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Effects of protein kinase A and G inhibitors on hippocampal cholinergic markers expressions in rolipram- and sildenafil-induced spatial memory improvement

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ABSTRACT

Although there are number of studies showing that phosphodiesterase (PDE) 4 and 5 inhibitors affect different kinds of memory, their effects on spatial memory consolidation in conjunction with the cholinergic activity in the hippocampus have not been studied before. In the present study firstly, rats were evaluated for the effects of different doses of the PDE4 inhibitor rolipram and the PDE5 inhibitor sildenafil on spatial memory consolidation in the water maze task. Rolipram or sildenafil was daily administered intraperitoneally 3 or 0 h after the last trial of training, respectively. Then in a separate related experiment the effect of the most efficient doses of rolipram or sildenafil accompanied by an intrahippocampally injected protein kinase A (PKA) or protein kinase G (PKG) inhibitor, respectively, was examined. Finally for determination of the hippocampal cholinergic activity the protein expression of hippocampal vesicular acetylcholine transporter (VAChT) and cholineacetyltransferase (ChAT) was measured. Rolipram at 0.03 mg/kg as well as sildenafil at 3 mg/kg increased spatial memory and their enhancing effect was completely blocked following inhibition of PKA and PKG, respectively. Furthermore, none of the treatments had a significant effect on the hippocampal ChAT and VAChT levels. Our data showed that rolipram and sildenafil enhanced spatial memory consolidation in an inverted U-shaped dose–response curve. This effect is dependent on the activity of cAMP/PKA- and cGMP/PKG-mediated pathways, respectively in the hippocampus. However, we did not find evidence for a chronic increase of cholinergic activity in the observed PDE inhibitor-induced memory improvement.

1. Introduction

Cyclic nucleotides as important and ubiquitous second messengers trigger a wide range of biological responses to stimulation of light, hormones, neurotransmitters and odorants (Campbell and Edwards, 2006; Frey et al., 1993; Rutten et al., 2007). They are strongly involved in brain neuroplasticity processes such as long-term potentiation (LTP), which is assumed to be the physiological substrate of memory (Bliss and Collingridge, 1993; Bliss and Lomo, 1973). Until now it is not clear how the different types of LTP are translated into memory consolidation, a step of memory lasting a few hours after acquisition when memories are transformed from an impermanent into a more stable status (Izquierdo et al., 2006).

Phosphodiesterase (PDE) is a superfamily of enzymes that catalyze cyclic nucleotides and are specifically distributed in the brain (Boswell-Smith et al., 2006; Lakics et al., 2010). PDE inhibitors increase intracellular levels of cAMP and cGMP. It has been found that specific PDE inhibitors improve different phases of memory consolidation probably by specific modulation of cyclic nucleotide-depending signaling (Blokland et al., 2006; Boess et al., 2004; Rutten et al., 2007). Currently, PDE inhibitors are considered a novel approach for treatment of cognitive disorders, and in particular memory improvement beyond the cholinergic-based strategies (Reneerkens et al., 2009; Rutten et al., 2007; Terry et al., 2011). Investigations on the involvement of PDEs in learning and memory have mainly focused on PDE2, PDE4, PDE5 and PDE10 (Terry et al., 2011) due to availability of selective inhibitors for these PDEs. There are in particular studies demonstrating the enhancing effect of PDE4 and PDE5 inhibitors on object recognition, social

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recognition, working and associative memory in different behavioral tasks (Barad et al., 1998; Baratti and Boccia, 1999; Devan et al., 2004; Egawa et al., 1997; Prickaerts et al., 2004, 2005; Rutten et al., 2005, 2006, 2007; Zhang et al., 2005).

Both cAMP and cGMP signaling pathways have been reported to be disrupted in Alzheimer's disease (AD) (Gong et al., 2004; Puzzo et al., 2009). Additionally, these studies showed that PDE4 inhibitor rolipram and PDE5 inhibitor sildenafil are able to attenuate memory dysfunction in AD mice.

There is a wealth of human and animal investigations indicating that cholinergic pathways play an important role in hippocampal plasticity and cognition (Drever et al., 2011; Sharifzadeh et al., 2006; Terry and Buccafusco, 2003). In addition, molecular studies in both AD animal models and human patients have shown a significant decrease in acetylcholine synthesis and release, choline reuptake as well as a decrease in the number of nicotinic and muscarinic receptors (Antoine et al., 2004; Auld et al., 2002). For instance in AD, memory loss is associated with a dysfunction of acetylcholine neurotransmission (Bartus et al., 1982). Secondly, the majority of clinically available treatments as cognitive enhancers or for the amelioration of AD symptoms act via increasing the activity of cholinergic pathways (Auld et al., 2002; Terry et al., 2011). Increases in the cholinergic activity in animal brain are being used to delineate the memory enhancing effect of substances by measuring the expression of cholineacetyl transferase (ChAT) and vesicular acetylcholine transporter (VAChT) as two well-known cholinergic markers (Azami et al., 2010; Parent and Baxter, 2004; Sharifzadeh et al., 2006).

Interestingly, despite the previous studies related to beneficial effects of PDE4 and PDE5 inhibitors on different kinds of memory processes, their effects on spatial memory consolidation and the possible involvement of a cholinergic mechanism had not been studied before. Therefore, in this study we examined the possible role of the cholinergic system in the PDEs induced memory improvement.

In the present experiment we first investigated the effects of different intraperitoneal (i.p.) doses of the PDE4 inhibitor rolipram and the PDE5 inhibitor sildenafil on spatial memory consolidation in the Morris water maze task. Drugs were daily administered 3 or 0 h after the last trial of training, respectively, based on previous studies (Rutten et al., 2007, 2009). Next we explored the molecular mechanisms in spatial memory consolidation by combining the most effective doses of rolipram or sildenafil with a PKA or PKG inhibitor, respectively, which were injected intrahippocampally (i.h.). Finally for determination of possible interaction between elevated cyclic nucleotides and the hippocampal cholinergic activity, the expressions of hippocampal cholineacetyltransferase (ChAT) and vesicular acetylcholine transporter (VAChT) were measured by western blotting analysis.

