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Role of Hippocampal CaMKII in Serotonin 5-HT_{1A} Receptor-Mediated Learning Deficit in Rats

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The serotonin 5-HT_{1A} receptor agonist, 8-OH-DPAT (8-hydroxy-2-di-*n*-propylamino-tetralin), impairs retention performance in a passive avoidance learning task in rats. In the hippocampus of rats trained on this procedure and killed 1 h after the acquisition trial, an increase in the membrane levels of both Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and phosphorylated CaMKII, as well as in total and Ca^{2+}-independent enzyme activity in tissue lysates was found. These effects were learning-specific as no changes in CaMKII levels or activity were found in rats receiving a footshock identical to the trained rats. The effect of training on CaMKII was prevented by a low 8-OH-DPAT dose. The 5-HT_{1A} agonist also reduced protein kinase A (PKA) activity and increased the membrane levels of phosphatase 1 (PP1) and PP1 enzyme activity in the hippocampus. All of the changes induced by 8-OH-DPAT were reversed by the selective 5-HT_{1A} antagonist WAY-100635, indicating receptor-specific effects. We suggest that 5-HT_{1A} receptor-mediated disruption of retention performance is a consequence of the reduced PKA activity and the ensuing enhancement in PP1 activity, possibly through decreased phosphorylation/activation of endogenous PP1 inhibitors, that cause a reduced activity of phosphorylated CaMKII, a key enzyme in early stages of memory formation. This study provides an *in vivo* molecular basis for the cognitive deficits induced by stimulation of hippocampal 5-HT_{1A} receptors.

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INTRODUCTION

Numerous intracellular signaling pathways with different time courses are activated during the acquisition and consolidation of memory. It seems that early changes are dependent on modifications of pre-existing proteins, probably affecting their phosphorylation state, whereas the later phase is associated to gene expression (Milner *et al*, 1998). The critical role of glutamate transmission in the early stages of hippocampal-dependent memory storage and in long-term potentiation (LTP) has been extensively documented (Bliss and Collingridge, 1993; Riedel *et al*, 2003). In synaptic plasticity underlying memory storage, other neurotransmitter systems may also exert an influence, probably through interactions with glutamate.

Serotonin (5-hydroxytryptamine, 5-HT) regulates different forms of synaptic plasticity both during development and in the adult brain (Mazer *et al*, 1997). In particular, a major role for the serotonergic system in modulating learning and memory has been suggested (eg review by Buhot, 1997). Yet, the nature of the role of the serotonergic system in cognition is not clear, and strategies in which whole brain 5-HT levels were increased or decreased have led to some inconsistent results (Buhot, 1997; Misane and Ögren, 2000). The diversity of 5-HT receptor subtypes has led to explore more specifically the contribution of 5-HT to cognitive processes. Among the 15 subtypes of mammalian 5-HT receptors, much attention has been paid to the 5-HT_{1A} receptor, highly expressed in limbic areas related to memory storage, particularly in the hippocampus (Pazos and Palacios, 1985). In general, systemic or intrahippocampal administration of the selective 5-HT_{1A} receptor agonist 8-OH-DPAT (8-hydroxy-2-di-*n*-propylamino-tetralin) before training impairs memory retention 24 h later in different experimental tasks, including passive avoidance (Carli *et al*, 1992; Otano *et al*, 1999; Misane and Ögren, 2000). This effect has been generally related to direct stimulation of postsynaptic receptors located on excitatory hippocampal neurons (Carli and Samanin, 1992; Mendelson *et al*, 1993). Serotonin, as well as the selective 5-HT_{1A} agonist 8-OH-DPAT, also inhibit LTP induction (Corradetti *et al*, 1992; Edegawa *et al*, 1998), an effect that can be blocked by 5-HT_{1A} receptor antagonists, indicating the role of this receptor subtype in 5-HT effects. Other reports have shown that 5-HT_{1A} receptor stimulation inhibits the induction of LTP by suppressing

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NMDA receptor function in postsynaptic neurons (Edegawa *et al*, 1999).

In LTP induction, there is an early activation of NMDA receptors. Subsequently, a transient Ca^{2+} entry activates Ca^{2+}-dependent enzymes, leading to long-lasting potentiation of synaptic efficacy. Of particular relevance is the activation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) that, after autophosphorylation at Thr286, induces crucial molecular events related to synaptic potentiation (Fink and Meyer, 2002), such as phosphorylation of the GluR1 subunit of the AMPA receptor (Barria *et al*, 1997) and delivery of AMPA receptors to synapses (Shi *et al*, 2001). The rise in Ca^{2+} ions may also activate enzymes such as cAMP-dependent protein kinase A (PKA) required for long-term stability of synaptic efficacy (Elgersma and Silva, 1999). Although 5-HT$_{1A}$ receptors are coupled to multiple cell signaling pathways, in the hippocampus, adenylyl-cyclase inhibition appears to be the initial target in 5-HT$_{1A}$ receptor-mediated effects (Schoeffter and Hoyer, 1988).

Even though other brain structures, such as the amygdala (Liang, 1999) or the frontal cortex (Santucci and Shaw, 2003), may contribute to early memory processing of passive avoidance, the sequence of molecular events in the hippocampus associated to learning and retention of passive avoidance tasks appears to be strikingly similar to those underlying other forms of learning and also LTP (reviewed in Izquierdo and Medina, 1997; Shobe, 2002). In an attempt to explore the mechanisms involved in the disruptive effect of 5-HT$_{1A}$ receptor activation on cognition, we sought for targets susceptible to modification by systemic administration of a 5-HT$_{1A}$ receptor agonist to rats subjected to passive avoidance training. On the basis of the presumed 5-HT$_{1A}$ receptor-mediated inhibition of PKA in the hippocampus, a crucial structure in cognitive processes (Milner *et al*, 1998), we have studied early changes in the expression levels and/or activity of kinases, PKA and CaMKII, and also of protein phosphatase 1 (PP1). PKA indirectly regulates CaMKII through endogenous PP1 inhibitors, and PP1 inhibition results in higher levels of activated CaMKII (Blitzer *et al*, 1998; Genoux *et al*, 2002). It was consequently expected that 5-HT$_{1A}$ receptor activation could lead to changes in CaMKII, a key enzyme in memory storage.

