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Involvement of hippocampal AMPA glutamate receptor changes and the cAMP/protein kinase A/CREB-P signalling pathway in memory consolidation of an avoidance task in rats

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Abstract

Training in step-down inhibitory avoidance (0.3-mA footshock) is followed by biochemical changes in rat hippocampus that strongly suggest an involvement of quantitative changes in glutamate AMPA receptors, followed by changes in the dopamine D_1 receptor/cAMP/protein kinase A (PKA)/CREB-P signalling pathway in memory consolidation. AMPA binding to its receptor and levels of the AMPA receptor-specific subunit GluR1 increase in the hippocampus within the first 3 h after training (20-70%). Binding of the specific D_1 receptor ligand, SCH23390, and cAMP levels increase within 3 or 6 h after training (30-100%). PKA activity and CREB-P levels show two peaks: a 35-40% increase 0 h after training, and a second increase 3-6 h later (35-60%). The results correlate with pharmacological findings showing an early post-training involvement of AMPA receptors, and a late involvement of the D_1/cAMP/PKA/CREB-P pathway in memory consolidation of this task.

Key words
- Glutamate AMPA receptors
- cAMP/PKA/CREB pathway
- Memory formation
- Rat hippocampus

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From mollusks to mammals, neural plasticity has an early protein synthesis-independent phase that lasts 1-3 h and directly depends on events related to cell excitation, and a late protein synthesis-dependent phase that lasts hours, days or more and is independent of ongoing electrical activity (1-5). The best studied form of plasticity is long-term potentiation (LTP) in the CA1 subregion of rat hippocampus. In the first 1-3 h, CA1 LTP is accompanied by enhanced α-amino-3-hydroxy-5-methyl-isoxazolepropionate (AMPA) receptor sensitivity (2) and binding properties (3). Persistence of LTP beyond 3 h requires activation of the cyclic adenylyl monophosphate (cAMP)-responsive element binding (CREB) family of proteins by the cAMP-dependent protein kinase A (PKA) (4,5). This late phase of LTP is regulated by D_1 dopamine receptors, which activate adenylyl cyclase (5). Gene transcription mediated by phosphorylated CREB (CREB-P) is also crucial for the maintenance beyond 3 h of serotonin-induced facilitation in *Aplysia* (6), of memory of odor avoidance in *Drosophila* (7), and memory of spatial tasks

in mice (8). We recently showed that activity of hippocampal AMPA receptors within the first 3 h (9,10), and cAMP/PKA-dependent processes later on (11) are crucial for long-term memory consolidation of a one-trial step-down inhibitory avoidance task in rats.

Here we study levels of the specific AMPA receptor subunit GluR1, AMPA binding to AMPA receptors, D_1 receptor binding properties, cAMP levels, PKA activity and CREB-P levels in rat hippocampus following inhibitory avoidance training.

Male Wistar rats (3-4 months old, 240-310 g) were divided into three groups: animals trained in inhibitory avoidance, naive controls and shocked controls. Trained animals were placed on a 2-cm high, 7 x 25 cm platform facing a 42 x 25 cm grid of 0.1-cm caliber stainless steel bars spaced 1 cm apart; upon stepping down on the grid they received a 0.3-mA, 2-s scrambled footshock (9-11). Shocked controls were placed directly on the grid and exposed to the footshock. Naive controls were just taken out of their home cage. All animals were sacrificed by decapitation.

