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Research report

Facilitatory effect of the intra-hippocampal pre-test administration of MT3 in the inhibitory avoidance task

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Abstract

The cholinergic system plays a crucial role in learning and memory. Modulatory mechanisms of this system in the acquisition and consolidation processes have been extensively studied, but their participation in the memory retrieval process is still poorly understood. Conventional pharmacological agents are not highly selective for particular muscarinic acetylcholine receptor subtypes. Muscarinic toxins (MTs) that are highly selective for muscarinic receptors were extracted from the venom of the mamba snake, like the toxin MT3, selective for the M4 receptor subtype. These toxins are useful tools in studies of the specific functions of the M4 mediated transmission. The M4 receptor selective antagonist MT3, given into the dorsal hippocampus before the test, have enhanced the memory retrieval of an inhibitory avoidance task in rats. MT3 had no effect in the habituation to a new environment, including basic motor parameters, meaning that the effect in he inhibitory avoidance is purely cognitive. Our results suggest an endogenous negative modulation of the cholinergic muscarinic system upon the retrieval of previously consolidated aversive memories, hereby shown by the facilitatory effect of MT3.

Keywords: Memory retrieval; Cholinergic muscarinic system; MT3; Inhibitory avoidance task; Hippocampus; Rat

1. Introduction

The cholinergic system plays a crucial role in learning and memory. Lesions of cholinergic nuclei, pharmacological manipulation of cholinergic receptors and enzymes, intracerebral transplantation of genetically modified cells that produce acetylcholine, and anatomical changes in cholinergic pathways during ageing have all been correlated with altered cognition mechanisms [2,37,40].

Muscarinic ACh receptors (MAChR), members of the seven-transmembrane protein receptor family coupled to G-proteins, are expressed widespread throughout the body, and are involved in many fundamental physiological processes in the central nervous system such as learning and memory [9,17]. Five subtypes of MAChR are expressed in the mammalian brain (M1–M5) and their coding genes have been cloned [12]. Upon agonist binding, M1, M3 and M5 subtypes preferentially interact whit G_q protein family, activating the inositol phosphate pathway, while subtypes M2 and M4 are usually coupled to adenylyl

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cyclase through G_i proteins, therefore inhibiting cAMP production [7,31].

There are differences in the concentration of receptor subtypes in different brain regions and more than one subtype is often expressed in the same cell [15]. The hippocampal formation of the rat was early suggested to have a high proportion of M1 and M4 receptors [9,15,18,20,27]. The hippocampus is the target of cholinergic fibers from the medial septum, an input known to be important for modulation both at the cellular and at the network levels, including the theta rhythm [10]. The septo-hippocampal cholinergic pathway also appears to be essential for memory formation and the cholinergic receptors activation might be involved with this and other kinds of synaptic plasticity [3,10].

The study of the MAChRs localization, quantification and function has faced difficulties due to the lack of selective ligands exclusively acting upon one or other receptor subtype. However, muscarinic toxins (MTs) extracted from the venom of *Dendroaspis* snakes, distinguish among some muscarinic receptor subtypes; for example, MT2 has a 4-fold higher affinity for M1 than for M4 receptor (Ki = 360 and 1200 nM, respectively), with rather low or negligible affinity for the other subtypes; while MT3 has 214-fold higher affinity for M4 than for M1 receptor (Ki = 1.2 and 250 nM, respectively). MT2 behaves as a M1 agonist and a M4 antagonist, while MT3 behaves as a very selective M4 antagonist [6,9,12,18,26,28,29].

Previous work has shown that the infusion of MT2 into the dorsal hippocampus of rats immediately after training modified performance in an inhibitory avoidance task. In the lowest dose, MT2 improved performance. On the other hand, pirenzepine, a relatively selective antagonist, was amnesic. These experiments have shown that the M1 receptor has an important positive role in memory consolidation for the inhibitory avoidance task [9]. Moreover, the infusion of the selective M4 receptor-selective antagonist MT3 into the hippocampus has an amnestic effect in the consolidation of this memory with aversive components [9,16].

