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Administration of muscarinic antagonists induce changes in passive avoidance learning and in synaptic transmission in the CA1 area of the hippocampus

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Muscarinic acetylcholine receptors (mAChR) are known to be related to learning and memory processes. Inactivation of mAChR by cholinergic antagonists have been shown to produce amnesia in a variety of behavioral tasks. In this study, we investigated the role of M1 and M2 AChR on passive avoidance learning and plasticity of synapses formed by Schaffer collaterals in freely moving rats. Experiments were performed using Wistar male rats. Seven days before testing, a recording electrode was lowered in the CA1 region under chloral hydrate anaesthesia to record the field excitatory postsynaptic potential (fEPSP) in response to Schaffer collateral stimulation. Selective M2 receptor antagonists methoctramine and selective M1 receptors antagonist pirenzepine were intraperitoneally injected immediately after training. The effects on memory retention were examined using passive avoidance training. We measured latency of the first entry into a dark compartment of the chamber. fEPSP amplitude and slope ratio were measured before shock presentation, 90 min after the shock, and 24 hour after the shock. Methoctramine significantly impaired behavior in the passive avoidance test but pirenzepine did not induce any changes compared to control. Our results showed that pirenzepine but not methoctramine suppressed the amplitude of fEPSPs. On the other hand, intracerebroventricular methoctramine administration impaired passive avoidance learning and increased the amplitude of fEPSP.

Key words: methoctramine, pirenzepine, mAChR, hippocampus, passive avoidance, memory consolidation

INTRODUCTION

Acetylcholine (ACh) is involved in modulation of neuronal excitability, synaptic transmission, and plasticity in the hippocampus (Cobb and Davies 2005) and classically excites pyramidal cells (Cole and Nicoll 1983, Dodd et al. 1981). The hippocampus is the main structure that is involved in different forms of learning, memory, and synaptic plasticity (Bliss and Collingridge 1993, Izquierdo et al. 2002). Previous studies have shown that each region of the hippocampus is associated with various types of memory, such as episodic, contextual or working memory. It is known that the CA1 region of the hippocampus is involved in memory formation processes. Immediate, but not delayed, intra CA1 infusions of

the selective muscarinic agonist oxotremorine improves memory, whereas scopolamine has an amnestic effect in passive avoidance (PA) learning (Izquierdo and Medina, 1997). Synaptic processes in this region are strongly regulated by cholinergic inputs and it appears that muscarinic but not nicotinic receptors play the leading role in the modulation of these processes (Dasari and Gullledge 2011, Fernández de Sevilla et al. 2002).

There are five subtypes of muscarinic acetylcholine receptors (mAChR) and all of them are expressed in the hippocampus (Drever et al. 2011, Flynn et al. 1995). The major subtypes of muscarinic receptors within the central nervous system (CNS) are muscarinic M1, M2 and M4 (Drever et al. 2011). Recent findings from single cell analysis revealed that, in the hippocampus, muscarinic M1 receptors are located on CA1 pyramidal cells. M2

receptors are expressed by interneurons (Zeisel et al. 2015) and are present on septo-hippocampal cholinergic terminals (Drever et al. 2011, Rouse et al. 2000, Zhang et al. 2002). According to Zeisel et al. (2015), M4 receptors localize not only to interneurons (like M2 receptors) but also to pyramidal cells. M1 receptors are coupled to $G_{q/11}$ -protein and muscarinic agonists can activate phospholipase C and increase the level of intracellular Ca^{2+} . In contrast, M2 and M4 receptors are coupled to $G_{i/o}$ -protein and their activation reduces activity of adenylate cyclase, which leads to reduction of cAMP levels (Drever et al. 2011, Lanzafame et al. 2003).

