Research

Differing time dependencies of object recognition memory impairments produced by nicotinic and muscarinic cholinergic antagonism in perirhinal cortex

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The roles of muscarinic and nicotinic cholinergic receptors in perirhinal cortex in object recognition memory were compared. Rats’ discrimination of a novel object preference test (NOP) test was measured after either systemic or local infusion into the perirhinal cortex of the nicotinic receptor antagonist methyllycaconitine (MLA), which targets alpha-7 (α7) amongst other nicotinic receptors or the muscarinic receptor antagonists scopolamine, AFDX-384, and pirenzepine. Methyllycaconitine administered systemically or intraperirhinally before acquisition impaired recognition memory tested after a 24-h, but not a 20-min delay. In contrast, all three muscarinic antagonists produced a similar, unusual pattern of impairment with amnesia after a 20-min delay, but remembrance after a 24-h delay. Thus, the amnesic effects of nicotinic and muscarinic antagonism were doubly dissociated across the 20-min and 24-h delays. The same pattern of shorter-term but not longer-term memory impairment was found for scopolamine whether the object preference test was carried out in a square arena or a Y-maze and whether rats of the Dark Agouti or Lister-hooded strains were used. Coinfusion of MLA and either scopolamine or AFDX-384 produced an impairment profile matching that for MLA. Hence, the antagonists did not act additively when coadministered. These findings establish an important role in recognition memory for both nicotinic and muscarinic cholinergic receptors in perirhinal cortex, and provide a challenge to simple ideas about the role of cholinergic processes in recognition memory: The effects of muscarinic and nicotinic antagonism are neither independent nor additive.

[Supplemental material is available for this article.]

Much evidence indicates that the perirhinal cortex is critically involved in object recognition memory (for reviews, see Murray and Bussey 1999; Brown and Aggleton 2001; Mumby and Pinel 1994; Ennaceur et al. 1996; Meunier et al. 1993, 1996; Mumby and Pinel 1994; Ennaceur et al. 1996; Winters et al. 2004) or by local perirhinal infusions of glutamatergic receptor antagonists (Winters and Bussey 2005; Barker et al. 2006b). The cholinergic system is widely hypothesized to play a prominent role in mechanisms of memory and attention (for reviews, see Voytko 1996; Sarter and Bruno 1997; Easton and Parker 2003; Sarter et al. 2003; Hasselmo and Giocomo 2006; Hasselmo and Stern 2006; Dani and Bertrand 2007). Nicotinic and muscarinic receptors are located on neurons in the cerebral cortex, including perirhinal cortex (Saleem et al. 2007). Nicotinic receptor subunits form ligand-gated ion channels (Sargent 1993), while muscarinic receptors are G-protein linked (Wess 1993). Most nicotinic receptor subtypes are permeable to Na+ and K+ ions, but the alpha-7 (α7) subtype is of particular interest because it is also permeable to Ca2+ ions (Seguela et al. 1993) and has been linked to second messenger pathways important in plasticity and memory formation (Bittner et al. 2007). Indeed, it has been claimed that α7 nicotinic receptors play a role in hippocampal LTP (Matsuyama et al. 2000; Chen et al. 2006) and the nicotinic antagonist methyllycaconitine (MLA) that acts against α7, as well as other nicotinic receptors (Mogg et al. 2002), has been shown to inhibit hippocampal LTP induction (Freir and Herron 2003; Chen et al. 2006). In perirhinal cortical slices, the broad spectrum muscarinic receptor antagonist scopolamine blocks LTD but not LTP (Warburton et al. 2003) and application of the cholinomimetic carbachol induces a long-lasting depression (Massey et al. 2001). The role of nicotinic, including α7 receptors in perirhinal plasticity, is unknown.

It has been reported previously that scopolamine can impair familiarity discrimination (Huston and Aggleton 1987; Ennaceur and Meliani 1992; Bartolini et al. 1996; Besheer et al. 2001; Norman et al. 2002; Warburton et al. 2003; Winters et al. 2007), including when infused directly into the perirhinal cortex in monkeys (Tang et al. 1997) or rats (Abe and Iwasaki 2001; Warburton et al. 2003; Winters et al. 2007). However, studies of nicotinic receptors have chiefly investigated agonist actions and none have determined the role of antagonists within the perirhinal cortex (Van Kampen et al. 2004; Boess et al. 2007; Pichat et al. 2007). In the experiments reported here, the effect upon recognition memory of MLA, which strongly antagonizes α7 nicotinic but may also antagonize other nicotinic receptors (Mogg et al. 2002), has been compared with that of the broad-spectrum muscarinic antagonist scopolamine. The actions were found to doubly dissociate across 20-min and 24-h time delays. As this result was unexpected and the effect of scopolamine was not in accord

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with a previous study, further experiments were performed with AFDX-384 (at a concentration designed to act also as a broad-spectrum muscarinic antagonist) (Dorje et al. 1991), scopolamine using variants of the basic experimental conditions, and the selective M1 muscarinic antagonist pirenzepine. Additionally, to determine whether impairment at both 20 min and 24 h could be produced by combining muscarinic with nicotinic antagonism, the effects of coadministration were sought. The findings established an unusual double dissociation in the effects of muscarinic and nicotinic antagonism that provides a challenge to simple ideas about the role of cholinergic processes in recognition memory.

