatus or training environment. All experimental procedures were approved by the Review Committee of the Biomedical Facility of University College Dublin and were carried out by individuals who held the appropriate license issued by the Irish Minister of Health.

Histology

Following transcardial perfusion with a 4% (w/v) paraformaldehyde/2% (w/v) glutaraldehyde solution at pH 7.4, brains were removed from the rats and kept overnight in the same fixative. A pre-

formed mold was used to make coronal cuts rostral to the cerebellum and caudal to the prefrontal cortex at a point approximately -3.3 mm with respect to Bregma. The two central blocks produced by this process were fixed separately to the stage of a vibroslicer -3.3 mm face uppermost, and sections of approximately 100 µm obtained in both rostral and caudal directions. The slices were collected into phosphate buffer (0.1 M) in preparation for processing into epoxy resin. This procedure resulted in all analyzed sections being coronally orientated. Despite such consistent orientation, however, the unbiasedness of the stereological method applied in this study was maintained (Mayhew, 1992). Subsequent to a 30-min post fixation in 0.1% (w/v) osmium tetroxide, the slices were dehydrated and flat embedded with epoxy resin by routine methods. Polymerization of the resin permitted the hippocampus to be excised from the brain slices and re-embedded in resin-filled capsules. Using an ultra-microtome, we identified the re-embedded hippocampus and isolated the dentate gyrus; semi-thin sections were taken. In serial sections stained with 1% toluidine, the changing form of the granule cell layer could be discerned. This distinctive change allowed a 'frame match' to be sought, whereby a series of 18 consecutive sections corresponded to the changing form of granule cell layer that was observed over the distance recorded for the serial sections (O'Connell et al., 1997). The total number of hyperchromatic cells per section was calculated as described previously (O'Connell et al., 1997).

For the ultrastructural analysis, from within the same defined dentate region, 10 serial ultrathin sections of gold interference color were cut and collected in pairs on coated slot grids (2 x 1 mm). Further semi-thin sections were then removed for a distance great enough to preclude double counting, yet sufficiently small to ensure that future ultrathin sectioning remained within the desired area. On average, two series of ultrathin

sections were taken per re-embedded dentate slice. Ultrathin sections were stained using uranyl acetate (5% (w/v) in dH₂O) and lead citrate (0.3% (w/v) in 0.1 M NaOH). Sections were examined in a JOEL 1200EX electron microscope at an accelerating voltage of 80 kV.

Stereology

The density of dendritic spines in the dentate middle molecular layer region was estimated using the double disector, an unbiased stereological method (Sterio, 1984). In this method two serial sections are photographed, the first of which is designated the reference section and the second the look-up section. Photomicrographs were printed to a final magnification of X23,000. Within an unbiased counting frame (Gundersen, 1977) of area 45 μm^2 , the number (Q) of spine necks present in the reference section but absent in the look-up section were counted. The number of spine neck counts per volume of tissue is given by:

$$N_v = Q/h \cdot a(\text{fra})$$

where

N_v is the numerical density (μm^{-3}) of spine necks;

h is the disector height (the distance between the two disector planes), and is equal to the thickness of the reference section;

$a(\text{fra})$ is the area of the counting frame (West & Gundersen, 1990).

Section thickness (h) was determined using the minimum fold technique (Small, 1968). Minimum folds were identified and photographed in ultrathin sections (at magnification X7,000), and measured on photographic prints of final magnification X17,000. The latter criteria were also used to

examine a calibrating grating replica of 1200 lines per mm, all measurements being adjusted to this standard. For each animal, disector pairs were chosen until a progressive mean test of Q values consistently showed the standard error of the mean to be less than 10% (Williams, 1977). The mean of each animal was then used to establish the final mean \pm SEM for each group. Differences between mean values were assessed using the Student t-test, a P value < 0.05 being considered significant.