Many studies, using brain slices, have shown that the involvement of muscarinic receptors in the induction of long-term potentiation (LTP), particularly in Schaffer collaterals (SC/C) and the CA1 region. It was shown that application of the muscarinic agonist carbachol (CCh) enhances LTP or induces LTP by itself (Auerbach and Segal 1994, 1996, Shinoue et al. 2005) and antagonists disrupt LTP (Sanchez et al. 2009). In slices of M2 knockout mice LTP was significantly reduced compared to wild types (Seeger et al. 2004). Participation of mAChR in the regulation of LTP was also shown in studies *in vivo*. Stimulation of the septum in anesthetized rats induced LTP in CA1 (Markevich et al. 1997, 2007) and this potentiation was blocked by the non-selective acetylcholine receptor antagonist scopolamine and M1 receptor antagonist pirenzepine (Ovsepian et al. 2004). M2 muscarinic antagonist administration enhanced synaptic transmission in the CA1 area *in vivo* (Hayes et al. 2008). However, all these studies did not reveal any relationships between changes in synaptic plasticity and behavior.

Many studies have suggested a role of muscarinic receptors in learning and memory processes. For instance, scopolamine and pirenzepine, antagonists of muscarinic receptors, significantly impaired short-term memory performance and spatial cognition in the delayed non-matching to position test and in the 8-arm radial maze task when given before testing (Aura et al. 1997, Mishima et al. 2000). M2- and M2/M4-knockout mice showed an impairment in fear-associated learning in a PA task (Tzavara et al. 2003). Nevertheless, some papers indicated that muscarinic M2 receptor selective antagonist methoctramine could improve short-term memory performance in delayed non-matching to position test (Aura et al. 1997). Only a few papers showed the relation between mAChR-dependent synaptic plasticity and learning. One of the papers showed that spatial working memory was affected by the M1 antagonist pirenzepine but the drug did not induce any changes in the CA1 LTP in anesthetized animals (Kikusui et al. 2000). Another study demonstrated that cholinergic facilitation of hippo-

campal LTP in the walking animals *in vivo* was blocked by pirenzepine (Doralp and Leung 2008).

Therefore, we designed our study to investigate the role of muscarinic M1 and M2 receptors in synaptic plasticity and reconsolidation of memory traces in freely moving rats. Our laboratory previously reported that the non-selective muscarinic antagonist scopolamine reduced the synaptic plasticity of the SC/C synapses but had no effect on the memory retention in the PA task (Dobryakova et al. 2014). Since scopolamine is a non-selective muscarinic antagonist, we analyzed the effects of two more specific drugs, methoctramine, a selective muscarinic M2 receptor antagonist, and pirenzepine, a selective muscarinic M1 receptor antagonist, on PA learning and synaptic plasticity in freely moving rats.

METHODS

The experiment was performed using adult male Wistar rats (250–300 g) received from Reasearch Center of Biomedical Technology RAMS, nursery “Pushchino”. A total of 78 rats were used in this study ($n=5-10$ /group). Animals were housed under standard conditions at $21\pm 1^{\circ}C$ with a 12 h light/dark cycle, food and water were provided *ad libitum*. All experiments were performed in accordance with the ethical principles stated in the European directive (86/609/EC) and were approved by the Ethical Committee of the Institute of Higher Nervous Activity and Neurophysiology of the Russian Academy of Sciences.

Stereotaxic surgery

Neurophysiological and behavioral studies were preceded by surgical operation. Rats were intraperitoneally (i.p.) anaesthetized with chloralhydrate (400 mg/kg) and mounted in a Kopf stereotaxic frame. A bipolar nickel-chrome electrode (diameter 80 micrometers) was implanted into the brain for Schaffer-collateral pathway (3.0 mm posterior, 3.0 mm lateral to bregma, approximately 2.8 mm ventral to dura) stimulation (Paxinos and Watson 1998). To record field excitatory postsynaptic potential (fEPSP) in response to paired pulse stimulation a recording nickel-chrome electrode was lowered into the CA1 area (2.7 mm posterior, 1.5 lateral to bregma, approximately 2.2 mm ventral to dura) (Paxinos and Watson 1998). The bregma and lambda were at the same horizontal level. No differential lead was used, therefore, one electrode in the frontal bone served as a ground and as a reference electrode. The electrodes were fixed to the skull using

quick-setting dental plastic (protakril M) and stainless steel screw. Rats were allowed to recover for 7 days after the surgery.

Passive avoidance (PA) learning

To study the effect of muscarinic antagonists on the formation of PA we used the dark-light chamber paradigm. The test apparatus (OpenScience, Russia) consisted of a plastic box equally divided into two compartments (30cm×30cm×30cm): one was white-colored and brightly illuminated and the other one was black-colored and dark. The two compartments were not separated by door.