Results

To establish the role of nicotinic and muscarinic receptors in recognition memory, the effects of systemic administration or local infusions of the nicotinic antagonist MLA or muscarinic antagonists into the perirhinal cortex were determined on the rats’ preferential exploration of a novel compared with a familiar object. Systemic injections or perirhinal infusions (see Fig. 1B for infusion sites) of vehicle or antagonist were made 30 min (systemic) or 15 min (local infusion) prior to the acquisition phase of the object recognition test with the choice (test) phase being at a delay of either 20 min or 24 h. The discrimination ratio (DR) at test was calculated by dividing the difference in time exploring the novel and the familiar object by the time taken exploring both objects.

All rats under control conditions showed significant discrimination at all time intervals (t-tests, $P \leq 0.05$). Additionally, the total time exploring objects in the acquisition or choice phases showed no significant differences dependent on whether the animals had received vehicle or cholinergic antagonists (Table 1).

Experiment 1 investigated the effect of nicotinic receptor antagonism.

Experiment 1: Object recognition memory following administration of the nicotinic receptor antagonist MLA

Figure 2 shows that MLA impaired recognition memory at a delay of 24 h, but not at a delay of 20 min, whether administration was systemic (87.5 μg/kg) or local via cannula infusion (0.0875 ng/μL) into the perirhinal cortex.

Analyzing across all of the MLA conditions, three factor ANOVA (delay [20 min or 24 h], administration route [systemic injection or intracerebral infusion], treatment [vehicle or drug]) revealed a significant effect of treatment ($F_{(1,40)} = 6.62, P = 0.01$), and a significant interaction of treatment by delay ($F_{(1,40)} = 4.61, P = 0.04$). Data for the two delays were therefore analyzed separately.

For the 20-min delay tests, two factor ANOVA (administration route, treatment) showed no significant effect of treatment ($F_{(1,20)} < 1, P = 0.8$) and no significant interaction of administration route by treatment ($F_{(1,20)} < 1, P = 0.4$). The MLA-treated animals showed significant discrimination in the test (DR > 0: t-test, systemic injection: $P = 0.001$; intracerebral infusion: $P = 0.001$).

For the 24-h delay tests, two factor ANOVA (administration route, treatment) showed a significant effect of treatment ($F_{(1,20)} = 15.17, P = 0.001$) and no significant interaction of administration route by treatment ($F_{(1,20)} = 2.00, P = 0.2$). Post-hoc analysis for the 24-h tests with ANOVAs showed a significant impairment in the MLA-treated rats compared with controls (systemic injection: $F_{(1,19)} = 7.74, P = 0.02$; intracerebral infusion: $F_{(1,11)} = 7.48, P = 0.02$); the MLA-treated animals did not show significant discrimination in the test (DR > 0: t-test, systemic injection: $P = 0.3$; intracerebral infusion: $P = 0.4$).

As previously reported work (Warburton et al. 2003; Winters et al. 2006) had not looked at the effects of muscarinic antagonism at both 20-min and 24-h delays under the same experimental conditions, to allow direct comparison with the effects of MLA, Experiment 2 sought the effects of the broad spectrum muscarinic antagonist scopolamine under the same experimental conditions as for MLA.

Experiment 2: Object recognition memory following administration of the muscarinic receptor antagonist scopolamine

Figure 3 reveals that scopolamine impaired recognition memory at a delay of 20 min, but not at a delay of 24 h, whether administration was systemic (0.05 mg/kg) or local via cannula infusion (0.05 ng/μL) into the perirhinal cortex.

Analyzing across all of the scopolamine conditions, three factor ANOVA (delay [20 min or 24 h], administration route [systemic injection or intracerebral infusion], treatment [vehicle or drug]) revealed a significant effect of treatment ($F_{(1,39)} = 13.67, P = 0.001$) and a significant interaction of treatment by delay ($F_{(1,39)} = 10.27, P = 0.003$). Data for the two delays were therefore analyzed separately.