2. Material and methods

2.1. Animals

Adult male Wistar rats (n = 120) weighing 200–220 g were obtained from Pasteur Institute of Iran. Animal were housed four in a cage and were randomly divided in different experimental groups and maintained at room temperature (25 ± 2 °C) under standard 12 h light – 12 h dark cycle. Rats were handled daily for training and tested in the light period (7 a.m–7 p.m). Food and water were freely available. Animals were subjected in accordance with recommendations of the Ethical Committee for the use and care of laboratory animals of Tehran University of Medical Sciences (No. 8069, July 2008).

2.2. Surgery

Rats were anesthetized with a mixture of ketamine (100 mg/kg, i.p.) and xylazine (25 mg/kg, i.p.) and placed in a stereotaxic instrument (Stoelting, Wood Dale, IL, USA). Guide cannulas were implanted bilaterally into the CA1 region and attached to the skull surface using dental cement and jeweler's screws. Stereotaxic coordinates for cannula placement were determined based on the Paxinos and Watson atlas (anterior–posterior, 3.8 mm; medio-lateral, ±2.2 mm; dorso-ventral, 2.7 mm from bregma) (Paxinos et al., 1980). Intra-hippocampal infusions were administered through guide cannulas (23 G) using injection needles (30 G) connected by polyethylene tubing to 5 μl Hamilton micro-syringes. The injection needle was inserted 0.5 mm beyond the tip of the guide cannula. The duration of the drug infusion was 2.5 min, and the needles were left in place for an additional 60 s to allow diffusion of solution away from the needle tip.

2.3. Behavioral procedure and treatments

The Morris water maze (MWM) task and particular environmental conditions have been described extensively (Choopani et al., 2008). A hidden platform which was located in the center of North-West quadrant (target quadrant) was the only escape from the water. Animals were trained to find the hidden platform for four subsequent days as described elsewhere (Hosseini-Sharifabad et al., 2011; Sharifzadeh et al., 2007a). Briefly, four trials per day were given with random starting positions. A video camera was located directly above the center of water maze pool, which recorded the rat swimming track. Recorded tracks were transformed to data of escape latency (time to reach the hidden platform), traveled distance (length of the swimming path), swimming speed and times spent in different zones of arena by EthoVision 3.1 tracking system (Noldus Information Technology, Wageningen, the Netherlands) to evaluate training efficacy and spatial memory performance (Azami et al., 2010; Hosseini-Sharifabad et al., 2011). The arena was divided to four equal quadrants and the platform containing quadrant named target quadrant. Target proximity annulus was conventionally defined as a two centimeter diameter circle around the platform in the image of arena.

The drugs were freshly dissolved in saline or DMSO (1% in saline). In the first step of the experiment, rolipram (Sigma, USA) (0.01, 0.03, 0.1, and 0.3 mg/kg) or sildenafil (Cayman Chemical, USA) (1.5, 3, and 4.5 mg/kg) was injected intraperitoneally 3 h or immediately after the last trial of training in each day, respectively. The control groups received daily saline (injection volume: 2 ml/kg) 3 or 0 h after last trial. In the second step, the different groups of animals that underwent stereotaxic surgery were treated according to one of the following conditions: rolipram (0.03 mg/kg, i.p.) and vehicle (DMSO 1%, 1 μl/side, i.h.) or rolipram (0.03 mg/kg, i.p.) and H89($C_{20}H_{20}BrN_3O_2S \cdot 2HCl \cdot xH_2O$) (Sigma, USA)(5 μmol/l, 1 μl/side, i.h.) 3 h, sildenafil (3 mg/kg, i.p.) and vehicle (DMSO 1%, 1 μl/side, i.h.) or sildenafil (3 mg/kg, i.p.) and KT5823 ($C_{29}H_{25}N_3O_5$) (Sigma, USA) (5 μmol/l,1 μl/side, i.h.) directly, saline (0.5 ml, i.p.) and vehicle (DMSO 1%, 1 μl/side, i.h.) as control animals 3 or 0 h, after the last trial of training in each day.

For evaluation of consolidation in treated animals a probe testing trial was carried out 48 h after the last block of training. In this trial, the hidden platform was removed, and animals were placed in the pool in opposite of target quadrant and allowed to swim freely for 90 s. Thirty minutes after the probe trial, the platform was elevated in the center of South-West quadrant above the water surface, and each rat was put in the water from each starting side randomly (visibility).

2.4. Histology

After behavioral evaluations, animals were randomly decapitated and their brains were removed for histological verification in a separate experiment. To make sure the cannula were implanted in the dorsal hippocampus, brain sections were taken, mounted on slides and stained with cresyl violet.