Behavioral procedures

On day 1, during the first testing, rats were placed into the light compartment and allowed to move freely between the two parts of the chamber for 5 min (habituation trial). After the habituation trial, the fEPSP initial slope was recorded for 30 min in an individual recording cage. Immediately after the fEPSP recording rats were placed into the same chamber (acquisition trial), behavioral conditions were similar to the habituation trial but entry into the dark compartment was paired with a 10-sec electric shock (0.5 mA) provided through the metal grid covering the floor of the test camera.

After the shock, rats were immediately removed from the apparatus, received an intraperitoneal injection (1 ml/kg) of saline, methoctramine hydrate 2 mg/kg (Sigma-Aldrich) or pirenzepine 1 mg/kg (Sigma-Aldrich) (dissolved in sterile 0.9% NaCl) and were placed into the recording cage for the fEPSP registration (90 min). On day 2, during the retention trial, no foot shock was given and the step-through latency was recorded as a measure of retention. Before and after that rats were placed into the recording cage for fEPSP registration (30 min).

In additional experiments, rats received intracerebroventricular (i.c.v.) infusions of methoctramine (12.5 µg/rats). Guide cannulas were lowered into the ventricle (0.8 mm posterior, 1.5 lateral to bregma) (Paxinos and Watson 1998). Drug (2 µl) was infused at a rate of 0.2 µl/min. Infusing cannula was attached to a polyethylene tubing (Corning Corporation, USA), which was attached to a 10 µl Hamilton syringe (Hamilton company, USA). Drug administration was controlled by a microinfusion pump (Stoelting Co., USA). The location of the infusion sites were then histologically confirmed.

Histology

Rats were anaesthetized with chloralhydrate (400 mg/kg, i.p.) followed by intracardiac perfusion of 0.9% NaCl and then 4% formalin (Sigma-Aldrich, USA). Brains were removed and placed in a 4% formalin solution in PBS for at least 5 days. After that, brains were placed in a 20% sucrose solution on PBS for at least 24 h. Coronal sections (20 µm thick, proximal to injection and electrodes tracks) were sliced at -18°C using a cryostat (Zeiss, Microm HM560). Cannula placements were verified using Nissl staining. Cannula and electrodes placement were found to be correct in 95% of the animals (within the limits of 1 mm³ of the insertion sites).

The methoctramine dose for intraperitoneal injections (2 mg/kg) was based on our pilot behavioral experiments. The dose of the drug for intracerebroventricular (i.c.v.) administration (12.5 µg/rat) was chosen from previous data (Aura et al. 1997). The dose of pirenzepine (1 mg/kg, i.p.) was selected on the basis of previous data (Witkin et al. 1988) and data of our pilot behavioral experiments in the PA task.

Electrophysiology

For habituation to the experimental conditions, each rat was housed in the individual recording cage for 30 min prior to the start of the field EPSPs recording session. The amplitude of the fEPSPs was measured in freely moving rats during the first 90 min after the trial and saline/methoctramine/pirenzepine injection (consolidation period) and 24 h after (memory retention test).

The fEPSP amplitude in the CA1 field evoked by stimulation of the Schaffer collaterals (interstimulus interval 30 msec; intertrain time 20 sec at intensity of 100–400 µA) was obtained from 10 successive stimuli and was recorded every 10 min. The test paired pulse intensity was set to evoke 40–50% of maximum fEPSP amplitude. The efficacy of synaptic transmission was evaluated based on changes in the amplitude characteristics of the evoked responses in the hippocampal CA1 field in response to test stimulation of the Schaffer collaterals.

Statistical analysis

All data are presented as mean ± SEM. Across groups of electrophysiological data, statistical significance between means was determined using a mixed-design analysis of variance followed (where applicable) by

Fisher's Least Significant Difference (LSD) *post hoc* test to reveal group differences on separate time intervals; for within the group comparisons, a paired Student's *t*-test was used. The differences in behavioral parameters were analyzed by the Mann-Whitney *U*-test and χ^2 criterion.

