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Role of hippocampal M_1 and M_4 muscarinic receptor subtypes in memory consolidation in the rat

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Abstract

Muscarinic receptors in the hippocampus are relevant to learning and memory, but the role of each subtype is poorly understood. Muscarinic toxins (MTs) from *Dendroaspis* snakes venom are selective for muscarinic receptor subtypes. MT2, a selective agonist for M_1 receptors, given into the hippocampus immediately after training, improved memory consolidation of an inhibitory avoidance task in rats, whereas the antagonist pirenzepine was amnestic, supporting a facilitatory role of M_1 receptors. Instead, MT3, a selective antagonist at M_4 receptors, caused amnesia. Neither M_1 nor M_4 receptor appeared involved in habituation to a new environment. Thus, our results suggest that memory consolidation of an inhibitory avoidance task in the rat involves the participation of both M_1 and M_4 hippocampal receptors, with a positive modulatory role.

Keywords: Muscarinic acetylcholine receptor; Memory; Hippocampus; Muscarinic toxins; M_1 receptor; M_4 receptor

1. Introduction

The classical muscarinic antagonist scopolamine, when systemically administered, causes amnesia for many behavioural tasks in rats, suggesting the involvement of cholinergic transmission mediated by muscarinic cholinoceptors in learning and memory processing (Izquierdo, 1989). When scopolamine was directly injected into the hippocampus immediately after training, caused retrograde amnesia, revealing that active muscarinic receptors in this structure are needed to consolidate the trace (Izquierdo et al., 1992). The existence of different muscarinic receptor subtypes (M_1–M_5) and their differential localization in the hippocampal formation are likely to account for the complex cholinergic modulation in this structure (Rouse and Levey, 1997). However, the role of each subtype is poorly understood in vivo due to the overlapping expression and the lack of pharmacological tools selective enough to distinguish among them. However, muscarinic toxins (MTs) from the venom of *Dendroaspis* snakes distinguish among some muscarinic receptor subtypes and are useful for behavioural studies in vivo (Jerusalinsky et al., 1993, 1997). For example, MT2 has a 4-fold higher affinity for M_1 than for M_4 receptor (K_i = 360 and 1200 nM, respectively), and rather low or negligible affinity for the other subtypes (Kornisiuk et al., 1995); in preliminary assays, MT2 caused facilitation of memory consolidation of an inhibitory avoidance task in male rats (Jerusalinsky et al., 1993); while MT3, another toxin from the same venom, with 200-fold higher affinity for M_4 than for M_1 receptor (K_i = 1.2 and 250 nM, respectively; Potter, 2001; Jerusalinsky et al., 1998)

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and negligible affinity for the other receptor subtypes, caused scopolamine-like amnesia in male rats (Jerusalinsky et al., 1998).

In the central nervous system, M_1, M_3 and M_5 receptors are mainly coupled to the phosphoinositide pathway through G_q protein, whereas central M_2 and M_4 receptors are preferentially coupled to the inhibition of stimulated adenylyl cyclase through $G_{i/0}$ (Nathanson, 2000).

In previous assays, it was shown that MT2 enhanced carbachol-induced phosphatidylinositol turnover in homogenates of rat cerebral cortex (Jerusalinsky et al., 1994) and it also behaved as an M_1 agonist in various preparations (Bradley, 2000; Jerusalinsky and Harvey, 1994; Jerusalinsky et al., 1994). Concerning the effect of the toxins at M_4 receptors, both MT2 and MT3 were able to fully antagonize the oxotremorine inhibition of adenylyl cyclase activity, previously stimulated by forskolin, in the hippocampus (Kornisiuk et al., 2001). Olianas et al. (1998) reported that MT3 partially antagonized the acetylcholine inhibition of adenylyl cyclase activity in some cell lines and regions of the central nervous system. In addition, it was shown that MT3 acted as an M_4 antagonist at the anococcygeus muscle preparation (Bradley, 2000).