Immunoblot analysis of phosphoCREB expression

The hippocampal dentate gyrus was bilaterally dissected at various times post-passive avoidance training and hand homogenized in 0.32 M sucrose containing 1 mM isobutylmethylxanthine. Nuclear extracts were prepared according to standard procedures. The protein concentration, determined according to the method of Lowry et al. (1951), was normalized in each sample to 1 mg/mL. Samples were boiled in sample buffer containing 40 mM dithiothreitol; proteins were separated by electrophoresis on 10% polyacrylamide minigels at 100 V constant voltage, followed by transfer to nitrocellulose membranes in a wet transfer system at 200 V for 1 h. Equal protein loading and transfer was verified by Ponceau S staining of the membrane. Both the Upstate and NEB antibodies recognize the 43-kDa CREB1 protein when it has undergone phosphorylation on the serine133 residue. The NEB antibody is also known to cross-react with the phosphorylated form of the related transcription factor ATF, which has a molecular weight of 38 kDa. Blocking conditions and antibody concentrations were as recommended by the manufacturers. All primary antibodies were incubated overnight at 4°C; peroxidase-conjugated secondary antibodies were incubated for 2 h at room temperature. Immunoreactivity was detected using an enhanced chemiluminescence system.

Semi-quantitative scanning densitometry

Semi-quantitative scanning densitometry was performed using the ImageQuant software system. Bands for analysis were outlined and their volumes integrated to obtain an indication of band intensity.

RESULTS

At the various post-training times, the animals were tested for recall of the passive avoidance task before being sacrificed. All animals employed in the studies achieved the criterion latency of 300s. Quantitative histological analysis was employed to determine the time-dependent expression of HC cells in the dentate gyrus following passive avoidance training (Fig. 1). Table 1 shows the

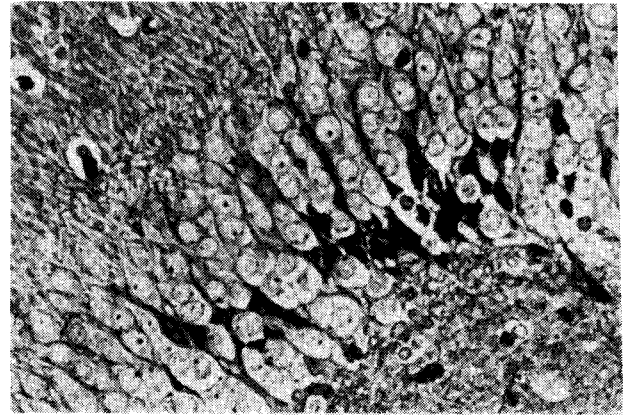


Fig. 1: Hyperchromatic granule cells in the adult rat dentate gyrus. Semi-thin coronal sections were stained with Toluidine Blue. The darkly staining neurons were those quantified in Table 1.

TABLE 1

Distribution of hyperchromatic cells in the dentate gyrus of naïve and trained adult rats following passive avoidance training.

Time post training (h)	Cell no.			n
	Dorsal blade	Ventral blade	Total	
Naïve (handled)	59.50±8.47	29.28±10.17	88.78±16.73	6
0	102.13±25.3	24.60±10.92	126.73±33.85	5
0.5	97.67±19.75	51.60±13.07	142.60±29.72	5
2	1.72±0.94*	3.33±1.25*	5.17±1.47*	6
3	36.44±9.09	10.61±3.60*	47.06±8.83*	6
4	3.27±1.23*	4.27±2.30*	7.53±2.31*	5
5	48.66±10.83	36.94±11.16	85.61±19.59	6
6	61.83±12.50	36.03±9.46	97.87±20.66	10
7	80.73±22.42	38.77±12.53	119.5±34.42	10
8	17.60±5.15*	4.53±2.27*	22.07±6.21*	5
12	11.67±3.65*	1.56±0.85*	13.22±3.77*	3
24	89.80±32.57	60.40±20.93	150.2±52.48	5
72	51.22±23.73	44.67±20.19	95.89±43.78	3