RESULTS

We tested PA behavior in five groups of rats that received methoctramine (2 mg/kg, *i.p.* and 12.5 μ g, *i.c.v.*), pirenzepine (1 mg/kg, *i.p.*) and saline (*i.p.* and *i.c.v.*) (Fig. 1). Since saline *i.c.v.* injected rats had PA learning parameters similar to saline *i.p.* rats, we combined the results of these two control groups. In contrast to both doses of methoctramine, which reduced the latency to enter the dark chamber, there were no differences between latencies obtained after injections of saline or pirenzepine (Fig. 1). Thus, selective M2 muscarinic antagonist methoctramine at doses of 2 mg/kg (*i.p.*) and 12.5 μ g (*i.c.v.*) was able to reduce latency to enter the dark chamber compared with selective M1 antagonist

pirenzepine and to control group (91.1 \pm 48.9 sec (*n*=8), 98.1 \pm 47.9 sec (*n*=8), 206.25 \pm 37.9 sec (*n*=12), 228.6 \pm 28.8 sec (*n*=19), accordingly, $\chi^2=5.53$, *P*<0.05).

Next, we examined the effects of muscarinic antagonists (methoctramine, pirenzepine) on fEPSP evoked by Schaffer collateral stimulation. Under our experimental conditions, fEPSPs were stable for at least 1 hour (data not shown). Mixed design ANOVA did not show significant effect of training on fEPSP amplitude ($F_{1,42}=1.0$, *P*<0.3). However, significant differences were found for the drug treatment ($F_{2,42}=3.41$, *P*<0.04). Furthermore, a significant time and drug interaction ($F_{24,504}=1.7$, *P*<0.02) suggested that decrement in fEPSP amplitude over time is related to the drug action. Subsequent ANOVA for the separate conditions followed by *post hoc* revealed that pirenzepine induced a significant reduction of the fEPSP characteristics over time (an average decrease of 15%, Fisher's LSD; *P*<0.05) (Fig. 2A, C).

Since systemic (*i.p.*) methoctramine injection did not affect the fEPSPs amplitude in the CA1 region of the hippocampus, we further used an intracerebroventricular methoctramine administration to clarify brain-spec-