Our goal in this study was to shed some light on the role of hippocampal muscarinic receptor subtypes in memory consolidation. The hippocampal formation of the rat was early suggested to have a high proportion of M_1 (Nathanson, 1987) and M_4 receptors (Jerusalinsky et al., 1998, 2000); the main cells, pyramidal neurons, granule cells and interneurons, were immunopositive for M_1 and M_4 receptors, while exhibiting M_2 weak staining (Rouse and Levey, 1997). Hence, we have used MTs with selectivity for M_1 and M_4 receptor subtypes to further study the involvement of these hippocampal receptors in memory consolidation of an inhibitory avoidance task in female rats.

2. Material and methods

Female Wistar rats (210–300 g) from our own colony were housed five to a cage, under a 12-h light/dark cycle at 25 °C, with water and food ad libitum. They were anesthetized by ketamine and xilazine (75 and 10 mg/kg ip, respectively), and were bilaterally implanted with 27-gauge guide cannulae, aimed 1.00 mm above the CA1 region of the dorsal hippocampus (at coordinates A: −4.2, L: ±3.0, V: 1.3 mm). Once recovered, the rats were trained in a step-down inhibitory avoidance task (Izquierdo et al., 1992). In the training session, a rat was placed on an isolated platform (2.5 cm high, 7.0 cm wide, 25.0 cm long) at the left side of a 50.0 cm long × 25.0 cm wide × 25.0 cm high acrylic box, with the floor made of parallel (0.1 cm caliber) bronze bars, spaced 1.0 cm apart. Latency to step-down placing the four paws on the grid (training latency) was measured; upon stepping down, the rat got a 3.0-s, 0.5-mA scrambled footshock. Immediately after training, a 30-gauge needle was fitted into the guide cannulae, protruding 1 mm beyond its tip, aimed to the pyramidal cell layer in CA1, in the dorsal hippocampus. Animals were divided into groups receiving bilateral infusions of 0.5 μl of either pirenzepine, MT2, MT3 (two different concentrations) or the vehicle (saline), immediately after the training session. A retention test was carried out 24 h later, without footshock and with the step-down latency (test latency) limited to a maximum of 300 s. Differences between training and test latencies were evaluated by Wilcoxon test and, among groups, by Kruskal–Wallis H test for independent samples. When significant differences were found, groups were further ordered by Mann–Whitney U test to compare the effect of each drug against the respective vehicle-injected group. Analysis was limited to those animals in which the cannula was found, on autopsy, to be within 1 mm of the correct coordinates (122 rats).

To evaluate the possible effect of the toxins upon locomotor activity and/or exploratory behaviour, either toxin was administered into the dorsal hippocampus of adult rats, immediately after leading them to freely explore an open field (60.0 cm long × 40.0 cm wide × 50.0 cm high) for 5 min (training).

The number of rearings and crossings from one quadrant to another (12 of 15.0 cm × 13.3 cm) was measured in the training as well as in the test session performed 24 h later. Differences were evaluated either by Student's t test or by one-way ANOVA according to the number of groups to be compared.

Experiments with rats were performed in strict accordance to the Brazilian law to the recommendations of the Brazilian Society for Neurosciences, Review Committee of the School of Veterinary, 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 1985).

3. Results

The rats were trained in a step-down inhibitory avoidance task and then, were divided in nine groups at random. There was no significant difference among all groups in training latencies to step-down from the isolated platform to the grid (Kruskal–Wallis, $P>.10$); the latency dispersions in the vehicle groups were wider than those previously observed for male rats.