Values are the mean ± SEM of hyperchromatic cell number per section and those differing significantly from the handled naïve control are indicated by * ($p \leq 0.05$).

frequency of HC cells in the dentate gyrus in naïve animals and at various times following passive avoidance training. Data from naïve animals and those sacrificed at 0, 0.5, and 24 h post-training is specific to the current study. The frequency of HC cells observed immediately after training (0 h) was indistinguishable from that observed in handled, experimentally-naïve animals. The results, when combined with the data points generated in our previous study (O'Connell et al., 1997), reveal two time intervals—one at 2 to 4 h and another at 8 to 12 h, in which the HC cell frequency is significantly reduced compared with that in naïve animals and at earlier post-training times. Importantly, this expanded data indicate that levels of HC expression at 5 to 7 h post-training do not differ significantly from the basal state.

In our previous studies, a transient episode of synapse formation was identified at the 6 h post-training period (O'Malley et al., 1998). As that study only compared spine density levels with those found at 1 and 72 h post passive avoidance training, we have now quantified this morphological parameter both in naïve animals and in those sacrificed at the 48-h post-training time to determine if spine formation corresponds to HC cell expression. The results showed that spine density in naïve animals did not differ significantly from that observed at 1 h following training and confirmed that spine levels at 6 h were an elevation over the basal value, and in this respect, differed from the temporal expression of HC cells (Table 2). Spine density tended to remain elevated at the 48 h post-training time but this elevation was not statistically significant from that observed at 1 h or in naïve animals, indicating that this morphological parameter is regulated monophasically during memory consolidation.

In the dentate, nuclear levels of the activated (phosphorylated) form of CREB showed two variations over the post-training range of 0 to 24 h, the first occurring at 2 to 3 h, the second at 12 h.

TABLE 2

Spine density in the mid-molecular layer of the hippocampal dentate gyrus in naïve and trained rats following passive avoidance training

Time post training (h)	Spine density/ μm^3
Naïve	0.789 \pm 0.11
1	0.74 \pm 0.14
3	1.11 \pm 0.10
6	1.70 \pm 0.27*
6 (passive)	0.75 \pm 0.19
48	1.09 \pm 0.12
72	0.86 \pm 0.11

Values are the mean \pm SEM of spine density per animal (n=3) and those differing significantly from naïve control are indicated by * ($p \leq 0.05$). Passive animals were exposed to the apparatus but received no footshock.

At each of the two time points, a comparative decrease in phosphoCREB expression occurred when compared with that at all other times analyzed (Fig. 2). The first decline in CREB phosphorylation may relate to a generalized reduction in the activity of the CREB/ATF family of transcription factors because ATF phosphorylation also appeared to decrease at this period, although this effect was not quantified. Nevertheless, reduced phosphorylation was specific to CREB at 12 h post-training. Thus, taken together, our results suggest that the variation in frequency of HC cells and CREB expression in the 0 to 24 h post-training interval is biphasic with simultaneous troughs at 2 h and at 12 h. This combined morphologic and biochemical evidence points to episodes of protein synthesis inhibition at 2 to 4 h and 8 to 12 h following acquisition of a passive avoidance task.