MATERIALS AND METHODS

Animals

Male Wistar rats (Harlan, Barcelona, Spain) weighing 200–220 g were used. Animals were housed in plastic cages with free access to food and water and maintained in a temperature-controlled environment (21–23°C) on a 12 h light/dark cycle. Behavioral experiments were always performed during the light period, from 0900 to 1500 hours. All procedures were in accordance with the guidelines established by the normative of the European Community of November 24, 1986 (86/609/EEC). This study was approved by the Ethical Committee of the University of Navarra (no. 016/01; March 6, 2001).

In biochemical studies, rats were killed by decapitation 1 h after training (ie 1 h after the footshock of the acquisition trial; see below). The corresponding controls received identical pharmacological treatments but were not subjected to passive avoidance training. In another control group, rats were directly placed on the grid floor and were killed 1 h after receiving an identical footshock to the trained rats. The brains were immediately removed and the hippocampi were dissected on ice. In enzyme activity studies, extracts were prepared immediately after the killing and hippocampus dissection. In most Western-blotting studies, hippocampi were frozen on dry ice after dissection and stored at −80°C until use. Given the rapid translocation of CaMKII from the cytosol to the synapses after decapitation (Suzuki *et al*, 1994), in some Western-blotting experiments, particularly with pCaMKII, the brains were frozen on dry ice before dissection. The results were virtually identical using either procedure.

Drugs and Chemicals

8-OH-DPAT and *N*-[2-(4-(2methoxyphenyl)-1-piperazinyl)ethyl]-*N*-(2-pyridinyl) cyclohexane carboxamide (WAY-100635) were from Sigma (USA). Nonidet P-40, leupeptin, and PMSF were from Roche Diagnostics (Germany). All other chemicals were from Sigma (USA). The sources of the antisera, the different enzyme assay kits, and radiochemicals are indicated in the corresponding sections.

Passive Avoidance Learning

This test was performed according to previously described procedures, using a two-compartment (white/dark) passive avoidance apparatus (Artaiz *et al*, 1995; Otano *et al*, 1999). The rat was placed in the illuminated area and 3 s later, the door was raised. During 90 s, the animal explored the apparatus freely (habituation trial). After 10 min, the rat was placed again in the illuminated chamber. When the rat entered the dark compartment, a guillotine door was closed and after 10 s, the animal was returned to its home cage. After 60 min, the animal was placed again in the white compartment. When the rat entered the dark chamber, the guillotine door was closed again and, after 10 s, an inescapable 2 mA scrambled electrical foot shock was delivered for 3 s through the grid floor using a shock generator (training trial). A retention trial was given 24 h after the acquisition trial by placing the rat in the illuminated compartment and measuring the response latency to re-enter the dark compartment using a cutoff time of 300 s. The 5-HT$_{1A}$ receptor agonist 8-OH-DPAT (0.1 mg/kg s.c.) was given 30 min before the acquisition trial. The 5-HT$_{1A}$ receptor antagonist WAY-100635 (0.5 mg/kg i.p.) was administered 15 min before 8-OH-DPAT. Control rats received saline injections by the same route. Animals were assigned at random to the different treatments.

Production of Protein Extracts

To obtain tissue lysates, the hippocampus was homogenized in five volumes of ice-cold lysis buffer containing 50 mM Tris-HCl (pH 7.7), 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 0.25% sodium deoxycholate (DOC), 2 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml

leupeptin, 1 mM Na_3VO_4, 2 mM NaF, and 2 mM $Na_4P_2O_7$, and incubated on ice for 30 min. Lysate was cleared by centrifugation at 14 000*g* for 20 min and the supernatant was aliquoted and frozen at −80°C. This lysate was used for the CaMKII and PP1 enzyme activity assays. For the PKA enzyme activity assay, the tissue was homogenized in ice-cold Tris-EDTA buffer (10 mM Tris-HCl pH 7.4 and 5 mM EDTA) containing protease and phosphatase inhibitors (1 mM EGTA, 0.1 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 0.1 mM Na_3VO_4, 1 mM NaF) and centrifuged at 700*g* for 10 min at 4°C. The supernatant was transferred to another tube and centrifuged again at 10 000*g* for 10 min. The resultant pellet was resuspended in 10 mM Tris-HCl buffer (pH 7.4) containing the mixture of enzyme inhibitors indicated and frozen at −80°C (Dunah *et al*, 2000). In both cases, protein concentration was determined using the Bio-Rad protein assay with bovine serum albumin as standard.

To obtain membrane-enriched proteins (P2 membrane proteins), the hippocampus was homogenized in ice-cold 10 mM Tris-HCl (pH 7.4)/5 mM EDTA buffer, containing 320 mM sucrose and the following protease and phosphatase inhibitors: 1 mM EGTA, 0.1 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 0.1 mM Na_3VO_4, and 1 mM NaF. The homogenate was centrifuged at 700*g* for 10 min, the supernatant was centrifuged again at 37 000*g* for 40 min at 4°C and the pellet (P2) was resuspended in 10 mM Tris-HCl in the presence of the indicated enzyme inhibitors (Dunah *et al*, 2000). The protein concentration was determined (Bio-Rad protein assay) and the aliquots were frozen at −80°C.