The participation of the cholinergic muscarinic system in memory consolidation was extensively studied, but there are few data concerning the function of this system in the memory retrieval processing. Recent studies have shown an enhanced effect on retention performance of a step-down inhibitory avoidance task by intra-hippocampal pre-test infusion of oxotremorine, a non-selective muscarinic agonist, and an amnestic effect of the non-selective muscarinic cholinergic antagonist scopolamine. These results indicate a positive role of the cholinergic muscarinic system on the retrieval process for this task [4]. However, the lack of selectivity of oxotremorine and scopolamine does not allow to answer which muscarinic receptor was involved.

The present work investigated the role of M4 muscarinic cholinergic receptor in memory retrieval by using a pre-test intra-hippocampal infusion of MT3. Two behavioral tasks were used, a step-down inhibitory avoidance (IA) and an open-field habituation (OF). The activity in the open-field test session may also be used as a motor/performance control for the drug effects.

2. Materials and methods

Ninety-five (95) male Wistar rats (age 2–3 months, weight 210–300 g) from our breeding colony were used. Animals were housed in plastic cages, 4–5 to a cage, under a 12 h light/dark cycle and at a constant temperature of 24 ± 1 °C, with water and food *ad libitum*. All animals were anesthetized by a mixture of ketamine and xilazine (i.p., 75 and 10 mg/kg, respectively) and bilaterally implanted with a 27-gauge guide cannulae aimed at AP −4.2 mm (from Bregma), LL ±3.0 mm, DV 1.5 mm, just 1.0 mm above area CA1 of the dorsal hippocampus (adjusted from Paxinos and Watson [28]).

Once recovered from surgery (48 h), the animals were submitted to a training session either in the step-down inhibitory avoidance (IA) or in the open-field habituation (OF) task; 24 h later they receive a bilateral intra-hippocampal infusion of the drug or its vehicle and 20 min later were tested for the corresponding task [30]. The IA task was carried out in an automatically operated, brightly illuminated box, in which the left extreme of the grid (42.0 cm × 25.0 cm grid of parallel 0.1 cm caliber stainless steel bars spaced 1.0 cm apart) was covered by a 7.0 cm wide, 5.0 cm high formica-covered platform. Animals were placed on the platform and their latency to step-down placing their four paws on the grid was measured. In the training session, immediately upon stepping down, the animals received a 0.5 mA, 3.0 s scrambled footshock. In the test session no footshock was given, and a ceiling of 180 s was imposed to the step-down latency. The OF was studied using a 50 cm high, 60 cm × 40 cm plywood box with a frontal glass wall and a linoleum floor divided in 12 equal rectangles. Animals were left there for 2 min both in the training and the test session, and the number of rearings and crossings between sectors were registered. The difference between the two sessions in the number of rearings and of crossings between rectangles, were considered a measure of habituation to the open-field: if the animals habituated to the field during the first session, they should recognize it as familiar and, in consequence, the number of rearings and crossings should decrease in the second session [31]. The number of crossings in the test session was also used as a control for the possible motor and general performance effects of the drug administered 24 h before.

At the time of the pre-test infusion, 30-gauge cannulae were fitted into the guide cannulae; the tip of the infusion cannulae protruded 1.0 mm beyond that of the guide cannulae and was, therefore, aimed at the pyramidal cell layer of CA1 in the dorsal hippocampus (Fig. 1), with 0.5 μl volume being administered at a 20 μl/h rate. The animals were divided into groups receiving bilateral infusions of 0.5 μl, either of MT3 (0.5, 1.0 and 2.0 mg/side—purified from lyophile by us, according to Jerusalinsky and Harvey [13]), or of its vehicle (phosphate buffered saline) administered 20 min before the test session (IA); only the dose effective in the IA task of each drug was tested in the OF task. The selected doses covered a range consistent with previous post-training studies [1,9].