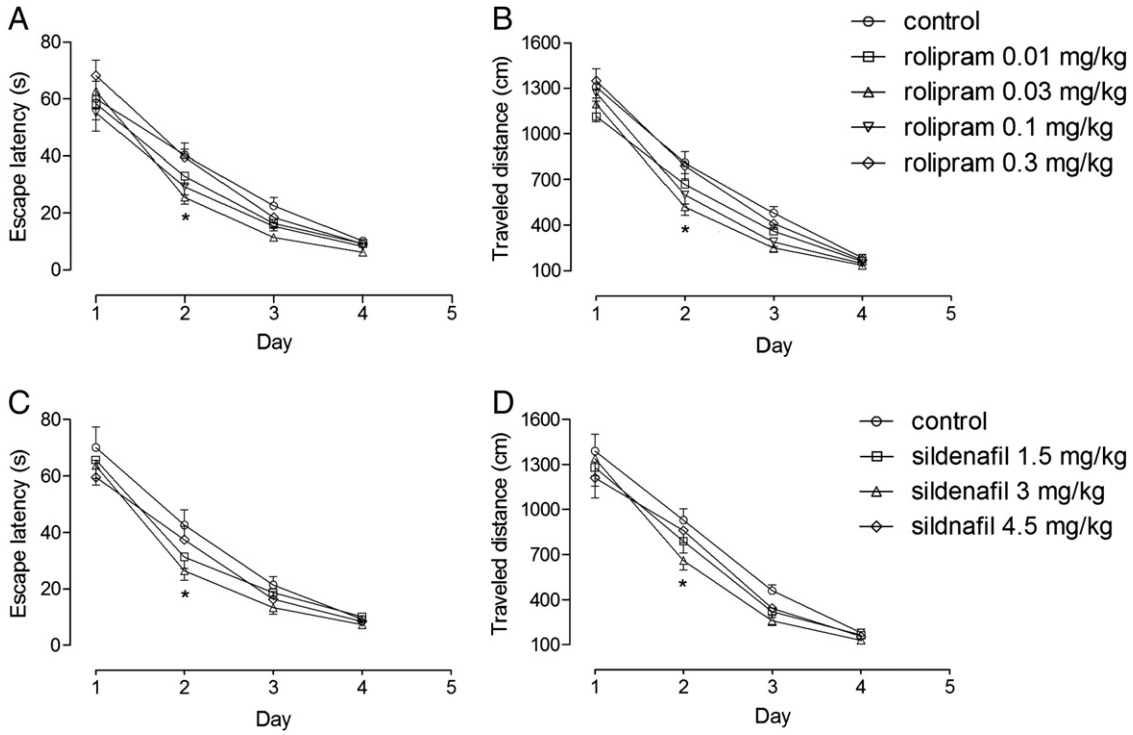

Fig. 1. Effect of post-training administration of rolipram and sildenafil on finding the hidden platform in the Morris Water Maze task.All groups of animals learned to find the hidden platform during the training process. There was a significant difference ($P<0.001$) in escape latency (A, C) and traveled distance (B, D) between the first and fourth day of training in each group. There are no significant differences in escape latency and traveled distance between the treated groups on the fourth day (A–D). On the second day, 0.03 mg/kg rolipram (A, B) and 3 mg/kg of sildenafil (C, D) showed shorter escape latencies and distances compared to respective control groups (*$P<0.05$). The results are presented as means ± S.E.M. for each group (n = 7).

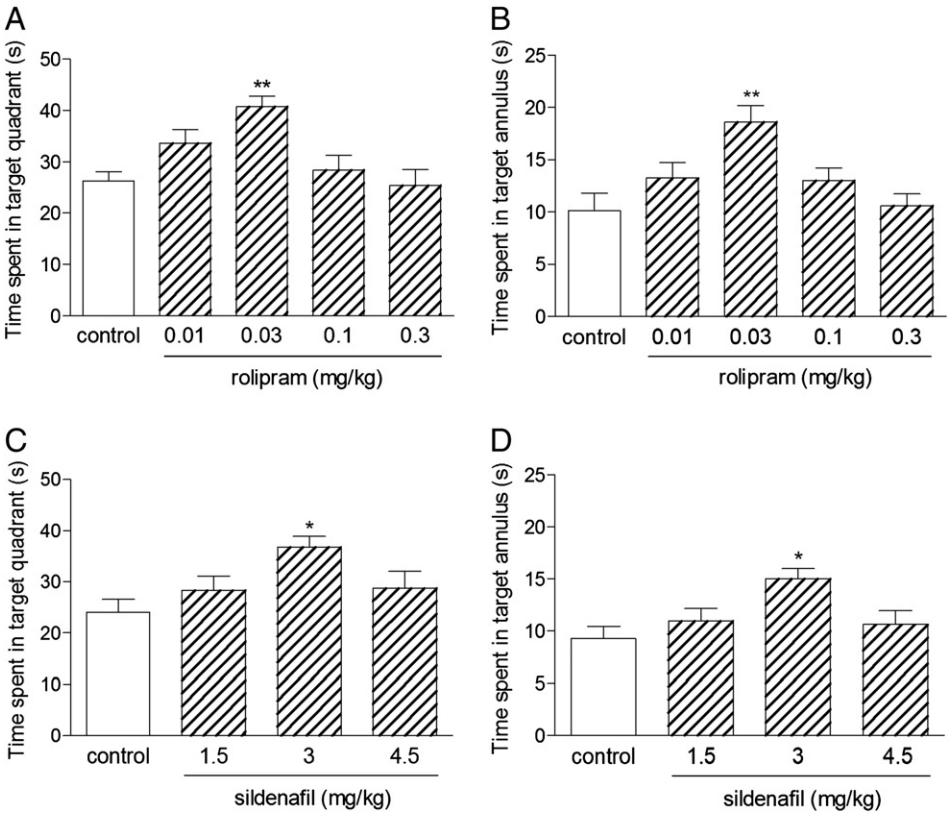

Fig. 2. Effect of post-training administration of rolipram and sildenafil on the time spent in the target quadrant and target proximity annulus in the MWM probe trial.Intraperitoneal administration of 0.03 mg/kg rolipram 3 h after the last trial of training in each day significantly increased the time animals spent in target quadrant (A) and target proximity annulus (B). This significant increasing effect on time spent in target quadrant (C) and proximity annulus (D) was shown when 3 mg/kg of sildenafil had been daily injected directly after the last training trial. Other studied doses of rolipram and sildenafil had no significant effect compared to control groups. Columns represent the means ± S.E.M (n = 7). (*$P<0.05$, **$P<0.01$; ANOVA with Newman-Keuls post-hoc correction).