Aliquots of the P2 membrane preparation were solubilized in nondenaturing conditions by adding 0.1 volumes of 10% DOC in 500 mM Tris-HCl, pH 9.0, followed by incubation at 36°C for 30 min. Then, 0.1 volumes of a buffer containing 1% Triton X-100, 500 mM Tris-HCl (pH 9.0), and the protease and phosphatase inhibitors was added and the preparations were centrifuged at 37 000*g* for 40 min at 4°C. Aliquots of the supernatant were frozen at −80°C. Other aliquots were solubilized in denaturing conditions by adding 0.1 volumes of 20% sodium dodecyl sulfate (SDS) containing 50% β-mercaptoethanol and boiled for 5 min. The denatured preparations were diluted 20-fold in 50 mM Tris-HCl (pH 7.4)/0.1% Triton X-100 buffer in the presence of protease and phosphatase inhibitors and centrifuged at 37 000*g* for 10 min at 4°C. Aliquots of the supernatant were frozen at −80°C.

Western Blotting

Tissue lysate or P2 membrane proteins (20 μg), the latter solubilized in either denaturing and nondenaturing conditions, were separated onto SDS-polyacrylamide gels. Samples were diluted in an equal volume of electrophoresis buffer and boiled for 5 min. Proteins were transferred to a nitrocellulose membrane (Hybond ECL, Amersham Biosciences) using a Trans-Blot® SD semidry (Bio-Rad) system for 30 min at 12 V. The membranes were blocked with 5% milk, 0.05% Tween-20 in PBS for 1.5 h at room temperature, followed by overnight incubation with the following primary antibodies: mouse monoclonal anti-CaMKII (1 : 10 000, Chemicon), mouse monoclonal anti-phospho-CaMKII(Thr286) (2 μg/ml, Upstate Biothechnology), rabbit polyclonal anti-PP1 (2 μg/ml, Upstate Biothechnology), and anti-α-tubulin (1 : 20 000, Sigma). The membranes were washed three times in PBS/Tween-20 at room temperature, and HRP-conjugated anti-rabbit or anti-mouse antibody (Dako; dilution 1 : 1,500, except for PP-1 that was 1 : 3000) was added and incubated for 60 min. Following two washes in PBS/Tween-20 and one in PBS alone, immunolabeled protein bands were detected using an enhanced chemiluminiscence system (ECL Amersham Biosciences), following an autoradiographic exposure to Hyperfilm™ECL (Amersham Biosciences). The quantification of the signals was determined by densitometry using the program Image-Master I-D (Pharmacia).

Enzyme Assays

Ca^{2+}/calmodulin kinase II assay. Analysis of total CaMKII activity was performed using the 'CaMKII Assay Kit' (Upstate Biotechnology) with autocamtide as a selective peptide substrate, in the presence of calcium and calmodulin. The reaction was carried in MOPS assay buffer containing 30–40 μg of tissue lysate, 100 μM autocamtide, 1 mM $CaCl_2$, 8 ng/μl calmodulin, 10 μl of a mixture containing PKA and PKC inhibitors (0.4 μM TYADFIASGRTGRRNAI and 0.4 μM RFARKGALRQKNV, respectively), and 10 μl of Mg^{2+}/ATP mixture containing 20 nCi/μl [γ-^{32}P]ATP (Amersham Biosciences). The mixture was incubated at 30°C for 10 min, and the phosphorylated substrate was separated from residual [γ-^{32}P]ATP using P81 phosphocellulose filters. The filters were rinsed three times with 0.75% phosphoric acid and one time with acetone, and the bound radioactivity was quantified in a scintillation counter. Blanks to correct for nonspecific binding of [γ-^{32}P]ATP to the phosphocellulose paper were run in parallel and CaMKII activity was expressed as pmol/min/μg protein. To determine Ca^{2+}-independent CaMKII activity, 1 mM EGTA was added instead of $CaCl_2$.

PP1 assay. Analyses were performed using the 'Protein Serine/Threonine Phosphatase (PSP) Assay Kit' (BioLabs) that quantifies the release of inorganic phosphate from a labeled protein. The substrate used was Myelin Basic Protein (MyBP), labeled with [γ-^{32}P]ATP following the kit recommendations. Tissue lysate (10 μl), prepared without phosphatase inhibitors, were added to 30 μl of assay buffer containing 2 nM okadaic acid and 50 μM FK-506 as PP2A and calcineurin inhibitors, respectively. After a preincubation for 5 min at 30°C, the reaction was started by adding 10 μl of substrate and incubated for 10 min at 30°C. The reaction was terminated by adding 200 μl of cold 20% trichloroacetic acid and, after centrifugation at 12 000*g*, radioactivity was counted in the supernatant. Activity of PP1 was expressed as pmol/min/μg protein.

PKA assay. PKA activity was measured by using the 'PKA Assay Kit' (Upstate Biotechnology) with kemptide as substrate. The reaction mixture contained 1.7 μM cAMP, 80 μM kemptide, 10 μl of a mixture containing inhibitors of CaMK and PKC, 10 μl of Mg^{2+}/ATP mixture containing [γ-^{32}P]ATP and 10 μl of tissue homogenate in a final volume

of 60 µl. After 10 min at 30°C, the reaction was terminated by spotting a 25 µl aliquot onto phosphocellulose P81 filters. After washing, the bound radioactivity was quantified with a scintillation counter, and PKA activity was expressed as pmol/min/µg protein.

Statistical Analysis

In biochemical studies, results, reported as means ± SEM, were analyzed using ANOVA, and *post hoc* comparisons were made using the Newman–Keuls test. In passive avoidance studies, nonparametric statistics was used (Kruskal–Wallis ANOVA followed by Mann–Whitney *U*-test).