Two groups were injected with pirenzepine, receiving either 0.5 or 2 μg per hippocampus immediately after the training session. The medians of test latencies of both pirenzepine-injected groups and the corresponding control of vehicle-injected rats are shown in Fig. 1A. Pirenzepine, 2 μg/hippocampus, but not 0.5 μg, was strongly amnestic, taking into account that the animals spent about the same time on the platform in the training as well as in the

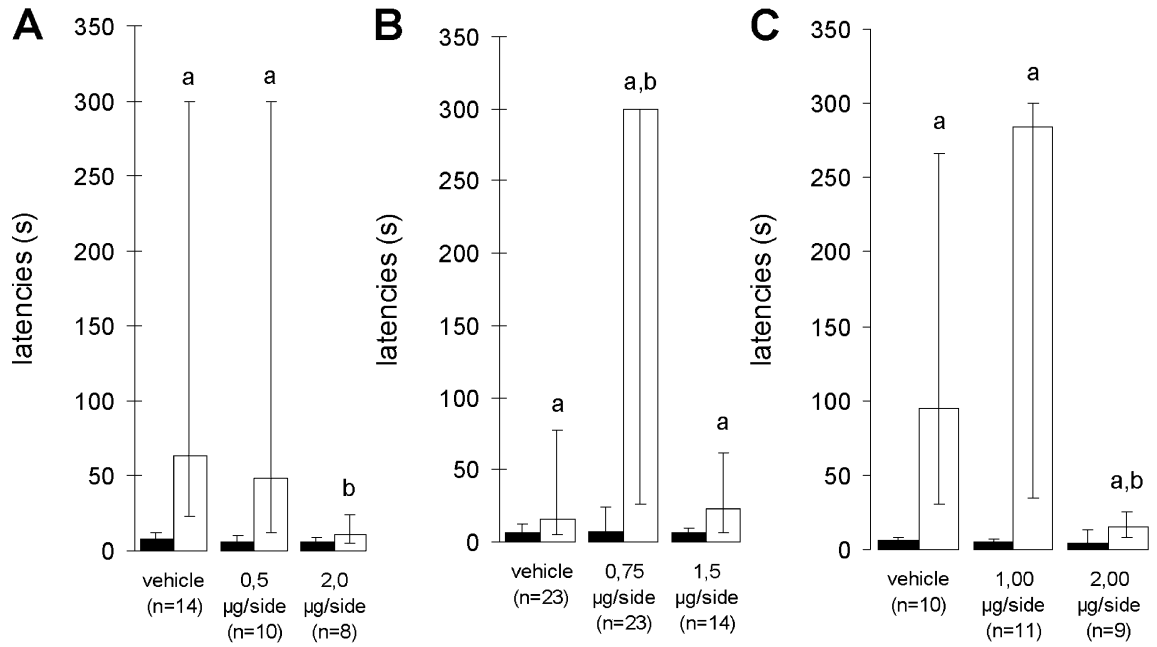

Fig. 1. Effects of selective muscarinic agents upon memory retention of an inhibitory avoidance task in the rat. Adult female Wistar rats were bilaterally injected into the dorsal hippocampus with either pirenzepine (A), MT2 (B), or MT3 (C), in two different doses (indicated under each bar), immediately after training. Bars represent the median (with the interquartile intervals) of training (black) and test (white) latencies. (a) Significant differences within groups by Wilcoxon ($P<.05$). (b) Significant differences between groups by Kruskal–Wallis ($P<.05$).

test sessions; hence, there were no significant differences between training and test latencies and test latencies were significantly shorter than those in the vehicle-injected rats, which remained for a longer time on the platform (post hoc Mann–Whitney, P=.0191).

The medians of test latencies for those animals treated with MT2 are represented in Fig. 1B. The rats injected with MT2, 0.75 μg/hippocampus, remained for a longer time on the platform in the test session; thus, the test latencies resulted significantly higher compared to the corresponding vehicle-injected group (Kruskal–Wallis, P=.0059), suggesting a facilitatory effect on retention. However, a higher dose of MT2 (1.5 μg/side) was ineffective, since test latencies were similar to those in the respective control group. Furthermore, we did not see any significant difference after injecting 3 μg of MT2 per hippocampus (test latency median after 3 μg MT2, 19.2 s [iq25/iq75, 14.7/61.5]; $n=7$).