2.5. Western blot analysis

The brains were dissected and bilateral hippocampi were removed in cold artificial cerebrospinal fluid. Hippocampal tissues were homogenized with a manual homogenizer (EF24837V, Kontes, USA). Homogenized tissues were incubated with 200 μl of lysis buffer (10 mmol/lTris pH = 8, 0.1% SDS, 0.5 mmol/l DTT, 1% NP40, 0.5% Na-deoxycolate, 0.5 mmol/l PMSF, 5 μg/ml leupeptin) at 4 °C for 30 min. The samples were centrifuged at 14,000 rpm and 4 °C for 5 min, and the supernatant was taken out. 20 μl of loading buffer (50 mmol/lTris pH = 6.8, 2% SDS, 4% 2-mercaptoethanol, 0.1% bromophenol blue, 10% glycerol) was added to the product and boiled for 8 min. The samples were centrifuged in 14,000 rpm for 5 min, and the supernatants were loaded (60 μg protein) onto Sodium Dodecyl Sulfate Poly Acrylamide Gel Electrophoresis (SDS-PAGE). Proteins were separated on the 10% SDS-PAGE and electrophoretically transferred to a polyvinylidenefluride membrane (Roche, Germany). Membranes were then immunoblotted with anti-ChAT (Millipore, USA) (1:1000), anti-VAChT (Abcam, USA) (1:1000), or anti-β actin (Santa Cruz, USA) (1:2000) antibodies, in separate steps. For detection of the antibody reaction, blots were incubated with horseradish peroxidase-conjugated anti-goat (Abcam, USA) or anti-rabbit (Roche, Germany) (1:10,000) antibodies (according to primary antibody source). Detection was carried out using a Chemiluminescence Western Blotting Kit (mouse/rabbit) (Roche, Mannheim, Germany) on BioMax film (Kodak, Rochester, NY). Immunoreactive bands were digitized using image J 1.410 (NIH, USA) and normalized to β-actin.

2.6. Statistical analysis

Results are expressed as mean ± S.E.M. The data of the training trials were analyzed using a two way analysis of variance (ANOVA) with days as repeated measures factor and treatments as between subjects' factor. For statistical analyses of probe and visibility trial as well as molecular data a one way ANOVA was used. For multiple comparisons we used the Newman-Keuls post hoc tests in both behavioral and molecular part of experiment. P-values less than 0.05 were considered as significant.

3. Results

3.1. Effect of training on finding the hidden platform during consecutive training days in the MWM task

All groups of animals learned the location of the hidden platform after 4 days of training. In each group, escape latency ($F(3,120) = 221.5$ $P<0.001$ for rolipram and $F(3,96) = 135.9$, $P<0.001$ for sildenafil) (Fig. 1A, C) and traveled distance ($F(3,120) = 241.9$, $P<0.001$ for rolipram and $F(3,96) = 190.7$, $P<0.0001$ for sildenafil) (Fig. 1B, D) decreased significantly after 4 days compared to the first day. Swimming speed did not reveal remarkable changes during the training ($F(3,120) = 0.91$, n.s. for rolipram and $F(3,96) = 1.09$, n.s. for sildenafil) (data not shown). During the second day of training, 0.03 mg/kg rolipram significantly decreased escape latency ($F(4,120) = 4.6$, $P<0.05$) and traveled distance ($F(4,120) = 4.8$, $P<0.05$)(Fig. 1A, B), just as 3 mg/kg sildenafil treated animals showed significant shorter escape latency ($F(3,96) = 2.5$, $P<0.05$) and distance swum ($F(3,96) = 2.6$, $P<0.05$) (Fig. 1C, D) compared to the respective control groups.

3.2. Effect of post-training administration of rolipram and sildenafil on time spent in the target quadrant and proximity annulus in a 90 sec probe trial test

Treatment 3 h after training with 0.03 mg/kg rolipram, significantly increased the time animals spent in the target quadrant ($F(4,30) = 6.4$,

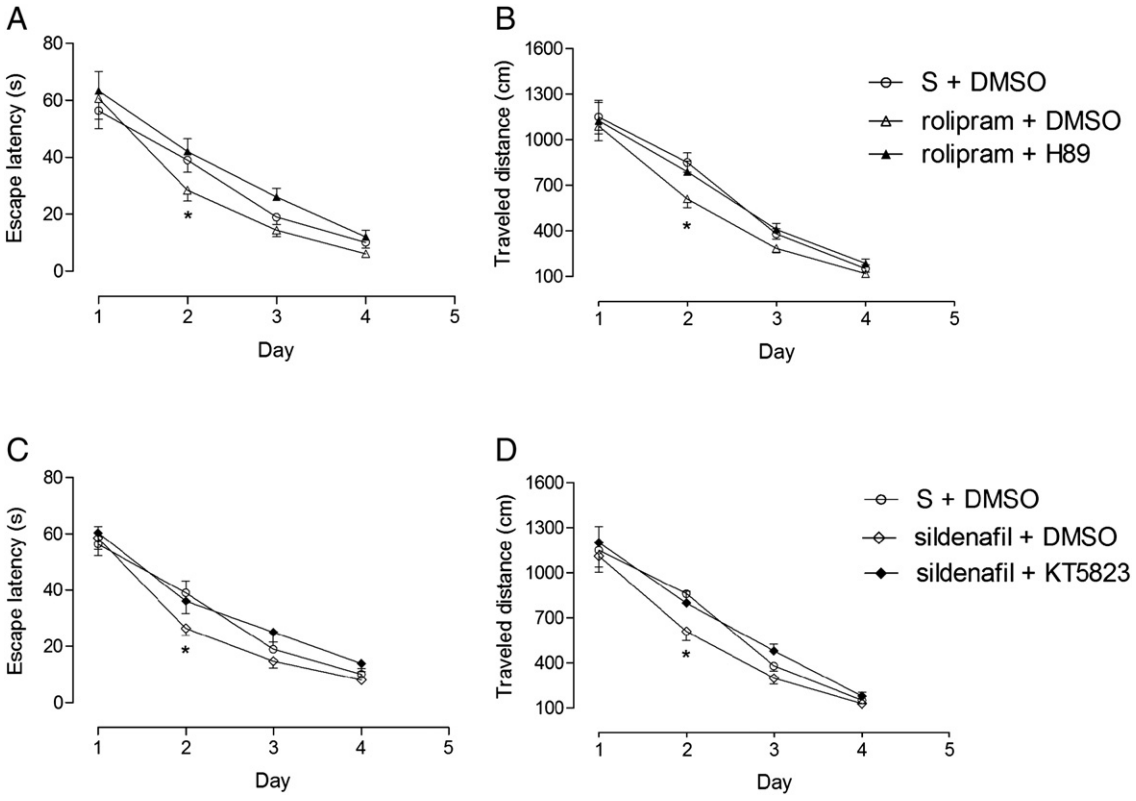

Fig. 3. Effect of post training administration of PKA inhibitor in rolipram-treated and PKG inhibitor in sildenafil-treated animals on finding the hidden platform in the Morris Water Maze task.The beneficial effect of rolipram (0.03 mg/kg) and sildenafil (3 mg/kg) on latency and distance swum at the second day of training were reversed to control levels by co-administration of H-89 (5 μM/side, i.h.) (A, B) and KT5823 (5 μM/side, i.h.) (C, D) respectively. The results are presented as means ± S.E.M. for each group (n = 7). (*$P<0.05$; two-way ANOVA) (DMSO; Dimethyl Sulfoxide, S; Saline).