For the 20-min delay tests, two factor ANOVA (administration route, treatment) showed a significant effect of treatment ($F_{(1,20)} = 15.49, P = 0.001$) and no significant interaction of administration route by treatment ($F_{(1,20)} < 1, P = 0.7$). The scopolamine-treated animals did not show significant discrimination in the test (DR > 0: t-test, intracerebral infusion: $P = 0.5$, systemic injection: $P = 0.5$).

For the 24-h delay tests, two factor ANOVA (administration route, treatment) showed no significant effect of treatment ($F_{(1,19)} < 1, P = 0.6$) and no significant interaction of administration route by treatment ($F_{(1,19)} = 1.19, P = 0.3$). The scopolamine-treated animals showed significant discrimination.
Table 1. Exploration of animals in the acquisition and choice phases

<table>
<thead>
<tr>
<th>Infusate type</th>
<th>Trial</th>
<th>Delay</th>
<th>MLA (systemic)</th>
<th>MLA (cannula)</th>
<th>scop (systemic)</th>
<th>scop (infusion)</th>
<th>AFDX-384</th>
<th>MLA + Scop</th>
<th>MLA + AFDX-384</th>
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<tr>
<td>Vehicle</td>
<td>Acquisition</td>
<td>20 min</td>
<td>32(3)</td>
<td>32(2)</td>
<td>32(3)</td>
<td>32(2)</td>
<td>25(3)</td>
<td>30(2)</td>
<td>24(1)</td>
</tr>
<tr>
<td>Vehicle</td>
<td>Acquisition</td>
<td>24 h</td>
<td>31(2)</td>
<td>37(1)</td>
<td>31(3)</td>
<td>26(3)</td>
<td>30(3)</td>
<td>29(3)</td>
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<tr>
<td>Vehicle</td>
<td>Choice</td>
<td>20 min</td>
<td>27(4)</td>
<td>21(3)</td>
<td>29(3)</td>
<td>15(2)</td>
<td>14(3)</td>
<td>17(2)</td>
<td>13(2)</td>
</tr>
<tr>
<td>Vehicle</td>
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<td>14(3)</td>
<td>9(2)</td>
</tr>
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</table>

Values shown are mean time exploring both objects in seconds (SEM) for vehicle or drug at each of the time-points investigated. For acquisition trials, three factor ANOVA (treatment, route/dose, delay) for Experiments 1 and 2, or two factor ANOVA for Experiments 3, 4A, or 4B, (treatment, delay) revealed no significant effects across any of the experimental groupings. Similarly, for choice trials, three factor ANOVA and two factor ANOVA revealed no significant effects across any of the experimental groupings.

in the test (DR > 0; t-test, intracerebral infusion: \( P = 0.001 \), systemic injection: \( P = 0.001 \)).

The deficit at 20 min for scopolamine replicated the findings of Warburton et al. (2003). However, the failure to find a clear impairment with scopolamine at a 24-h delay conflicts with the findings of Winters et al. (2006). Winters et al. (2006) investigated the effects of scopolamine using a Y-maze and Lister Hooded rats. In studies reported in the Supplementary Material (Experiments 1A, B; Figs. 1A, B), the same pattern of memory loss, with impairment at 20 min, but unimpaired performance at 24 h, was found using a Y-maze or using Lister Hooded rats, thereby confirming the findings of Experiment 2. As the pattern of scopolamine-induced impairment was unusual, Experiment 3 tested broad-spectrum muscarinic antagonism produced by a different compound, AFDX-384.

Experiment 3: Object recognition memory following intraperirhinal infusion of the muscarinic receptor antagonist AFDX-384

To determine whether the unusual pattern of impairment produced by scopolamine was replicable with a different muscarinic antagonist, experiments were undertaken using the AFDX-384 at a dose sufficiently high to antagonize all muscarinic receptor subtypes (Dorje et al. 1991). Administration was by local perirhinal infusion (12 ng/μL), and Dark Agouti rats were tested in the arena.

Figure 4 reveals that AFDX-384 produced the same pattern of memory deficit as scopolamine: impairment at 20 min, but not 24 h. An overall analysis by two factor ANOVA (delay [20 min or 24 h], treatment [vehicle or drug]) revealed a near-significant effect of treatment \( F(1,14) = 4.35, P = 0.06 \) and a near-significant interaction of treatment by delay \( F(1,14) = 4.16, P = 0.06 \). To allow closer comparison with the findings for scopolamine, data for the two delays were analyzed separately.

For the 20-min delay, the post hoc analysis showed significant impairment for the muscarinic antagonist-treated animals compared with controls (ANOVA: \( F(1,8) = 7.64, P = 0.02 \); the AFDX-384-treated animals did not show significant discrimination (DR = 0; t-test: \( P = 0.4 \)).