RESULTS

Passive Avoidance

On the training trial, latencies to enter the dark chamber were in general in the range of 1.6–22.5 s, and were not significantly modified after any drug treatment (Table 1). The training latencies of rats used for biochemical experiments were within the same range. The 5-HT$_{1A}$ receptor agonist 8-OH-DPAT (0.1 mg/kg s.c.), administered 30 min before the acquisition trial, significantly reduced retention latency 24 h later (Table 1). The 5-HT$_{1A}$ receptor antagonist WAY-100635 (0.5 mg/kg i.p.), given 45 min before the acquisition trial, did not show any intrinsic effect on passive avoidance performance but fully prevented the retention impairment induced by 8-OH-DPAT (Table 1).

CaMKII Levels and Enzyme Activity

Passive avoidance training did not modify CaMKII immunoreactivity in tissue lysates (Figure 1a). In the P2 membrane preparation, avoidance training induced a significant increase (~32%; $p<0.01$) in CaMKII levels, which was not observed in shocked untrained rats. In trained rats, there was a significant treatment effect ($F_{3,49}=22.69$, $p<0.0001$). The effect of training was counteracted by 8-OH-DPAT (0.1 mg/kg) and the effect of the 5-HT$_{1A}$ agonist was in turn reversed by the selective antagonist WAY-100635 at a dose (0.5 mg/kg) without any intrinsic effect (Figure 1b). Neither avoidance training nor

Table 1 Impairment of Passive Avoidance Retention in Rats by 8-OH-DPAT and Antagonism of the Effect of 8-OH-DPAT by WAY-100635

Treatment	Training latency (s)	Retention latency (s)
Control	12.3 ± 2.4	269.5 ± 24.4
WAY-100635	14.2 ± 4.1	277.1 ± 31.5
8-OH-DPAT	10.8 ± 3.2	61.0 ± 25.4*
WAY-100635 + 8-OH-DPAT	9.8 ± 3.5	260.5 ± 33.0†

8-OH-DPAT (0.1 mg/kg s.c.) was given 30 min and WAY-100635 (0.5 mg/kg i.p.) 45 min before the training session. Retention was measured 24 h later. Values are means ± SEM of 10–30 animals. *$p<0.01$ *vs* control; †$p<0.01$ *vs* 8-OH-DPAT (Kruskal–Wallis ANOVA followed by Mann–Whitney *U*-test).

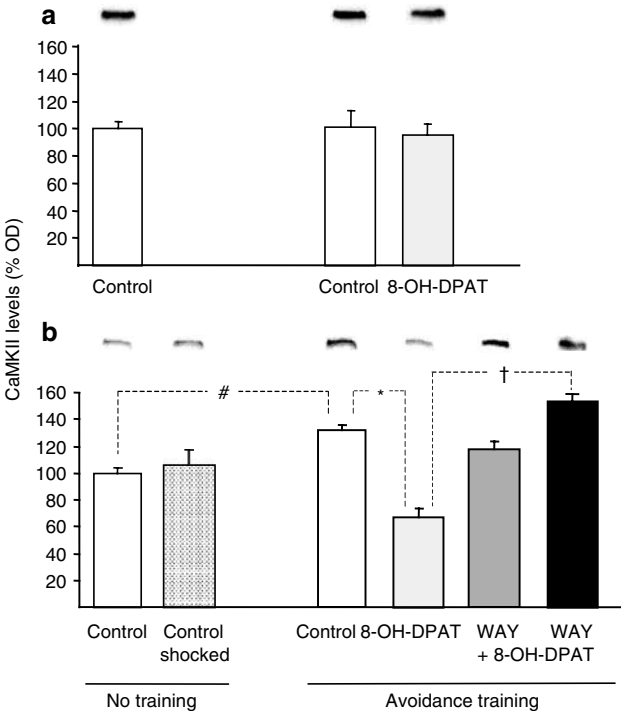

Figure 1 Ca^{2+}/calmodulin kinase II (CaMKII) levels in tissue lysates (a) and in solubilized P2 membrane preparations (b) from the hippocampus of rats killed 1 h after passive avoidance training and of the corresponding nontrained controls. 8-OH-DPAT (0.1 mg/kg s.c.) was given 30 min and WAY-100635 (0.5 mg/kg i.p.) 45 min before the acquisition session. All experiments were performed in triplicate. Values, means ± SEM of 16–20 animals (controls) or 8–12 animals (all other groups), are expressed as percentage of optical density (OD) values of control nontrained rats. #$p<0.01$ *vs* control nontrained rats; *$p<0.01$ *vs* control trained rats; †$p<0.01$ *vs* 8-OH-DPAT-treated rats. Representative scanned hybridized bands are shown at the upper part of the figures. WAY = WAY-100635, 0.5 mg/kg i.p.

8-OH-DPAT treatment produced any change in α-tubulin levels in tissue lysate or in the P2 membrane preparation (not shown).

pCaMKII(Thr286) levels were also measured in tissue lysates and in denaturing membrane extracts. In both cases, phosphorylated protein immunoreactivity was increased to a quite similar extent (~36 and 25% in tissue lysates and membrane extracts, respectively) after passive avoidance training and not after a noncontingent footshock (Figure 2a, b). In tissue lysates, 8-OH-DPAT prevented the effect of training, and the effect of 8-OH-DPAT was reversed by WAY-100635 ($F_{3,37}=9.47$, $p<0.0001$). Qualitatively identical effects were found in membrane extracts ($F_{3,44}=7.28$, $p<0.0001$). In trained rats, the ratio pCaMKII/CaMKII in tissue lysates was 1.34, and went down to 0.85 after 8-OH-DPAT administration. The ratio membrane pCaMKII/tissue lysate CaMKII was 1.24 in control trained rats and 0.99 in rats receiving 8-OH-DPAT.