$P<0.01$) and target annulus ($F(4,30)=5.4$, $P<0.01$) during the probe testing trial (Fig. 2A, B). This time was also increased following the administration of 3 mg/kg sildenafil given immediately after the last trial during each training session, both in target quadrant ($F(3,24)=3.8$, $P<0.05$) and annulus ($F(3,24)=4.4$, $P<0.05$) (Fig. 2C, D). Other dosages of rolipram (0.01, 0.1 and 0.3 mg/kg) and sildenafil (1.5 and 4.5 mg/kg) did not show any differences as compared to controls (Fig. 2A–D). Swimming speed was not significantly different in rolipram ($F(4,30)=0.52$, n.s.) or sildenafil ($F(3,24)=0.12$, n.s.) treated animals compared to respective controls (data not shown).

3.3. The interactive effect of PKA inhibitor on rolipram and PKG inhibitor on sildenafil-enhanced spent time in target quadrant and proximity annulus

Post-training bilateral infusion of the PKA inhibitor H89 (5 μM/side i.h.) reduced the rolipram-induced (0.03 mg/kg) decrease in swim latency ($F(2,72)=3.8$, $P<0.05$) and distance ($F(2,72)=3.2$, $P<0.05$) during the second day of training (Fig. 3A, B), as well as the increase in time spent in target quadrant ($F(2,18)=16.2$, $P<0.001$) and annulus ($F(2,18)=9.3$, $P<0.01$) to baseline level (Fig. 4A, B). Similarly, when the PKG inhibitor KT5823 (5 μM/side i.h.) and sildenafil (3 mg/kg i.p.) were co-administered, swim latency ($F(2,72)=2.9$, $P<0.05$) and distance swum ($F(2,72)=3.9$, $P<0.05$) during second day of training (Fig. 3C, D) and the time spent in target quadrant ($F(2,17)=8.5$, $P<0.01$) and annulus ($F(2,17)=7.4$, $P<0.01$) (Fig. 4C, D) was inverted to control levels. Again, none of the experimental groups showed a significant difference in swimming speed ($F(3,24)=0.52$, n.s.) (data not shown).

3.4. Treatment effect on performance in a visual discrimination task

Escape latencies to finding the visible platform during visual discrimination task showed no significant differences in the different rolipram-treated ($F(4,30)=0.77$, n.s.) and sildenafil-treated ($F(3,24)=0.26$, n.s.) groups compared to their respective controls (Fig. 5A, B). Also combined PDE treatments with H89 or KT5823 did not affect performance in comparison to control groups ($F(2,18)=0.22$, n.s. and $F(2,18)=0.1$, n.s., respectively) (Fig. 5C, D).

3.5. Effect of rolipram and sildenafil treatment in combination with MWM training on hippocampal ChAT and VAChT expression

The training process significantly increased hippocampal ChAT ($F(5,18)=5.5$, $P<0.01$) (Fig. 6A, B) and VAChT ($F(5,18)=6.6$, $P<0.01$) (Fig. 6A, C) protein level expressions in comparison to untrained animals. Neither rolipram (0.03 mg/kg, i.p.) nor sildenafil (3 mg/kg) treatment increased the expression of these cholinergic related proteins compared to trained animals. Also co-treatment with H89 (5 μM/side) and KT5823 (5 μM/side) did not show any significant change in the expression of these markers in rolipram and sildenafil treated groups respectively (Fig. 6A–C).

4. Discussion

In this study we examined the effects of different doses of phosphodiesterase 4 and 5 inhibitors (rolipram and sildenafil, respectively) on spatial memory consolidation in the MWM task. Our data showed that