For binding studies, brains were removed and frozen at -70ºC. Sagittal 12-µm sections were cut with a cryostat, thaw-mounted onto chrome-aluminum gelatin-coated slides and kept at -70ºC until incubation. For [^{3}H]AMPA binding, sections were preincubated at 30ºC in 50 mM Tris-HCl buffer, pH 7.4, for 30 min, and then incubated at 4ºC for 4 h in 100 mM of the buffer plus 100 mM KCSN and 70 nM [^{3}H]AMPA (53 Ci/mmol, NEN, Boston, MA). Nonspecific binding was measured in the presence of 1 mM L-glutamate. For [^{3}H]SCH23390 binding (12), sections were incubated in 50 mM Tris-HCl buffer, pH 7.4, with 4 nM [^{3}H]SCH23390 (72.5 Ci/nmol, NEN) at room temperature for 1 h. Incubations were terminated by washing the sections 3 times with the buffer and then in ice-cold water. [^{3}H]SCH23390 and [^{3}H]AMPA binding was measured by densitometric analysis of radioautographies as described elsewhere (10).

GluR1 was measured in synaptic plasma membranes (13) incubated overnight with a polyclonal antibody (Chemicon, Hercules, CA, USA). After addition of 50 µl pansorbin, the immune complex was collected by centrifugation, resuspended in 30 µl of 1 x Laemmli sample buffer, heated first at 50ºC for 30 min and then at 100ºC for 2 min, and then subjected to SDS-PAGE and immunoblot (alkaline phosphatase coupled with IgG goat antibody 1/3200, BioRad, Temecula, CA, USA). Densitometry was performed using an MCID image analysis system (5.02 V, Image Research Inc., Toronto, Canada).

For cAMP assays (9,14) the hippocampus was quickly dissected out, placed in 0.5 M sodium acetate buffer with 1-methyl-3-butylxanthine for 1 min at 60ºC, and then homogenized and centrifuged at 12,500 rpm for 10 min. A radioimmunoassay was carried out on the supernatant using [^{125}I]cAMP (25,000-28,000 cpm, NEN) and a specific anti-cAMP antibody (Sigma Chemical Co., St. Louis, MO). cAMP-specific phosphodiesterase activity (PDE) was measured in samples incubated at 37ºC for 10 min in 80 mM Tris buffer (10 mM $MgCl_2$, 16 mM ß-mercaptoethanol, 2 mM ATP, 200 µM cAMP) plus 70,000 cpm [^{3}H]cAMP. Samples were then boiled for 1 min and incubated with 50 µl 5'-nucleotidase (1 mg/ml) at 30ºC for 10 min and 1 ml of a 1:3 mixture of Dowex X50 resin/methanol was added and centrifuged at 3,000 rpm for 15 min. Supernatants were collected and counted (15).

For PKA activity, hippocampi were homogenized in 20 mM Tris-HCl buffer, pH 7.4, with 0.5 mM IBMX, 10 mM dithiothreitol, 5 mM NaF, 10 mM EDTA, 10 mM EGTA and a mixture of protease inhibitors (16). After centrifugation at 2,800 rpm for 10 min, supernatants were collected and 10-µl aliquots (2 mg protein/ml) were incubated at 30ºC for 5 min in buffer containing 30 µM kemptide, 10 mM cAMP and 200 µM [γ-^{32}P]ATP (200-300 cpm/pmol, NEN). The

reaction was stopped on phosphocellulose strips which were washed 3 times with 75 mM phosphoric acid. Filters were dried and counted.

For CREB-P immunocytochemistry (17) rats were anesthetized with chloral hydrate and perfused with 4% paraformaldehyde in phosphate-buffered saline for 5 min. Brains were removed and exposed for 4 h at 4ºC and 20-µm sagittal sections were cut with a cryostat and incubated with anti-CREB-P antibody (0.5 µg/ml) for 16 h at 4ºC. Specific immune complexes were visualized with an avidin biotin detection system (Vector, Brethon, UK) and measured densitometrically as described elsewhere (10).

Data on GluR1 levels, AMPA and SCH23390 binding are shown in Figure 1, and data on cAMP levels, PDE and PKA activity and CREB-P levels are shown in Figure 2.