In enzyme activity assays, total CaMKII activity in the hippocampus of control untrained rats was 0.38 ± 0.02 pmol/min/µg protein (mean ± SEM of 20 rats). This enzyme activity was significantly increased by training (mean ± SEM = 0.53 ± 0.03; $n=20$; $p<0.01$) and was not modified by a noncontingent shock. The effect of training

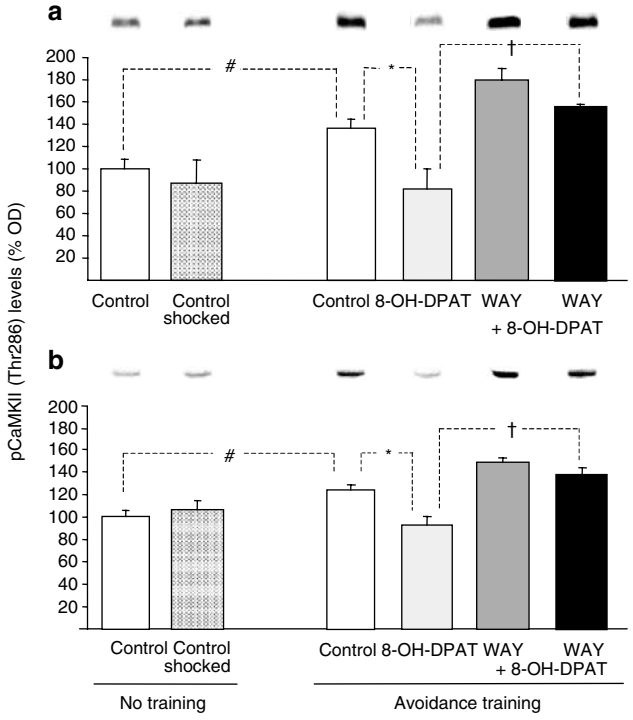

Figure 2 Levels of pCaMKII (Thr-286) in tissue lysates (a) and in solubilized P2 membrane preparations (b) from the hippocampus of rats killed 1 h after passive avoidance training and of the corresponding nontrained controls. 8-OH-DPAT (0.1 mg/kg s.c.) was given 30 min and WAY-100635 (0.5 mg/kg i.p.) 45 min before the acquisition session. All experiments were performed in triplicate. Values, means ± SEM of 16–20 animals (controls) or 8–12 animals (all other groups), are expressed as percentage of optical density (OD) of control nontrained rats. $^{\#}p<0.05$ *vs* control nontrained rats; $^{*}p<0.05$ *vs* control trained rats; $^{\dagger}p<0.05$ *vs* 8-OH-DPAT-treated rats. Representative scanned hybridized bands are shown at the upper part of the figure. WAY = WAY-100635, 0.5 mg/kg i.p.

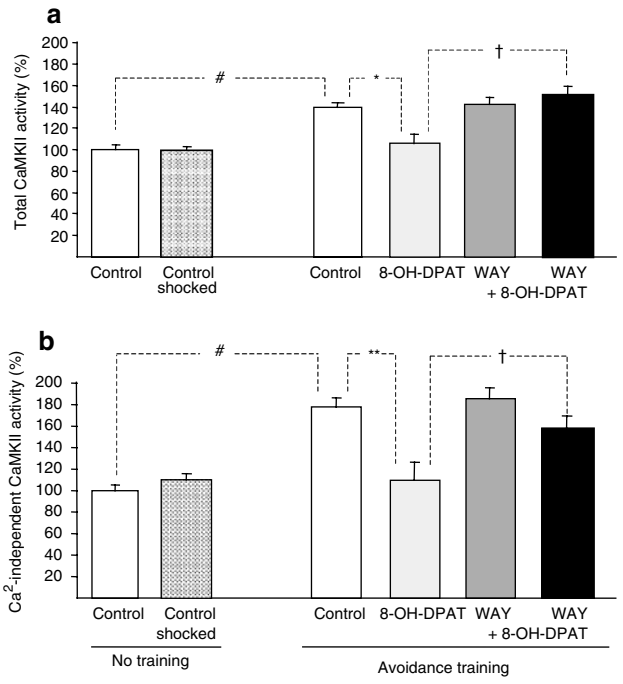

Figure 3 Total Ca^{2+}/calmodulin kinase II (CaMKII) activity (a) and Ca^{2+}-independent CaMKII activity (b) in tissue lysates from the hippocampus of rats killed 1 h after passive avoidance training and of the corresponding nontrained controls. 8-OH-DPAT (0.1 mg/kg s.c.) was given 30 min and WAY-100635 (0.5 mg/kg i.p.) 45 min before the acquisition session. All experiments were performed in triplicate. Values, means ± SEM of 16–20 animals (controls) or 8–12 animals (all other groups), are expressed as percentage of CaMKII activity (pmol/min/μg protein) in control nontrained rats. $^{\#}p<0.01$ *vs* control nontrained rats; $^{*}p<0.05$, $^{**}p<0.01$ *vs* control trained rats; $^{\dagger}p<0.05$ *vs* 8-OH-DPAT-treated rats. WAY = WAY-100635, 0.5 mg/kg i.p.

was again prevented by 8-OH-DPAT and the effect of the 5-HT$_{1A}$ agonist reversed by WAY-100635 ($F_{3,50}=3.52$, $p=0.022$; see Figure 3a).