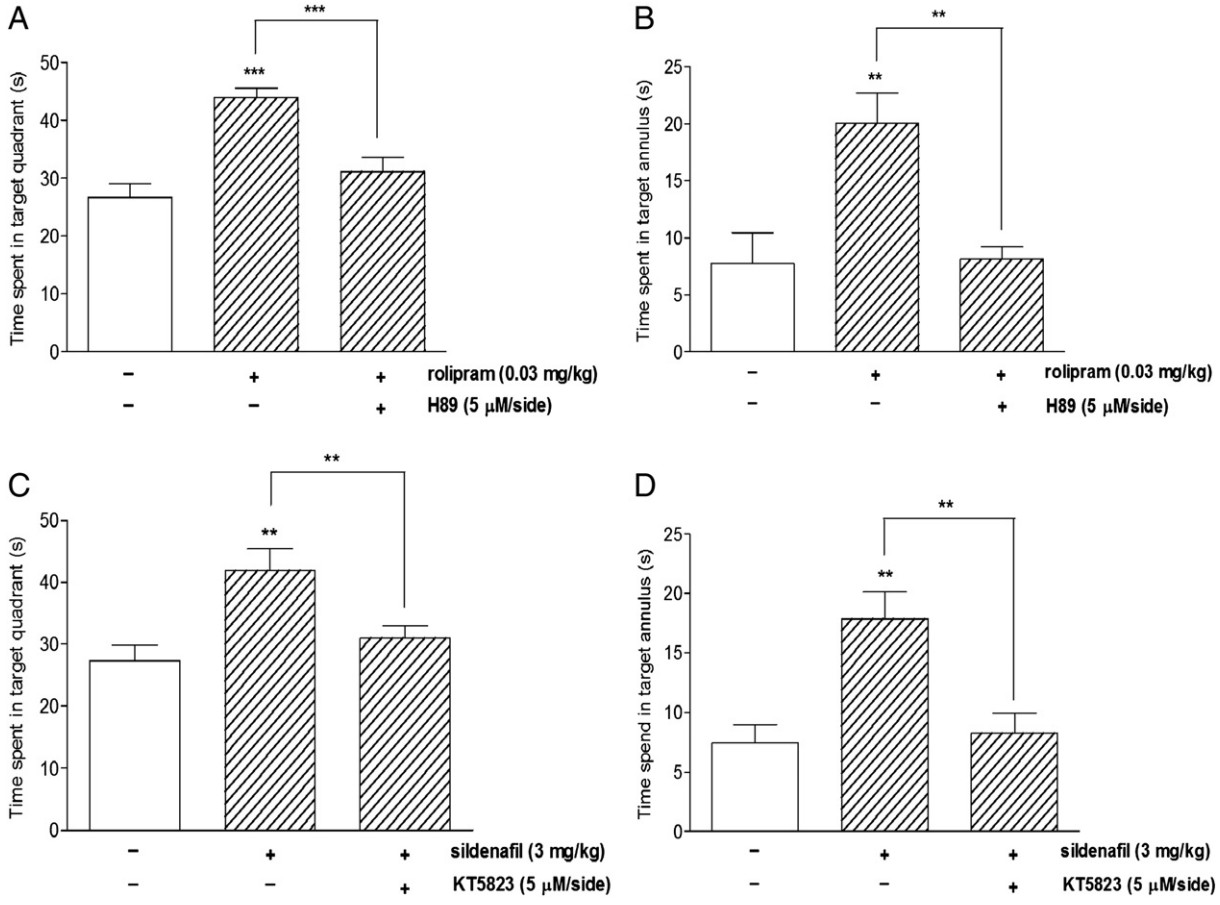

Fig. 4. Effect of post training administration of PKA inhibitor in rolipram-treated and PKG inhibitor in sildenafil-treated animals on time spent in target quadrant and proximity annulus.Intrahippocampal infusion of H-89 (5 μM/side) three hours after training significantly reversed enhancing effects of rolipram (0.03 mg/kg) on time spent in target quadrant (A) and proximity annulus (B) to control levels. Infusion of KT5823 (5 μM/side, i.h.) directly after the last trial of training significantly blocks the enhancing effect of sildenafil (3 mg/kg i.p.) on time spent in target quadrant (C) and proximity annulus (D) to normal level. Columns represent the means ± S.E.M (n = 7). (**$P<0.01$, ***$P<0.001$; ANOVA with Newman-Keuls post-hoc correction).

animals in all treatment conditions learned how to locate the platform after 4 days of training. However, animals that were treated with rolipram (0.03 mg/kg) and sildenafil (3 mg/kg) showed a steeper learning curve, suggesting an improvement in spatial memory consolidation. During the probe trial, which was carried out 48 h after the last training trial, the same animals (rolipram, 0.03 mg/kg; sildenafil, 3 mg/kg) showed a marked increase in time spent in the area where the platform used to be when compared to other conditions, indicating that PDE inhibitor treatment leads to superior spatial memory formation. These findings add to the accumulating evidence provided in current literature demonstrating cognition-enhancing effects of PDE inhibitors. Beneficial effects of PDE4 and PDE5 inhibitors on memory have been described in different behavioral paradigms and species, as well as in healthy subjects as in deficit models (Reneerkens et al., 2009).

None of the treatment conditions had an influence on swimming speed, demonstrating the absence of long-term deleterious effects of the studied PDE inhibitors on animal's muscle and motor function. However, it has been shown that some PDE inhibitors in higher doses cause side-effects, including sedative effects (Rutten et al., 2006), which might explain why the effect of rolipram and sildenafil was reduced when increasing the administered dose. No differences between control and experimental groups were detected with respect to non-spatial discrimination. Therefore, it is conceivable that the observed differences in MWM performance can neither be attributed to alterations of other non-mnemonic factors such as motivation or sensory processes.

Manipulating PDEs enables us to influence the potency of cyclic nucleotide signaling. Rolipram prevents cAMP degradation and consequently increases its level in presynaptic and postsynaptic neurons by inhibition of PDE4. Sildenafil elevates intracellular cGMP levels through inhibition of PDE5 (Bender and Beavo, 2006; Blokland et al., 2006; Boswell-Smith et al., 2006; Rutten et al., 2005, 2007). Cyclic nucleotides are important second messenger molecules in many cellular processes, including synaptic plasticity. Both cAMP and cGMP play a key role in hippocampal LTP (Boess et al., 2004; Frey et al., 1993; Prickaerts et al., 2002a; Rutten et al., 2007, 2008). Cyclic GMP and cGMP-related signaling processes are important during the early phase of LTP (E-LTP) which lasts less than 3 h, does not require gene transcription, and depends on the activation of a NO/GC/cGMP/PKG signaling cascade (Bernabeu et al., 1997b; Boulton et al., 1995; Campbell and Edwards, 2006; Monfort et al., 2002; Rutten et al., 2007; Zhuo et al., 1994). However, cAMP appears to be more relevant in the late phase of LTP (L-LTP) that lasts longer than 3 h, requires gene transcription and can be mediated either by a cAMP/PKA/CREB or cGMP/PKG/CREB dependent cascade (Bernabeu et al., 1997a; Bolshakov et al., 1997; Nguyen et al., 1994; Rutten et al., 2007). Indeed, previous studies have found that in order to be effective, cGMP-targeting drugs should be administered during the early consolidation phase, while cAMP-targeting treatments are most potent when given during the later stages of memory consolidation (Bernabeu et al., 1997a; Prickaerts et al., 2002a; Rutten et al., 2007, 2009). Along these lines, we injected sildenafil immediately or rolipram 3 h after the completion of training trials to study the cGMP- and cAMP-mediated mechanisms in more detail. We showed that the memory enhancing effect of rolipram was reversed to control levels by hippocampal inhibition of PKA immediately after the administration of rolipram, 3 h after training. Also, hippocampal inhibition of PKG immediately after the injection of sildenafil significantly reduced the sildenafil-induced memory improvement to respective control values. These results indicate that the enhancing effects of rolipram as a PDE4 inhibitor and sildenafil as a PDE5 inhibitor on spatial memory consolidation are mediated by PKA and PKG in the hippocampus, respectively. This is in agreement with previous studies that showed cAMP/PKA/CREB and cGMP/PKG/CREB signaling pathways lead to facilitation of LTP and memory formation (Bernabeu et al., 1997a; Kleppisch and Feil, 2009; Prickaerts et al., 2002a, 2002b;