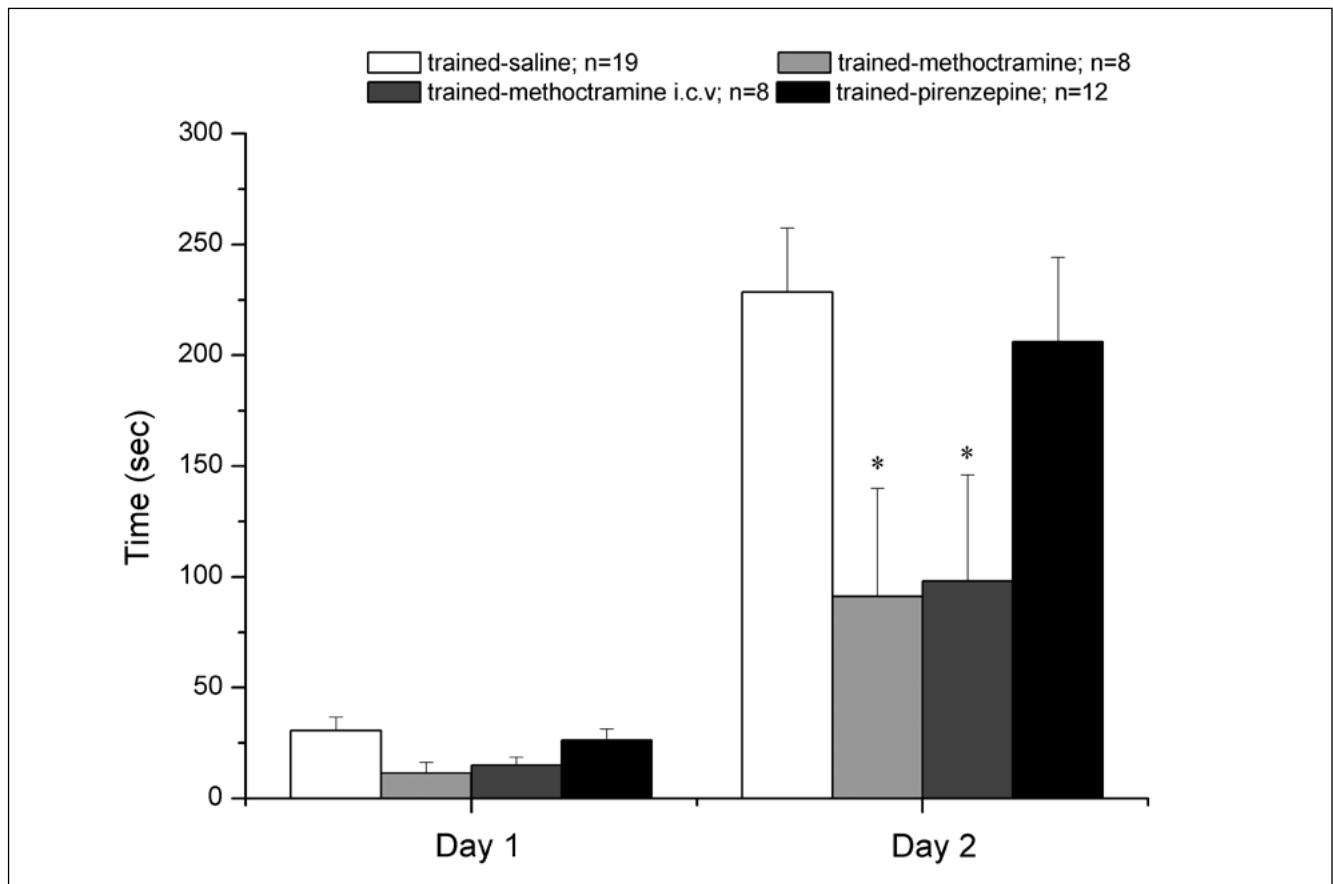


Fig. 1. Effect of methoctramine (*n*=8) and pirenzepine (*n*=12) on passive avoidance learning. Trained rats that received systemic (2 mg/kg) or *i.c.v.* (12.5 μ g/rat) methoctramine (*n*=8) had a significant shorter latency to enter the dark chamber than trained-saline (*n*=19) or trained-pirenzepine rats. *, significant difference against the saline group, *p*<0.05.

ificity of the M2 muscarinic antagonist action. Similar to i.p. injected animals, there were no significant differences in the amplitude of the fEPSPs between the untrained and trained rats ($F_{1,24}=0.16$, $P<0.7$). However, there was a main effect of drug ($F_{1,24}=1.4$, $P<0.05$). The rats from i.c.v. methoctramine treated group had significantly higher amplitude of fEPSPs compared to control (Fig. 2B, D). A significant interaction between drug and time ($F_{12,288}=2.33$, $P<0.01$) were found for the fEPSP amplitude. The amplitude in methoctramine-treated rats were higher and increased over time independently of training conditions.

We also assessed the effects of the antagonists on fEPSPs during the retention period (24 hour after the training and before and after placement into the light-dark chamber). At 24 hours, the fEPSP magnitude was not significantly different from the basal values in saline-treated trained and untrained rats. However, we found that, at 24 hours, in untrained rats i.p. methoctramine significantly increased the fEPSP amplitudes before placement into the light-dark chamber

($115.7\pm 6.6\%$ compared with basal level, $P<0.05$; Fig. 3A). In trained rats treated with methoctramine, the fEPSP amplitude also tended to increase ($120.1\pm 10.0\%$ before camera session compared with baseline, $P<0.06$; Fig. 3B). In pirenzepine-treated untrained and trained animals the magnitude of fEPSPs was higher compared to the basal level but these changes were not significant (Fig. 3A, B).

DISCUSSION

Here, we investigated the role of specific mAChR ligands on memory consolidation in the passive avoidance task and their relationship with fEPSP in the CA1 area in freely moving rats after intraperitoneal administration of the drugs. Our results have shown that i.p. injections of the selective M2 antagonist methoctramine induced a deficit in a PA learning. However, antagonism of M1 muscarinic ACh receptors by pirenzepine did not affect memory in this task. Pre-