Statistical analysis of the behavioral data (latencies to step-down in IA and number of rearings and crossings in OF) was limited to the animals with correct placements of the cannula (Fig. 1)—those animals were 83 out of 95 operated, as described in Izquierdo et al. [12]. Since the step-down latencies have not passed a normality test (Kolmogorov–Smirnov test with Lilliefors' correction), differences among groups were evaluated by a Kruskal–Wallis ANOVA with Dunn's all pair-wise multiple comparison *post-hoc* test; training versus test latencies were correspondingly compared by the Wilcoxon signed ranks test. In the OF task, as crossings and rearings were normally distributed, groups were compared by Student's *t*-test; training versus test latencies were correspondingly compared by the paired *t*-test.

Experiments with rats were performed in strict accordance to the Brazilian law, to the recommendations of Brazilian Society for Neurosciences (SBNeC) and the Brazilian College of Animal Experimention (COBEA), the Review Committee of the School of Veterinary at the University of Buenos Aires and the International Brain Research Organization (IBRO), and are in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (publication no. 85-23, revised in 1985).

3. Results

Fig. 2 shows the inhibitory avoidance task results for the MT3 injected groups. As data were not normally distributed

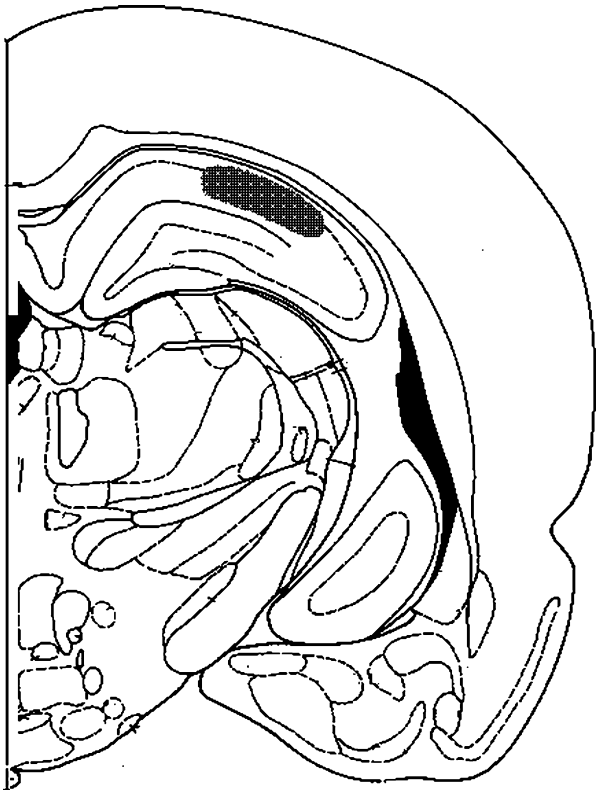

Fig. 1. Drawing representing AP plane −4.2 mm adapted from the Atlas of Paxinos and Watson [27] showing the extent of the area reached by our infusions in the rat dorsal hippocampus (stippled areas represent typical regions of accepted animals, as dyed by 0.5 μl of 2% methylene blue in saline infused through the same cannulae).

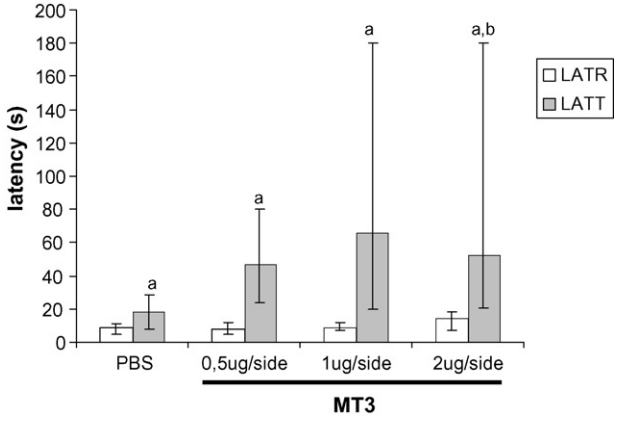

Fig. 2. Effect of MT3 in the step-down inhibitory avoidance task. Data expressed as median and interquartile intervals (training session in white; test session in gray). *N*s per group, respectively, 21, 15, 11 and 16. Kruskal–Wallis test shows no significant difference among training session latencies ($P = 0.786$). (a) Each of the four experimental groups have shown a significant difference between training and test sessions latencies ($P < 0.05$, Wilcoxon test). (b) Only the 2.0 μg/side group of MT3 show a significant difference in the test session latency compared to the control group ($P < 0.05$, Dunn test). LATR: training session latency; LATT: test session latency.