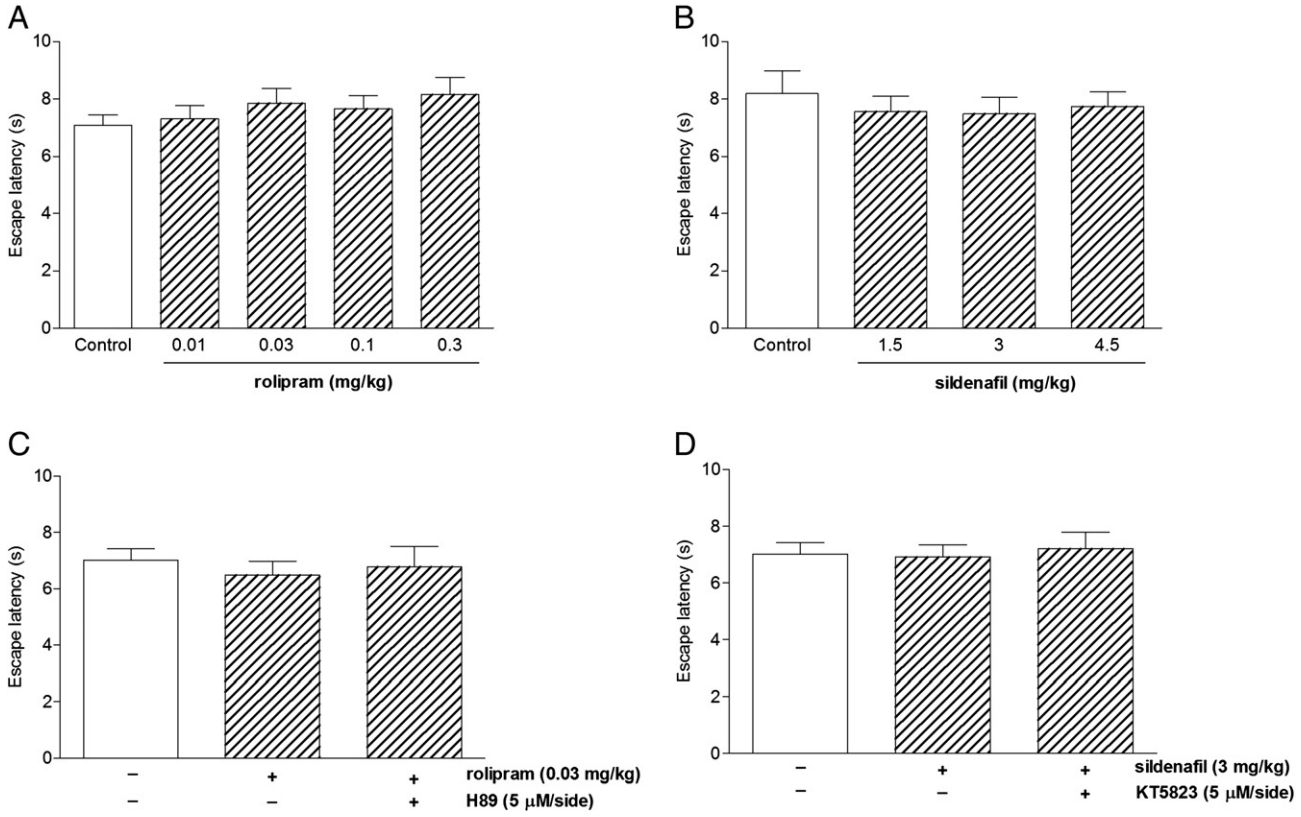

Fig. 5. Escape latency during visual discrimination task.Animals in all treatment conditions showed no difference in escape latency to find a visible platform when compared to the respective control groups (A–D). Columns represent the means ± S.E.M (n = 7).

Rutten et al., 2007, 2009). Downstream CREB phosphorylation in cyclic nucleotide signaling might lead to alterations in expression of plasticity-relevant proteins, which would instigate long-lasting changes in synaptic connectivity (Bernabeu et al., 1997a; Kleppisch and Feil, 2009; Prickaerts et al., 2002a, 2002b; Rutten et al., 2007, 2009). Within this context, possible mechanisms for PDE inhibitors-induced memory enhancement include the activation of cyclic nucleotide-gated ion channels, neurogenesis, anti-oxidant capacities and modulation of neurotransmitters activity (Abdollahi et al., 2003; Ferrari et al., 2002; Matsumoto et al., 2009; Uthayathas et al., 2007; Zhang et al., 2006). Our findings emphasize the potential of PDE inhibitors as an interesting new target for cognition improvement.