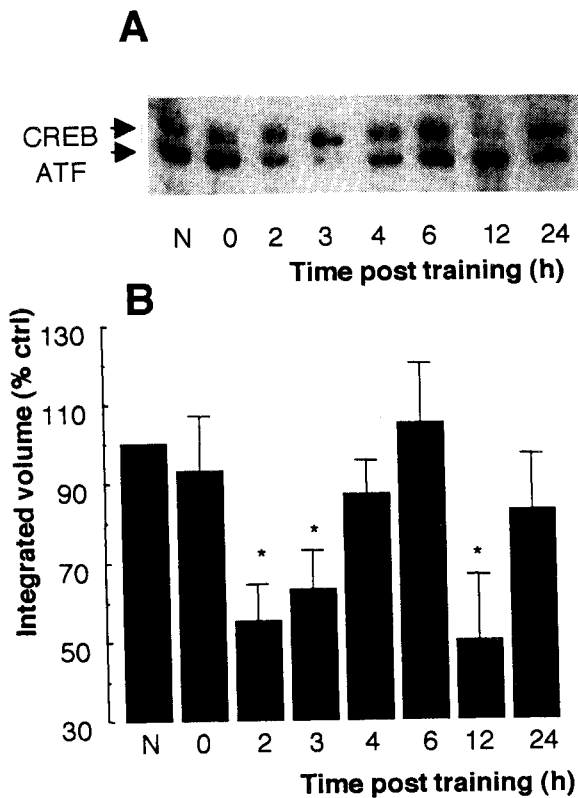


Fig. 2: Regulation of phosphoCREB expression in the hippocampal dentate gyrus following passive avoidance training. Animals were sacrificed at the indicated times post-training, the dentate gyrus was homogenised and immunoblot analysis with antibody to phosphoCREB was performed on nuclear extracts. Panel A shows a representative immunoblot. Semi-quantitative scanning densitometry is shown in panel B, where values are normalized to % naïve control and represent the mean \pm SEM ($n=3$). Values differing significantly from control are indicated by *.

DISCUSSION

In our previous report, we described the appearance of a population of hyperchromatic (HC) cells in the hippocampal dentate gyrus at 5 to

7 h following avoidance learning (O'Connell et al., 1997). In that study, the HC cell number in the early post-training period (2 h) was interpreted to reflect basal frequency. However, reappraisal of our previous data with that obtained in the present study revealed no HC cell frequency increase in the 5 to 7 h post-training period. Rather, a decrease in HC cell frequency occurred at the 2 to 4 h and 8 to 12 h post-training periods. Thus, a revised interpretation of our composite data points to a clearing event before a wave of protein synthesis at 5 to 7 h post-training.

This view broadly supports growing evidence in various learning models that long-term memory consolidation is controlled by negative, as well as by positive, regulatory mechanisms (reviewed in Abel & Kandel, 1998). More specifically, in *Aplysia* and *Drosophila*, transcriptional inhibition of cell adhesion molecules (CAMs) correlates with activity-dependent synaptic growth (Bailey et al., 1992; Mayford et al., 1992; Schuster et al., 1996). Moreover, we demonstrated that in the rat, a similar clearance of NCAM from the cell surface is initiated 3 h post passive avoidance training (Foley et al., 2000). Other proteins also function as inhibitory restraints on memory storage and are similarly targeted for degradation, as evidenced by the transient upregulation of C-terminal hydrolase, the rate-limiting protein in the ubiquitination pathway, in the same time period (Abel et al., 1998; Foley et al., 2000; Hedge et al., 1997). Another major target for learning-associated proteolysis is the regulatory subunit of protein kinase A (PKA), the removal of which results in persistent PKA activity (Hedge et al., 1993).

In view of its transcription activating function and conserved role in memory consolidation, we attempted to further characterize protein synthetic events by mapping the temporal regulation of CREB activity in the hippocampal dentate gyrus after avoidance training. Our findings suggest a correlation between phosphoCREB and hyper-

chromatic cell frequency in this brain region. Phosphorylation of CREB initially decreases in the 2 to 3 h post-training period, recovers between 4 to 6 h, and again decreases at the 12 h post training period. Overall, these findings are consistent with the established role of phospho-CREB in learning. For example, in a transgenic mouse in which CRE is linked to a *lacZ* reporter gene, both long-term LTP and a contextual fear conditioning paradigm stimulated *lacZ* expression, indicating that CREB-mediated gene transcription is involved in long-term memory consolidation (Impey et al., 1996, 1998). Our findings also concur with the observations of Guzowski and McGaugh (1997), who demonstrated that antisense-mediated disruption of CREB perturbed consolidation for a spatial learning paradigm when the animals were recalled at 48 h, but not in the first 4 h post-training period. Furthermore, phosphoCREB expression in the hippocampus of those animals was significantly reduced at 6 h post-training in the amnesic, antisense-treated group, suggesting that transcriptional activation of CREB is required at this timepoint. Increased levels of hippocampal phosphoCREB have been detected in the CA1 region at 3 and 6 h after training in an inhibitory avoidance task, although earlier timepoints were not investigated (Bernabeu et al., 1997). The observation of decreased phosphoCREB expression in the 12 h post-training period is unique. This decrease may precede a much later requirement for protein synthesis, as may be associated with the involvement of L1 in the 15 to 18 h post-training period of avoidance learning in the chick (Tiuнова et al., 1998).