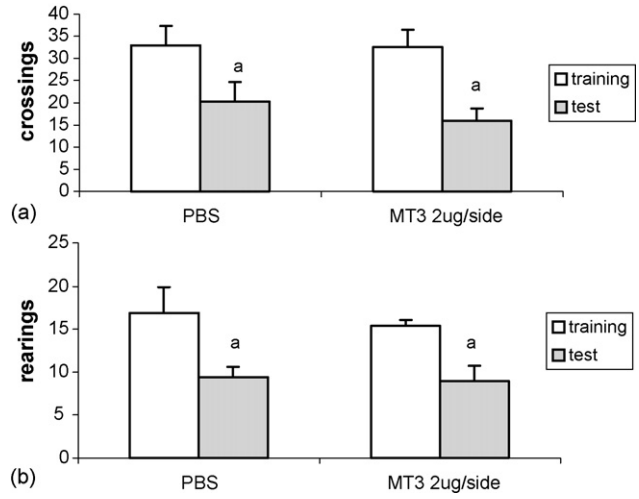

Fig. 3. (a and b) Absence of effect of MT3 in the open-field task. Data expressed as mean ± S.E.M. *N*s per group, respectively, 9 and 11. Number of (a) crossings and (b) rearings in the test are significantly different from the corresponding training values for both groups (paired *t*-test, $P < 0.05$), but MT3-treated group (in the same dose proven effective in the inhibitory avoidance task) was not significantly different from the control one ($P > 0.05$, Student's *t*-test).

(Kolmogorov–Smirnov normality test, $P > 0.200$), nonparametric tests were used.

The highest dose of MT3 (2 μg/side) administered at 20 min pre-test enhanced the performance of the animals compared to the control and the groups which received lower doses ($P < 0.05$, Dunn's all pair-wise multiple comparison *post-hoc* test, after a Kruskal–Wallis ANOVA with $P = 0.011$). The increase in the performance with 1 μg/side dose was not statistically significant (Fig. 2). Groups were comparable because there were no significant differences among training session latencies ($P = 0.229$, Kruskal–Wallis ANOVA); all groups have displayed normal learning, as each test latency was significantly larger than the corresponding training one ($P < 0.005$ for all doses, Wilcoxon signed ranks test).

Fig. 3 shows the open-field task results for animals injected with MT3 with the dose that was found to be effective in the inhibitory avoidance task (2.0 μg/side). Compared to controls, the drug-treated animals have shown no significant differences in the number of rearings or crossings neither in the training nor in the test sessions ($P > 0.05$, Student's *t*-test). Both variables (rearings and crossings) were significantly lower in the test than in the training session for the MT3-treated rats cannula ($P = 0.002/0.014$) and the respective control group ($P = 0.025/0.027$) evaluated by the paired *t*-test.

Additionally, the fact that there were no differences in the number of crossings between groups suggests that neither locomotor activity nor exploratory effects have been caused by the pre-test MT3.

4. Discussion

Our results show that MT3 (2 μg/side) has caused a facilitation of the retrieval of the inhibitory avoidance task when administered 20 min before test (Fig. 2). However, no effect was

found in the less aversive, exploratory open-field habituation task (Fig. 3). The unaltered number of crossings in the open-field, for the effective dose in the IA task, supports the idea that the effect of the drug in the IA is basically cognitive, neither a motor nor an exploratory effect.