Ca^{2+}-independent CaMKII activity was more sensitive than total enzyme activity to the effect of avoidance training, which induced a marked increase (~78%) in the activity of the autonomous enzyme from 0.096 ± 0.005 pmol/min/μg protein (mean ± SEM of 16 rats) to 0.171 ± 0.014 (Figure 3b). The ratio autonomous/total CaMKII activity was also increased (~28%) by avoidance training. 8-OH-DPAT prevented the effect of training on Ca^{2+}-independent CaMKII activity and WAY-100635 antagonized the effect of 8-OH-DPAT ($F_{3,38}=4.37$, $p=0.01$; Figure 3b).

In untrained rats, which were killed as in the above experiments 90 min after the same 8-OH-DPAT dose (0.1 mg/kg), no effect of drug treatment on CaMKII levels or enzyme activity was found. In P2 membranes, protein levels, expressed as percentage of optical density values of control untrained rats, were 107.5 ± 7.9 for CaMKII and 95.0 ± 8.1 for pCaMKII(Thr286) (means ± SEM of eight rats). CaMKII enzyme activity in tissue lysates, expressed also as percentage of activity in control untrained rats, was 93.2 ± 5.1 and 94.8 ± 7.7 for total and Ca^{2+}-independent CaMKII activity, respectively (means ± SEM of eight rats).

PP1 Levels and Enzyme Activity

Passive avoidance training did not induce any significant effect on PP1 immunoreactivity, neither in tissue lysates nor in P2 membrane preparations (Figure 4a, b). No significant increase in PP1 levels in tissue lysates was either found after 8-OH-DPAT administration to trained rats (Figure 4a). A significant treatment effect on membrane PP1 levels was found in trained rats ($F_{3,44}=14.08$, $p<0.0001$). Membrane PP1 levels were significantly increased by 8-OH-DPAT (~57% increase), and the effect of 8-OH-DPAT was prevented by the antagonist WAY-100635 (Figure 4b).

In enzyme activity assays, the method was initially validated with the phosphatase inhibitor okadaic acid (1 μM) and different PP1 concentrations to determine the linear range of dephosphorylation. As above, avoidance training did not produce any change in PP1 enzyme activity, which was enhanced by 8-OH-DPAT. There was a significant treatment effect ($F_{3,50}=12.32$, $p<0.0001$). The effect of 8-OH-DPAT was again prevented by the 5-HT$_{1A}$ antagonist WAY-100635 (Figure 5).

PKA Activity

The specificity of the method was initially checked by using the PKA inhibitor peptide (PKI) that reduced kinase activity

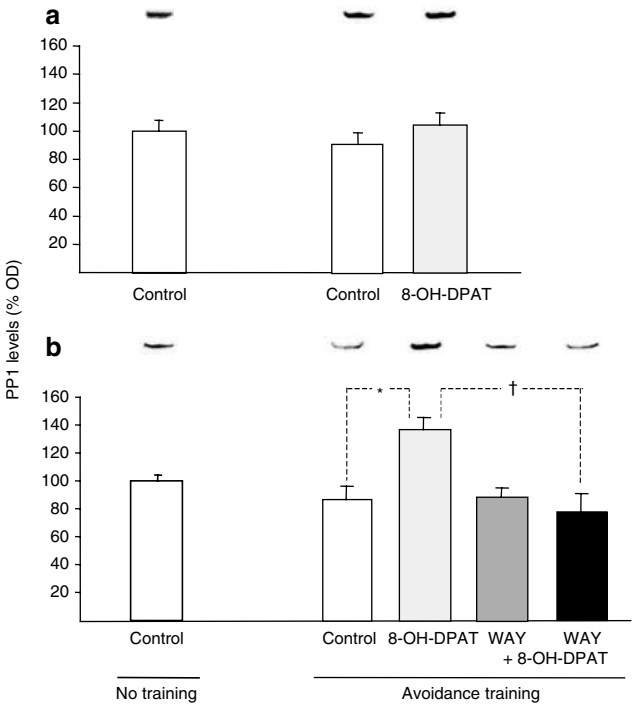

Figure 4 Phosphatase 1 (PP1) levels in tissue lysates (a) and in solubilized P2 membrane preparations (b) from the hippocampus of rats killed 1 h after passive avoidance training and of the corresponding nontrained controls. 8-OH-DPAT (0.1 mg/kg s.c.) was given 30 min and WAY-100635 (0.5 mg/kg i.p.) 45 min before the acquisition session. All experiments were performed in triplicate. Values, means ± SEM of 16–18 animals (controls) or 8–12 animals (all other groups), are expressed as percentage of optical density (OD) values of control nontrained rats. $^{*}p<0.01$ *vs* control trained rats; $^{\dagger}p<0.01$ *vs* 8-OH-DPAT-treated rats. Representative scanned hybridized bands are shown at the upper part of the figures. WAY = WAY-100635, 0.5 mg/kg i.p.

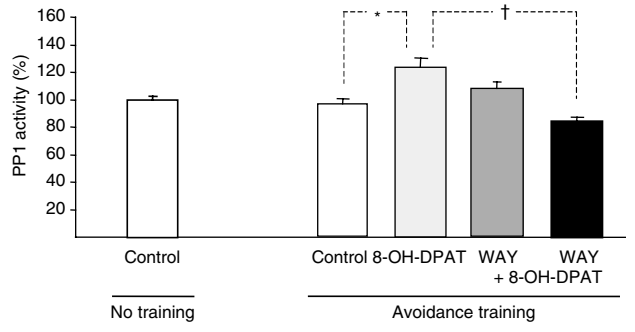

Figure 5 PP1 activity in tissue lysates from the hippocampus of rats killed 1 h after passive avoidance training and of the corresponding nontrained controls. 8-OH-DPAT (0.1 mg/kg s.c.) was given 30 min and WAY-100635 (0.5 mg/kg i.p.) 45 min before the acquisition session. All experiments were performed in duplicate. Values, means ± SEM of 18–22 animals (controls) or 8–12 animals (all other groups), are expressed as percentage of PP1 activity (pmol/min/μg protein) in nontrained control rats. $^{*}p<0.01$ *vs* control trained rats; $^{\dagger}p<0.01$ *vs* 8-OH-DPAT-treated rats. WAY = WAY-100635, 0.5 mg/kg i.p.