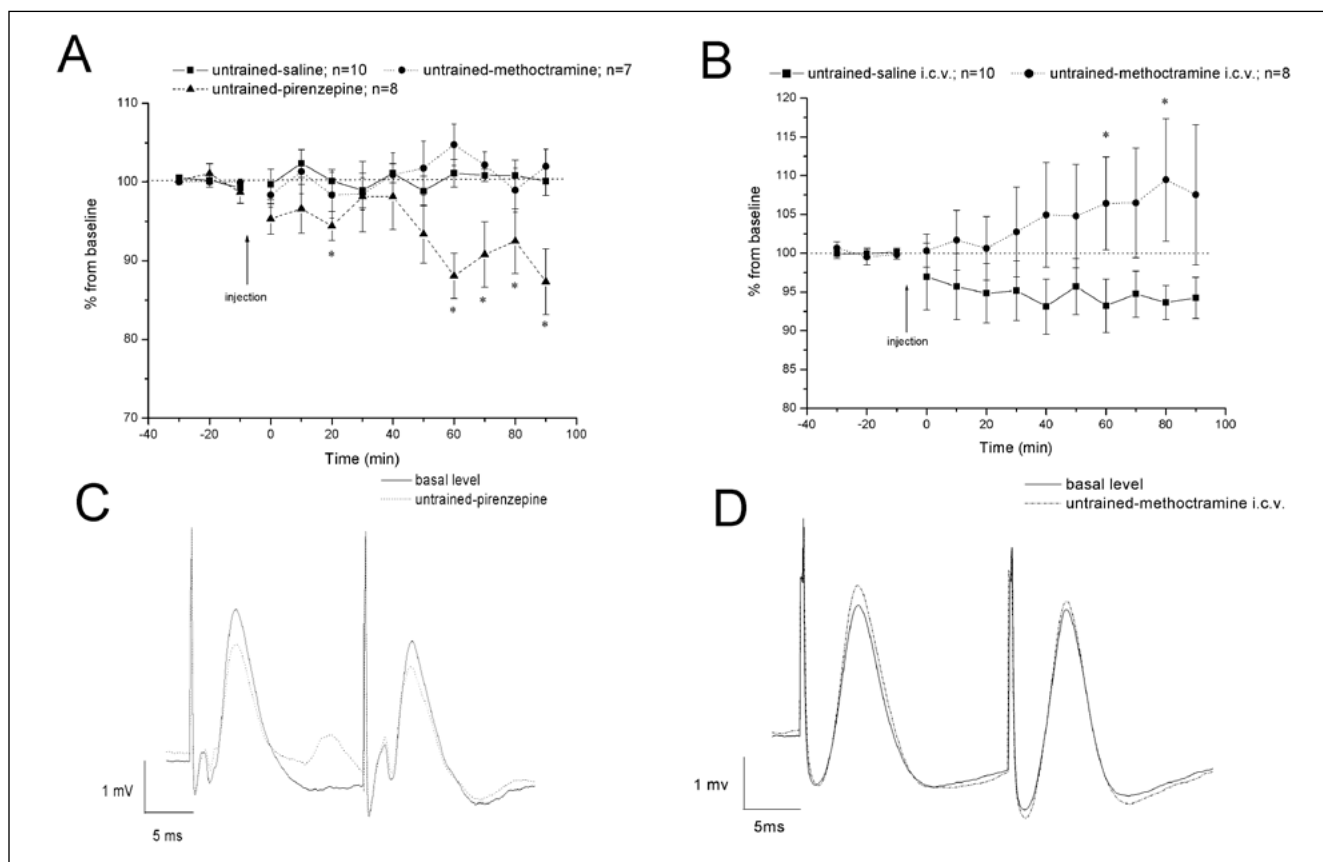


Fig. 2. Effect of methoctramine and pirenzepine on field excitatory postsynaptic potential (fEPSP) in the hippocampal CA1 region. (A), the time course of fEPSP in methoctramine (n=7), pirenzepine (n=8) and saline groups (n=10) for 90 min after intraperitoneal injection in untrained rats. (B) The time course of fEPSP in i.c.v. methoctramine and i.c.v. saline groups (n=8) for 90 min in untrained rats. (C, D) The fEPSP in vivo varies as a function of the drug treatment in the intact rat CA1 region. Each point represents the mean \pm S.E.M. percentage of basal fEPSP amplitude at 0 min; *, significant differences against the saline group, $P<0.05$.

vious studies have reported that different muscarinic antagonists were used to modulate behaviour and induced similar effects in various behavioral tests. For instance, posttraining scopolamine administration inhibited long-term memory in inhibitory avoidance task (Bianchin et al. 1999). Methoctramine and pirenzepine disrupted operant responding (Cousens and Beckley 2007, Witkin et al. 1987, 1988).