Maximum [^{3}H]AMPA binding increased within the first 2 h after training in CA1 and this increase was correlated with increased GluR1 levels measured in the same area (Figure 1). The changes suggest translocation of the receptor (18) since they are too rapid to be accounted for by synthesis and are consistent with similar findings on CA1 LTP (2,3), with the retrograde amnestic effect of the infusion of an AMPA antagonist into CA1 being observed within the first 2 h after training (9). In another study (10) we observed post-training increases of [^{3}H] AMPA binding in CA2 and CA3 lasting 24 h; it is possible that those very late changes were related to receptor synthesis (9,10).

8-Br-cAMP and the adenylyl cyclase activators, forskolin and SKF38393 (a selective dopamine D_1 receptor agonist), infused 3 or 6 h post-training into the rat hippocampus, enhance retention of the avoidance task, whereas the PKA inhibitor, KT5720, or the D_1 antagonist, SCH23390, is amnestic (11). In the present report we show that, indeed, there was enhanced binding of labeled SCH23390 to the receptor in the hippocampus 3 or 6, but not 0, 1 or 9 h after training (Figure 1).

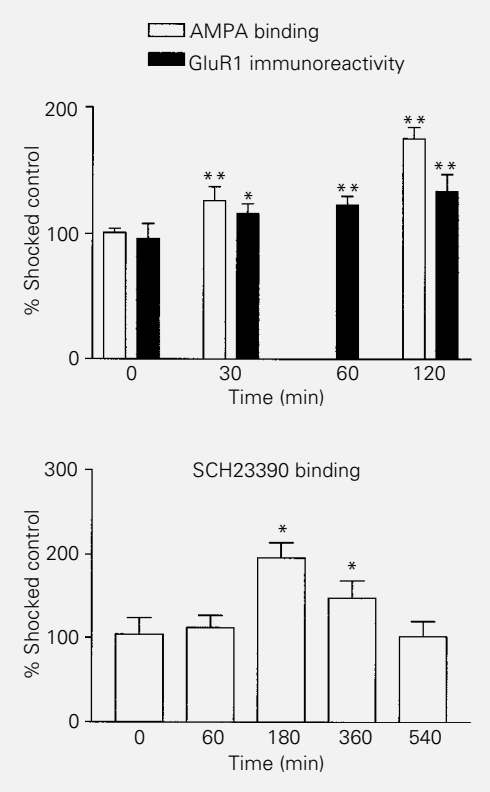

Figure 1 - In this and in the following figure, data are from animals sacrificed at various times after step-down inhibitory avoidance training (abscissae, in min) and are reported as % values of shocked controls (ordinates). *Top panel*, GluR1 levels measured by immunoblot and [^{3}H] AMPA binding to AMPA receptors in the CA1 area of the hippocampus at different times after training. *P<0.05, **P<0.01 compared to shocked controls (Newman-Keuls after ANOVA). *Bottom panel*, Mean ± SEM densitometric values of [^{3}H] SCH23390 binding to the hippocampal pyramidal cell layer at different times after training. There was maximum binding at 180 min, a slight decrease at 360 min, and a return to control values at 540 min. *P<0.01 compared to shocked controls (Newman-Keuls after ANOVA). Number of independent experiments ranged between 5-8 per group.

cAMP levels increased 3 or 6 but not 0 or 9 h post-training and this increase was not accompanied by detectable changes in PDE, and therefore may be attributed to activation of adenylyl cyclase. Two peaks of PKA activity and CREB-P levels were observed: one 0 h after training, and a second, higher one at 3 and 6 h after training (Figure 2). All effects were learning-specific: in all cases, differences between naive and shocked controls were nonsignificant (data not shown; see also 9,10).