In the present study, at a synaptic level both protein synthesis and phosphoCREB expression at 6 h post training correlated with increased spine density in the dentate gyrus. Thus, our results suggest the existence of a post-learning temporal synchronism among the three variables of CREB phosphorylation, HC cell number, and spine

formation. The decreased level of phosphoCREB and HC cell number, seen both before and after the 5 to 7 h post-training period, implies a redeployment of cellular synthesis machinery for the purpose of spine formation. A comparison with naïve animals and those assessed at later post-training times indicates that the synaptic growth in this period is transient and represents a true increase over basal levels without a prior synaptic clearance. In vitro models of synaptic growth have shown that CREB phosphorylation is directly correlated with increased spine density, and that PKA inhibition prevents spine growth (Murphy & Segal, 1997). Thus, it is likely that the transient nature of synaptic elaboration may be directly regulated by the CREB-mediated transcription of essential proteins. In light of the established morphoregulatory role of CAMs, these proteins must represent major candidates for mediating morphological synaptic change (Doherty et al., 1995). In both avian and mammalian learning models, the action of CAMs has been isolated to a critical 6 to 8 h post-training period of glycoprotein synthesis that coincides with synapse elaboration (Arami et al., 1996; Doyle et al., 1992; Murphy & Regan, 1998; Scholey et al., 1993; Skibo et al., 1998). Moreover, long-term potentiation produces an increase in NCAM 180 positive spine synapses (Schuster et al., 1998). Combined with the findings of the present study, it is plausible that decreased protein synthesis is CAM related, with downregulation facilitating the loosening of synaptic contacts before altering the network properties of neural circuits. Subsequently, protein synthesis in the 5 to 7 h post-training period contributes to the synaptic growth that is necessary for memory consolidation. But the transient role of the hippocampus in memory (Kim & Fanslow, 1992; Zola-Morgan & Squire, 1990) predicts that such synaptic stabilization is temporary. Thus, phosphoCREB downregulation at 12 h supports this view, whereby this episode of memory consolidation

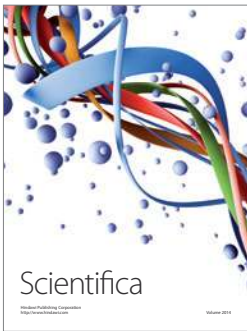
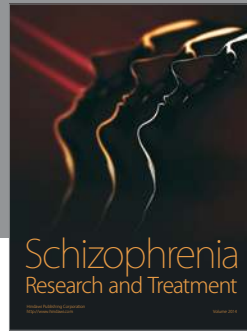
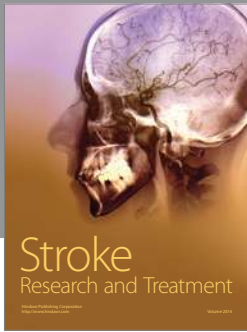
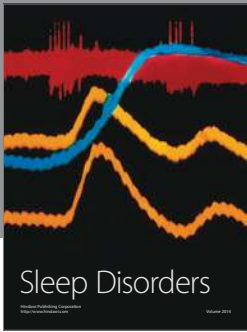
within the hippocampus, centered on the 6 h post-training period, is accompanied by decreased protein synthesis and a repression of CREB-mediated transcription.

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