Thus, the effect of methoctramine may be explained by the M2 receptor localization and selective M2 antagonist action (Drever et al. 2011). M2 receptors are present on cholinergic terminals where they regulate ACh release (Zhang et al. 2002). Therefore, we assumed that regulation of ACh release is associated with modulation of behavioral processes underlying performance of the PA task. In support of our i.p. results we have shown that

i.c.v. injection of methoctramine (12.5 μ g) also impaired PA learning. Thus, our data suggest that M2 receptors mediate a memory consolidation processes and that methoctramine at the doses used act via centrally located receptors.

In contrast to the previous studies where the M1 antagonist, pirenzepine, impaired long-term memory in operant task in rats (Witkin et al. 1987, 1988), in our paradigm of fear-associated learning pirenzepine did not induce any changes. The lack of the drug effects on PA learning may be ascribed to its hydrophilic properties, which limited CNS access of the drug via the blood brain barrier. It also appears that learning in this task is less sensitive to a low dose of i.p. pirenzepine administration. It was previously shown that the effects of i.p. pirenzepine in a PA task are dose-dependent and a much higher systemic dose is needed to disrupt PA learning (Worms et al. 1989). Further experiments are needed to determine the effects of i.c.v. pirenzepine for a proper comparison with the methoctramine data. However, in the studies mentioned above pirenzepine was injected 30 min before the task and it was not clear which phase of learning was affected: perception, memory formation or memory consolidation. Here, we injected the drug during the consolidation period, hence, our results suggest that pirenzepine does not affect consolidation of fear-associated memory. The differences in these results might be related to different experimental conditions.

It was previously hypothesized that cholinergic system is involved in memory and learning processes via modulation of synaptic plasticity. Thus, fEPSP characteristics can change during different stages of learning. However, even during *in vivo* studies the effect of the cholinergic agents on LTP was not tested simultaneously with behavior (Hoelscher et al. 1997, Markevich et al. 1997). Here we used the PA learning which is fear-associated and hippocampus-dependent test. We suggested that changes in hippocampal CA1 fEPSPs induced by muscarinic antagonists are dependent of the changes in learning processes in our behavioral test.

The lack of differences in the parameters of PA learning does not exclude the possibility that pirenzepine can affect synaptic transmission. Our data indicate that systemic administration of pirenzepine decreased fEPSP 90 min after drug administration. This findings is in agreement with previous studies showing that M1-selective antagonists pirenzepine and telenzepine suppressed the amplitude of LTP (Luo et al. 2008). This indicates that pirenzepine, at the dose used (1 mg/kg, i.p.), binds directly to M1 receptors and affects basal synaptic transmission in the CA1 hippocampal region.

In contrast to the M1-selective antagonist, i.p. administration of methoctramine did not induce any