On the other side, the injection of 2.0 μg/hippocampus of MT3 (Fig. 1C) was amnestic, since test latencies, though slightly longer than training latencies, resulted significantly shorter than test latencies for the vehicle-injected rats (Kruskal–Wallis, P=.011). However, the administration of half the dose of MT3 (1.0 μg/hippocampus) did not cause any significant effect on retention.

In the open-field task, animals injected with MTs in the doses effective in the inhibitory avoidance task show no significant differences in the number of both rearings and crossings in the test session, compared to control animals; both variables were significantly lower in the test than in the training session (Table 1). Hence, no locomotor activity or exploratory effects have been detected neither for MT2 nor for MT3.

Table 1
Crossings and rearings in the test session of an open-field task, after treatment with either MT2 or MT3 in the doses effective in the inhibitory avoidance task

Treatment	Crossings (mean ± S.E.M.)		Rearings (mean ± S.E.M.)	
	Training	Test	Training	Test
Vehicle (0.5 μl) ($n=22$)	61.3 ± 18.1	52.5 ± 14.6[a]	17.8 ± 5.6	14.4 ± 5.7[a]
MT3 (2.0 μg/side) ($n=14$)	66.6 ± 12.5	50.0 ± 20.1[a]	18.6 ± 4.8	12.4 ± 7.2[a]
Vehicle (0.5 μl) ($n=16$)	81.2 ± 12.5	41.4 ± 20.1[a]	18.6 ± 4.8	12.4 ± 7.2[a]
MT2 (0.75 μg/side) ($n=16$)	75.6 ± 4.7	49.4 ± 6.5[a]	18.6 ± 1.0	11.0 ± 1.4[a]

The toxins were administered intrahippocampus, immediately posttraining, and the test session was performed 24 h later.

[a] Test latencies are significantly different from the training values (paired t test, $P<.01$); training and test session latencies do not differ between control and treated groups for both toxins (Student's t test).

4. Discussion

Although the five muscarinic receptors are expressed in the hippocampal formation of the rat, there is a high proportion of M_1 (Nathanson, 1987) and M_4 receptors (Jerusalinsky et al., 1998, 2000) localized in the main cells.

The muscarinic toxin MT2 was previously shown to be selective for the M_1 receptor subtype, exhibiting 4-fold lower affinity for M_4 receptors (Kornisiuk et al., 1995). MT3 is highly selective for the M_4 subtype (Jerusalinsky et al., 1998, 2000; Potter, 2001) and both MTs have negligible binding to M_2, M_3 and M_5 receptors (Jerusalinsky et al., 1998; Kornisiuk et al., 1995). Both toxins have antagonist-like activity at M_4 receptors, while MT2 also behaves as an M_1 agonist. Either toxin, infused into the dorsal hippocampus of rats immediately after training, modified performance in an inhibitory avoidance task, depending on the dose. In the lowest dose, MT2 improved performance. On the other hand, the relative M_1 selective antagonist pirenzepine was amnestic. Taking into account the selectivity profile of MT2 and its agonistic action at M_1 receptors, our results strongly support that hippocampal M_1 receptors are necessary for and have a facilitatory effect on the memory consolidation process (Jerusalinsky et al., 1993, 1994). However, considering that affinities of pirenzepine were about 4- to 12-fold higher for M_1 than for M_4 receptors (Felder et al., 2001), at higher concentrations this antagonist could also block M_4 receptors. Although 0.75 μg of MT2 into the dorsal hippocampus facilitated consolidation (Jerusalinsky et al., 2000), at a higher dose the effect disappeared, suggesting that the toxin was also acting at another site, likely blocking the M_4 receptor. It was shown that MT2 behaved as a muscarinic antagonist at hippocampal M_4 receptors, counteracting the muscarinic inhibition of forskolin-stimulated adenylyl cyclase (Kornisiuk et al., 2001). In addition, MT1, another related toxin with a similar pharmacological profile, also showed a facilitatory effect on memory consolidation only at the lower doses, having no evident effect at higher ones (Jerusalinsky et al., 2000). A plausible explanation for this would be that although a lower dose of MT2 (and also MT1) activates M_1 receptor, which would facilitate consolidation, at a higher dose, MT2 also blocks M_4 receptors, likely counteracting and/or masking that facilitatory effect. The putative contribution of M_4 receptor blockade to the impaired performance was discriminated by the use of MT3. This toxin, highly selective for M_4 receptors, impaired retention for the same task in adult male rats (Jerusalinsky et al., 1998), causing a scopolamine-like retrograde amnesia, an observation here confirmed and extended to females. Hence, MT3's antagonistic action at M_4 receptors (Bradley, 2000; Olianas et al., 1998) should account for its amnestic effect. Therefore, the results with higher doses of MT2, in addition to the amnesia caused by MT3, support the involvement of M_4 receptors of the hippocampus in the consolidation of a memory trace. On the other hand, neither MT2 nor MT3 (in the dose effective at the inhibitory avoidance task) showed any evident effect in both crossings and rearings measured in a novel environment (Table 1), whereas scopolamine was amnestic (Jerusalinsky et al., 1998). Hence, the MTs did not appear to cause any evident effect on locomotor activity or exploratory behaviour, at least in the conditions of our experiments (Table 1), suggesting that neither M_1 nor M_4 receptors in the dorsal hippocampus are required for memory consolidation of habituation to a new environment.