In this study the expression of hippocampal ChAT and VAChT was also assessed to detect changes in hippocampal cholinergic activity following the administration of PDE inhibitors in consolidation and therefore to evaluate the contribution of the cholinergic system in rolipram- or sildenafil-induced memory enhancement. We found that training in the MWM caused a remarkable increase in hippocampal ChAT and VAChT expression. This is consistent with the results of previous studies indicating the crucial role of the cholinergic system in spatial learning and spatial memory retention (Azami et al., 2010; Hosseini-Sharifabad et al., 2011; Sharifzadeh et al., 2005, 2007b). One of the major finding of the present study is that PDE inhibitors did not cause significant alterations in the expression of cholinergic

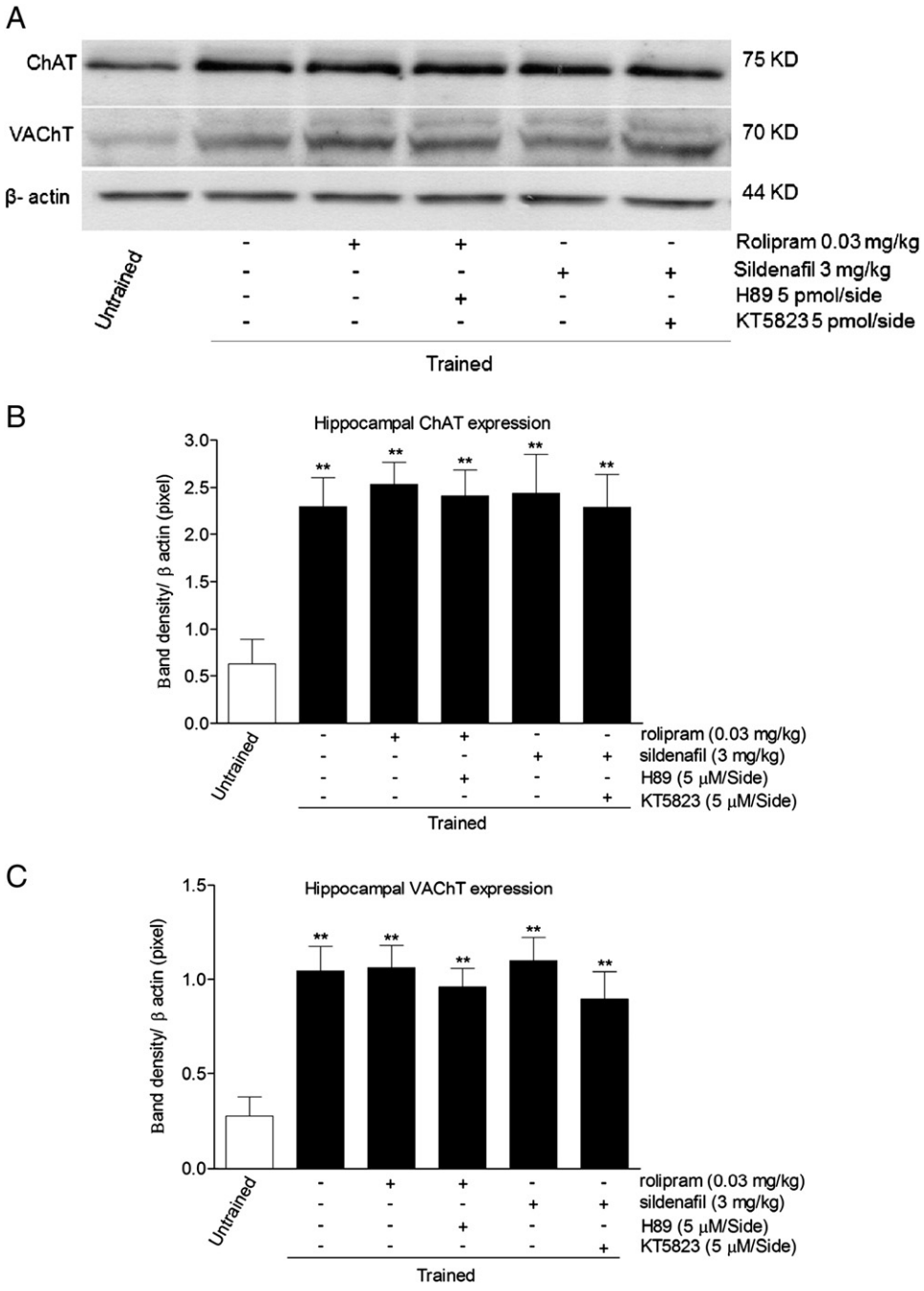

Fig. 6. Effect of rolipram and sildenafil on hippocampal ChAT and VAChT expression in trained animals administered separately or in combination with PKA and PKG inhibitor respectively.The training process significantly increased hippocampal ChAT (A, B) and VAChT (A, C) protein level expressions in comparison to untrained animals. Neither rolipram (0.03 mg/kg) nor sildenafil (3 mg/kg) showed an increase in hippocampal ChAT (A, B) and VAChT (A, C) expression compare to trained animals. Administration of H-89 (5 μM/side) in rolipram-treated and KT5823 (5 μM/side) in sildenafil-treated animals did not show a significant change in hippocampal ChAT (A, B) and VAChT (A, C) expression. Columns represent the means ± S.E.M of at least seven animals in each group. (**$P<0.01$; ANOVA with Newman-Keuls post-hoc correction).

markers during consolidation stage of memory. This result is different with the antagonization of scopolamine-induced memory deficits after PDE inhibitors administration (Devan et al., 2004; Egawa et al., 1997; Rutten et al., 2006). Thus, the later results might suggest that the cholinergic pathways in the hippocampus are not primarily important for consolidation-promoting effects of PDE inhibitors. It can be deduced that PDE inhibition induces a more global effect by affecting second messenger systems, which is in contrast to the presently approved cognition enhancers that mainly focus on cholinergic activity via augmentation of acetylcholine levels in the synaptic cleft (Bartus et al., 1982; Terry et al., 2011). However, the treatment period was relatively short, i.e. 4 consecutive days, and the hippocampal tissue was collected 50 h after the last treatment. Thus, it is not unlikely that we failed to detect an effect on the cholinergic measures because we did not assess acute effects within 24 h after PDE treatment on the cholinergic markers, while actual chronic effects might require a more extended period of drug administration.

In the present study, we demonstrate that PDE4 and PDE5 inhibitors enhance spatial memory consolidation, and that the induced memory improvement is dependent on the activity of cAMP/PKA- and cGMP/PKG-mediated pathways, respectively. We could not find evidence for a downstream involvement of chronic alterations in the cholinergic activity after PDE-inhibition treatment. Future research should be aimed at further elucidating the underlying mechanisms of cognition enhancement after PDE inhibition treatment.

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