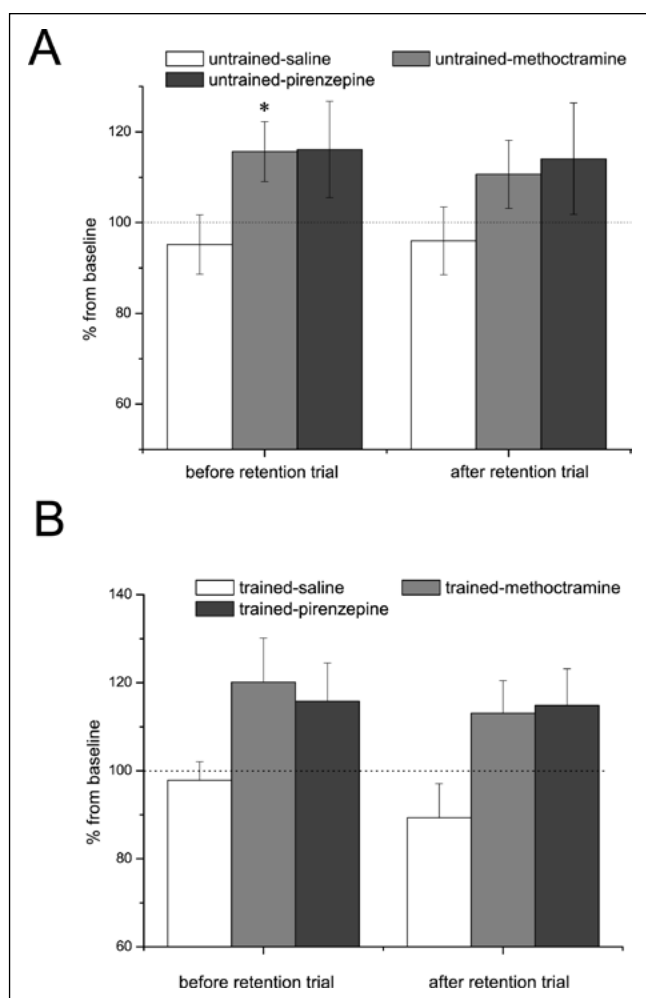


Fig. 3. The effect of methoctramine (2 mg/kg) (n=6) and pirenzepine (1 mg/kg) (n=6) on the Schaffer collaterals fEPSP amplitude in untrained (A) and trained rats (n=8 in methoctramine group, n=7 in pirenzepine treated rats) (B), in 24 hours after the injection (mean \pm S.E.M., N=6–9/group) before and after the testing of memory retention (retention trial). *, significant difference, P<0.05.

changes in fEPSP characteristics 90 min after the injection. However, methoctramine significantly increased the fEPSP amplitude 24 h after injection compared to baseline. Furthermore, i.c.v. injected drug caused a persistent enhancement in fEPSPs amplitude immediately after injection. According to previously published data, several M2-selective antagonists including methoctramine (AF-DX 116 and gallamine) also induced the same effect (Hayes et al. 2008, Li et al. 2007). A possible mechanism of this effect is the inhibition of M2 autoreceptors, regulating ACh release (Hayes et al. 2008, Li et al. 2007). M2 receptors are located on septo-hippocampal cholinergic nerve terminals (Rouse et al. 2000, Zhang et al. 2002). Therefore, the blockade of these autoreceptors induce an increase in ACh release (Carey et al. 2001, Vannucchi et al. 1997). Another possible mechanism of the persistent enhancement of fEPSPs amplitude is that methoctramine can inhibit M2 receptors on glutamatergic terminals (Drever et al. 2011). It was previously shown that antagonism of M2 receptors can increase endogenous glutamate release in the hippocampus (Marchi and Raiteri 1989).

Methoctramine is considered as a selective M2 receptor antagonist, however it may block M4 subtype as well. M4 receptors are also expressed on interneurons and on pyramidal cells (Drever et al. 2011, Volpicelli and Levey 2004) and can activate the same cascade. It was shown that concurrent activation of both muscarinic receptors subtypes may be required for changes in memory formation (Leaderbrand et al. 2016) and plasticity. However, the affinity of methoctramine to M4 is smaller compared to M2 and its contribution to the observed effects needs further investigations.

Overall, our results suggest that methoctramine affected memory consolidation in the PA task but enhanced the amplitude of basal synaptic transmission. Meanwhile, pirenzepine did not change consolidation of fear-associated memory but reduced the fEPSP amplitude. Our findings are in agreement with Kikusui et al. (2000) who have shown that spatial working memory in allocentric place discrimination task is affected by muscarinic antagonists but is independent from CA1 LTP. However, it has been discussed in several previous studies that pirenzepine blocked the facilitation of LTP induced during walking in freely behaving rats (Doralp et al. 2008) and affected LTP that is required for the formation of place representation (Dragoi et al. 2003). Thus, it is possible that the described changes in the efficacy of synaptic transmission and PA learning could be mediated by different mechanisms and are dependent on doses and types of methoctramine and pirenzepine administration. Perhaps, the behavioural effects of antagonists and synaptic changes have different sensitivity to the doses used.

In conclusion, our results showed that memory consolidation after PA learning is dependent on the muscarinic cholinergic system but it had no direct connection with the hippocampal CA1 fEPSP.

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