We had previously proposed for the first time that M4 receptors are involved in memory consolidation since post-training administration of MT3 in the very same structure and behavioral task used in the present work was amnestic [16]. It has been shown that muscarinic transmission suffers impairments with aging and also in some degenerative diseases where cognitive functions are altered [5,21]. Mulugeta et al. have shown that M4 receptors were specifically lost in CA4 and DG of Alzheimer's patients brains [24].

Since MT3 is highly selective for the M4 receptor subtype – with an antagonist-like activity – and has a negligible binding to M1, M2, M3 and M5 receptors [6,9,12,18,26,28,29], our results suggest a negative modulator role for the M4 receptors in the dorsal hippocampus, at least during the memory retrieval process. Notice that M2 and M4 muscarinic receptors could have an inhibitory role [14,19,37] and can be expressed as heteroreceptors at the pre-synaptic terminals of either inhibitory or excitatory neurons [33,34].

Compared to the ever growing literature about the molecular events underlying the consolidation phase of memory formation [14,23], little is known about the molecular requirements of memory retrieval [36]. Most studies point to an essentially diffuse modulatory role of the cholinergic muscarinic system upon cognitive functions [36]. In both the hippocampus and the amygdala, modulatory actions on memory may be exerted either by extrinsic muscarinic pathways acting upon the "executive" glutamatergic and GABAergic neurons, or by intrinsic projections from cholinergic interneurons, as it seems to be the case in the limbic system (*ibidem*). Concerning our results, it must be taking into account that muscarinic heteroreceptors may be expressed at the pre-synaptic terminals of either inhibitory or excitatory neurons [33,34]; furthermore, it might be speculated that their expression would take place in the glutamatergic neurons of the perforant path, an important input projection to the hippocampal formation [14,34,35,38].

According to immunohistochemical studies, muscarinic M1 and M4 receptor subtypes are localized in the CA and DG hippocampal regions, and M2 subtype is mainly expressed in non-pyramidal cells [32,34]. The fibers of the non-pyramidal pathways—alveus, fimbria and hippocampal comissure, contain M4 [22].

Here we reported the facilitatory effect of MT3 upon retrieval. The molecular mechanisms involved in memory retrieval of hippocampally modulated behavioral tasks seem to be basically similar to those involved in memory formation, though there appear to be some differences [36]. Therefore, the old tenet that retrieval must be a function of, or involve mechanisms similar to the consolidation process, might be at least incomplete (*ibidem*). It might be speculated that the fact that they react in opposite ways in these different circumstances might be due to a modification in the circuitry involved in these processes.

In this sense, one possible explanation raises from the fact that M4 receptors may be located pre-synaptically in the hippocampus, acting as homoreceptors controlling acetylcholine release [33,38]. Since nothing is said about *when* are they expressed in this brain structure, we may speculate that it could be an experience-triggered event.

Despite the fact that the literature is scarce on this subject, we have previously shown that two other muscarinic toxins acting intra-hippocampally as selective agonists, MT1 [11,15] and MT2 [9], induced memory facilitation when administered after training, possibly acting upon M1-bearing glutamatergic neurons [20]. Since MT3 was amnestic when administered post-training in the hippocampus [16], we could think that its M4 target would not be located in the same pathway above mentioned, neither post-, nor pre-synaptically.

Both possibilities – M4 plasticity as hetero- or homoreceptors – are logically feasible and further investigation is necessary to clarify this point. Evidence concerning the possibility of plastic modifications of these receptors is limited: there was an early report that muscarinic receptors undergo rapid changes after an acute stress [8], as well as it was recently shown that particularly M4 in the entorhinal cortex suffers the influence of adrenal hormones [25].

Finally, it must be pointed out that MT3 caused no evident effects in the open-field habituation task (Fig. 3a and b). Hence, the M4 receptors in the dorsal hippocampus seem not to be involved in memory retrieval process for this task suggesting that the muscarinic system demands some degree of aversiveness in order to be recruited, a phenomenon also observed regarding other neuromodulatory systems [1,9,39].

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