by 90–100%. Passive avoidance training significantly increased enzyme activity (Figure 6). As in previous experiments, footshock did not produce by itself any effect. In trained rats, there was a significant treatment effect ($F_{3,53}=12.98$, $p<0.0001$). A decrease in PKA activity

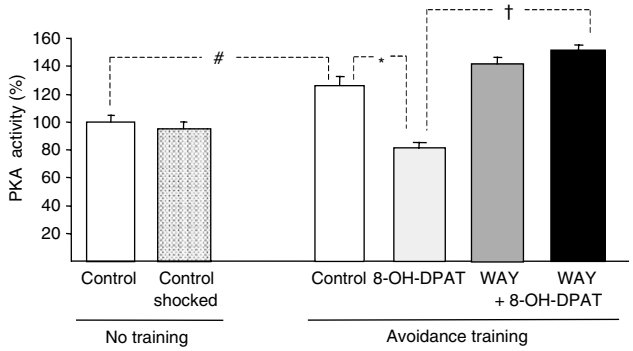

Figure 6 PKA activity in tissue homogenates from the hippocampus of rats killed 1 h after passive avoidance training and of the corresponding nontrained controls. 8-OH-DPAT (0.1 mg/kg s.c.) was given 30 min and WAY-100635 (0.5 mg/kg i.p.) 45 min before the acquisition session. All experiments were performed in triplicate. Values, means ± SEM of 18–22 animals (controls) or 8–12 animals (all other groups), are expressed as percentage of PKA activity (pmol/min/μg protein) in nontrained control rats. $^{\#}p<0.05$ *vs* control nontrained rats; $^{*}p<0.05$ *vs* control trained rats; $^{\dagger}p<0.01$ *vs* 8-OH-DPAT-treated rats. WAY = WAY-100635, 0.5 mg/kg i.p.

(−35%) was induced by 8-OH-DPAT (0.1 mg/kg) and this effect was reversed by the selective antagonist WAY-100635, 0.5 mg/kg (Figure 6).

DISCUSSION

An increase in CaMKII catalytic activity and in the expression of the autophosphorylated form of the enzyme was found in the hippocampus of rats 1 h after training on a passive avoidance task, and not in rats receiving an identical noncontingent footshock. This increase was prevented by pretraining administration of the 5-HT$_{1A}$ receptor agonist, 8-OH-DPAT, at a dose impairing retention performance. The effects of 8-OH-DPAT on CaMKII activity and retention performance were reversed by the highly selective 5-HT$_{1A}$ receptor antagonist WAY-100635. This antagonist also reversed the 8-OH-DPAT-induced enhancement in PP1 activity, which is probably a determinant of lower phosphorylated CaMKII levels, indicating receptor-specific effects. The results suggest that the 5-HT$_{1A}$ receptor-mediated disruption of retention performance is a consequence, at least in part, of the reduced phosphorylating activity of CaMKII, a critical mediator of neuronal plasticity and memory.

The impaired retention found after administration of a low dose of 8-OH-DPAT, 0.1 mg/kg, is in keeping with many previous data demonstrating deficits not only in passive avoidance (Carli *et al*, 1992; Misane *et al*, 1998; Otano *et al*, 1999; Santucci and Shaw, 2003) but also in other cognitive tasks such as spatial learning in a water-maze or visual learning in a Y-maze (Carli and Samanin, 1992; Kant *et al*, 1998; Cassaday *et al*, 2000). Previous studies from other laboratories (Misane and Ögren, 2000), as well as our previous experiments (Otano *et al*, 1999), had shown that nonspecific effects, such as changes in locomotor activity or in pain threshold, did not account for the 8-OH-DPAT-induced impairment in retention performance. The dose-related retention impairment induced by systemic

pretraining administration of 8-OH-DPAT is prevented by selective 5-HT$_{1A}$ receptor antagonists, in particular WAY-100635 (Misane and Ögren, 2000, and references therein contained), and seems to be related to an activation of postsynaptic 5-HT$_{1A}$ receptors. In this context, a recent clinical study suggests that postsynaptic 5-HT$_{1A}$ receptors in human hippocampus exert a negative influence on human memory function (Yasuno *et al*, 2003). This finding is obviously in line with the suggested potential utility of 5-HT$_{1A}$ receptor antagonists in the treatment of cognitive dysfunction (Schechter *et al*, 2002).

Abundant evidence indicates that CaMKII is an essential biochemical substrate for the early phase of memory formation (Fink and Meyer, 2002). Intracellular calcium elevation after appropriate stimuli causes the enzyme autophosphorylation at Thr286 followed by translocation of the kinase to the postsynaptic density where it binds to the NR1 and NR2B subunits of the NMDA receptor (Leonard *et al*, 1999; Shen and Meyer, 1999). This binding locks CaMKII in an active, calcium/calmodulin-independent state (Bayer *et al*, 2001) and provides spatial control of phosphorylation of different postsynaptic proteins implicated in memory formation, particularly the GluR1 subunit of the AMPA receptor (Barria *et al*, 1997). Consequently, the autophosphorylated enzyme has been proposed to serve as a 'memory molecule' for recent synaptic activity. This process is under regulation by dephosphorylation that leads to the dissociation of CaMKII from the postsynaptic density (Yoshimura *et al*, 1999). Although CaMKII may be dephosphorylated by either PP1 or PP2A, this process is almost exclusively mediated in the postsynaptic density by PP1 (Strack *et al*, 1997).