The observed dissimilar medians for latencies in the inhibitory avoidance task (Fig. 1) appeared gender-related, as nonsynchronized female rats usually exhibit larger dispersions than males, according to our experience (Jerusalinsky et al., 1993, 1998). Despite the larger dispersions, the effects of the drugs were robust enough, resulting in statistical significance.

Altogether, our results strongly suggest the necessary participation of both, M_1 and M_4 receptors in the dorsal hippocampus as positive modulators of memory consolidation of an inhibitory avoidance task in the rat.

At variance with these results, it was reported that methoctramine (icv), a relatively selective M_2 antagonist, improved memory (Aura et al., 1997); but there were no reports on direct administration of methoctramine into the hippocampus. Both M_2 and M_4 receptors preferentially couple to G_i proteins to mediate inhibition of adenylyl cyclase. M_4-selective MTs, with negligible binding to M_2 receptor (Kornisiuk et al., 1995, 2001; Olianas et al., 1998; Potter, 2001), were able to antagonize the muscarinic inhibition of adenylyl cyclase in the hippocampus (Kornisiuk et al., 2001). However, each receptor subtype could be acting through other different mechanisms (Nathanson, 2000). This gives rise to a complex question that deserves further investigation.

5. Conclusions

1. MT2, a muscarinic toxin with agonist-like action at M_1 receptors, facilitated memory consolidation of an inhibitory avoidance task in the rat when given into the dorsal hippocampus immediately after training, while the muscarinic antagonist pirenzepine caused retrograde amnesia; these results support the participation of M_1 receptors in the dorsal hippocampus as positive modulators of memory consolidation of this task.
2. However, at a higher dose of MT2, the facilitation disappeared. Therefore, MT2 must be acting at another site. Both toxins, MT2, selective for M_1 and M_4 receptor subtypes, and MT3, selective for M_4, behave as muscarinic antagonists at M_4 receptors. Thus, a higher dose of MT2 probably blocks the M_4 receptor, masking the facilitation caused by M_1 activation. This result, in addition to the amnesia caused by MT3, strongly supports the participation of M_4 receptor of the dorsal hippocampus as a positive modulator of memory consolidation of an inhibitory avoidance task in the rat.
3. The blockade of M_1 or M_4 receptors in the dorsal hippocampus did not affect crossings and rearings in a new environment.

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