The data suggest that an increase of AMPA receptor function, followed by activation of the D_1/cAMP/PKA/CREB-P pathway, is crucially involved in memory consolidation of inhibitory avoidance learning in the rat. Previous studies on full retrograde amnesia by intrahippocampal infusion of an AMPA antagonist within the first 3 h after training (9) or of PKA inhibitors or SCH23390 (11) within the following 3 h

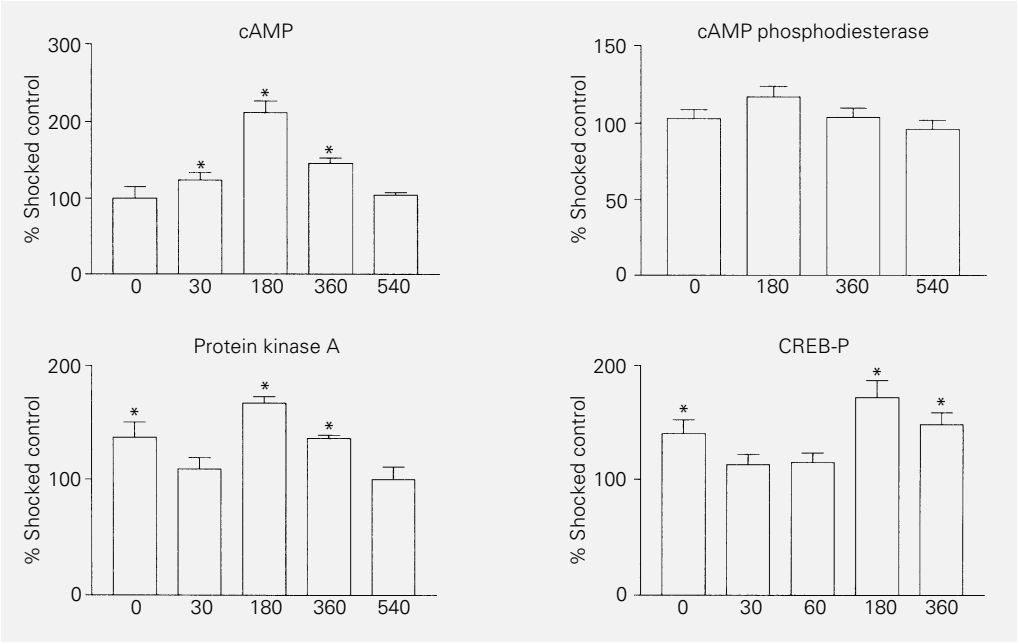

Figure 2 - *Top left panel*, Mean ± SEM cAMP levels in extracts of rat hippocampus measured with a specific antibody (Sigma). There was a peak at 180 min, a slight decrease at 360 min, and a return to control values at 540 min. *P<0.02 by Newman-Keuls after ANOVA; N = 5 independent experiments done in duplicate. *Top right panel*, Mean ± SEM cAMP phosphodiesterase activity (PDE) measured by the hydrolysis of [^{3}H]cAMP. No changes were detected at any time after training; N = 6 independent experiments done in duplicate. *Bottom left panel*, Mean ± SEM levels of PKA activity measured by the phosphorylation of kemptide. There was a peak immediately after training, followed by a return to control values, and a second, higher peak at 180 and 360 min. *P<0.01 by Newman-Keuls after ANOVA; N = 5 independent experiments done in triplicate. *Bottom right panel*, Mean ± SEM of CREB-P immunoreactivity obtained by densitometric analysis of specific immune complexes of CREB-P with a specific antibody (provided by Prof. D.D. Ginty) visualized in histological sections with an avidin biotin detection system. As with PKA, there were two peaks of CREB-P: the first 0 min after training, and the second at 180 min. *P<0.01 by Newman-Keuls after ANOVA; N = 5 independent experiments done in duplicate.

indicate that both processes are essential for memory formation.

The first, early peak of PKA and CREB-P precedes the onset of the cAMP increase and dopaminergic regulation and may be related to the production of early post-training protein messengers recently suggested for spatial learning in the rat hippocampus (19). The second PKA/CREB-P peak at 3-6 h was accompanied by high cAMP levels and enhanced [^{3}H]SCH23390 binding, and may correlate with enhanced glycoprotein synthesis and synaptic structural changes as described in rat hippocampus 5-7 h after training (20).

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