In keeping with previous studies (Cammarota *et al*, 1998), we found that passive avoidance training increased CaMKII levels in membrane preparations without any change in tissue lysate levels, as well as pCaMKII(Thr286) levels, suggesting a CaMKII translocation to postsynaptic sites. The increase in enzyme activity was also learning-specific, that is, was not influenced by a noncontingent shock, and was much more pronounced for the activated, Ca^{2+}-independent enzyme. Since novelty may influence memory of a passive avoidance task (Izquierdo *et al*, 1999), the eventual effect on CaMKII of a noncontingent shock after different times of exploration of the dark chamber remains to be established in future studies. While the amount of CaMKII in tissue lysates was unchanged by training, pCaMKII(Thr286) levels were correlated with enzyme activity. It is possible that the lack of correlation between CaMKII levels and total enzyme activity in tissue lysates results from the lower phosphorylation of CaMKII at Thr305/306. These phosphorylations are inhibitory and prevent localization of CaMKII to the postsynaptic density and kinase activity (Hudmon and Schulman, 2002; Lu *et al*, 2003). All of the learning-associated modifications in CaMKII were effectively prevented by pretraining administration of 8-OH-DPAT, an effect related to 5-HT$_{1A}$ receptor stimulation because it was antagonized by WAY-100635. Similar results were obtained in a recent *in vitro* study on rat prefrontal cortex pyramidal neurons where 5-HT and 5-HT$_{1A}$ receptor agonists reduced CaMKII activity and AMPA receptor GluR1 phosphorylation (Cai *et al*, 2002).

Of particular relevance in learning and memory is the regulation of CaMKII function by the cAMP-PKA signaling pathway. PKA indirectly regulates CaMKII by phosphorylation/activation of endogenous inhibitors of PP1 such as Inhibitor-1 and DARPP-32 (dopamine and cAMP-regulated phosphoprotein of M_r 32 kDa). These proteins act, when phosphorylated, as potent inhibitors of PP1. Inhibitor-1 is highly expressed in the dentate gyrus of the hippocampus (Gustafson *et al*, 1991), whereas DARPP-32 is enriched in striatum, although moderate to high levels are also found in several extrastriatal regions, such as the hippocampus (Ouimet *et al*, 1984). Mice lacking Inhibitor-1 display deficits in LTP induction (Allen *et al*, 2000), while an enhancement in different forms of hippocampal-dependent memory has been found in mice expressing inducibly a constitutively active form of Inhibitor-1 (Genoux *et al*, 2002). It has been established that phosphorylated Inhibitor-1, through PP1 activity blockade, maintains an active state of CaMKII at the postsynaptic location (Endo *et al*, 1996; Shobe, 2002) which may contribute to the effect of this phosphatase inhibitor on memory. It has been shown, on the other hand, that activation of DARPP-32 and the ensuing inhibition of PP-1 activity are of critical importance for the expression of two opposing forms of brain synaptic plasticity, LTD (long-term depression) and LTP (Calabresi *et al*, 2000). Also, DARPP-32 knockout mice exhibit impaired reversal learning in a discriminated operant task (Heyser *et al*, 2000). As already mentioned (see Introduction), in the hippocampus 5-HT$_{1A}$ receptors appear to be predominantly coupled through G_i proteins to adenylyl cyclase (Schoeffter and Hoyer, 1988), so it is possible that stimulation of these receptors indirectly modify CaMKII activation. Consonant with this possibility and with the above-mentioned *in vitro* study of Cai *et al* (2002), the selected 8-OH-DPAT dose produced a significant reduction of PKA activity in rats trained on the passive avoidance task and also a significant increase of PP1 enzyme activity in tissue lysates and protein levels in membranes. A critical event in the retention deficit induced by this 5-HT$_{1A}$ agonist could be a reduced Inhibitor-1/DARPP-32 phosphorylation as a consequence of the lower PKA activity. In line with this hypothesis, a recent study showed that agonists at 5-HT$_{1B}$ receptors, also coupled negatively to adenylyl cyclase, decreased *in vitro* the phosphorylation of DARPP-32 at the PKA site (Svenningsson *et al*, 2002).

The reduction of CaMKII activation induced by 8-OH-DPAT in trained rats may influence some of the molecular events related to the kinase activation and involved in later stages of memory formation, such as the increase in NMDA receptor function and the delivery of AMPA receptors to the synapses (Cai *et al*, 2002). In this regard, it is of interest that the 5-HT$_{1A}$ antagonist, WAY-100635, prevents the cognitive deficits caused by NMDA (Boast *et al*, 1999; Carli *et al*, 1999) or AMPA receptor blockade (Frechilla *et al*, unpublished observations). These findings suggest a close relationship between 5-HT$_{1A}$ and glutamate receptors (cf Edegawa *et al*, 1999). Postsynaptic 5-HT$_{1A}$ receptors are found in dendritic spines (Kia *et al*, 1996), where glutamate receptors are also concentrated. In the postsynaptic density, highly enriched in NMDA receptors (Kennedy, 1997), the NR1 and NR2B subunits bind to different proteins. Some of them are kinases and

phosphatases, such as CaMKII and PP1, involved in the modulation of receptor response (Kornau *et al*, 1995; O'Brien *et al*, 1998). We here show that these enzymes may be affected in intact rats through 5-HT$_{1A}$ receptor-mediated mechanisms. The present study provides evidence for early molecular events accounting for the impaired passive avoidance retention induced by 5-HT$_{1A}$ receptor stimulation and suggests the possible beneficial effect of 5-HT$_{1A}$ antagonists in the treatment of cognitive disorders.

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