For the 24-h delay, the post hoc analysis showed no significant impairment for the muscarinic antagonist-treated animals compared with controls (ANOVA: \( F(1,7) = <1, P > 0.9 \); the AFDX-384-treated animals showed significant discrimination (DR > 0; t-test, AFDX-384: \( P = 0.006 \)).

To further investigate the unusual pattern of impairment produced by scopolamine and AFDX-384, the experiment was repeated with the selective M1 muscarinic receptor antagonist pirenzepine. Pirenzepine produced the same pattern of memory deficit as scopolamine and AFDX-384: impairment at a 20-min, but not a 24-h delay (see Supplemental Material).

Figure 2. Effect of methyllycaconitine (MLA) on object recognition memory at 20-min or 24-h delays tested in Dark Agouti rats in the arena. (A) Effect of systemic injection of MLA (87.5 μg/kg, ip). MLA impaired memory after a 24-h (A2) but not a 20-min (A1) delay. (B) Effect of intraperirhinal infusion (0.0875 ng/μL) of MLA. Again, MLA impaired memory after a 24-h (B2), but not after a 20-min (B1) delay. (*) \( P < 0.05 \).
of both types of antagonist should produce an impairment at muscarinic antagonism, it was predicted that coadministration MLA + 

Figure 3. Effect of scopolamine on object recognition memory at 20 min and 24 h tested in Dark Agouti rats in the arena. (A) Effect of systemic injection of scopolamine (0.05 mg/kg, ip) prior to object recognition memory testing. Scopolamine impaired memory at a 20 min (A1) but not at a 24 h (A2) delay. (B) Effect of intraperihinal infusion of scopolamine (0.05 ng/μL) prior to object recognition memory testing. Scopolamine impaired memory at a 20-min (B1) but not at a 24-h (B2) delay. (*) Significance of ANOVA test at P < 0.05.

Given the different impairments found with nicotinic and muscarinic antagonism, it was predicted that coadministration of both types of antagonist should produce an impairment at both long and short delays: this was tested in Experiment 4.

Experiment 4: Object recognition memory following combined intraperihinal infusion of MLA and a muscarinic antagonist

To test the expectation of impairment at both delays, MLA was coinfused with scopolamine or AFDX-384 at the doses previously used. Figure 5 shows the results after Dark Agouti rats were tested in the square arena. Analyzing across all of the conditions, three factor ANOVA (delay [20 min or 24 h], drug combination [MLA + scop, MLA + AFDX-384], treatment [vehicle or drug]) revealed a significant effect of treatment (F(1,35) = 18.81, P < 0.001) and a significant interaction of treatment by delay (F(1,35) = 4.02, P = 0.05). For the 20-min delay test, two factor ANOVA (drug combination [MLA + scop, MLA + AFDX-384], treatment [vehicle or drug]) showed no significant effect of treatment (F(1,17) = 2.69, P = 0.12) and no significant interaction of treatment by drug combination (F(1,17) = <1, P = 0.7). For the 24-h delay test, two factor ANOVA (drug combination [MLA + scop, MLA + AFDX-384, MLA + pirenzepine], treatment [vehicle or drug]) showed a significant effect of treatment (F(1,19) = 20.37, P < 0.001) and no significant interaction of treatment by drug combination (F(1,18) < 1, P = 0.7). Hence, in each case the pattern of impairment mirrored that produced by MLA with impairment only at the 24-h delay rather than there being an impairment at both delays.

The results for the individual drug coadministrations are as follows. At the 20-min delay there was no significant effect of treatment (all Ps > 0.1) for MLA plus scopolamine or MLA plus AFDX-384. In each case the drug group showed significant discrimination (DR > 0; P > 0.01). At the 24-h delay there was a significant effect of treatment for MLA plus scopolamine (ANOVA, F(1,11) = 12.93, P = 0.004), and MLA plus AFDX-384 (ANOVA, F(1,7) = 9.82, P = 0.02). In both cases the drug group failed to show significant discrimination (DR > 0; P > 0.2).

Taken together, the results for coapplication of MLA and muscarinic antagonists (scopolamine or AFDX-384) provide evidence against an additive effect for antagonism of nicotinic receptors and muscarinic receptors. Coinfusions of MLA and scopolamine, or MLA and AFDX-384 all blocked memory after a 24-h but not a 20-min delay. Hence, addition of the muscarinic antagonist did not alter the profile of impairment produced by MLA administered alone, so that MLA appeared to override the effect of muscarinic antagonism.

Discussion

The results demonstrate a double dissociation in the effects of nicotinic and muscarinic receptor antagonism on object recognition memory measured at 20-min and 24-h delays. Nicotinic antagonism does not impair memory measured after 20 min, but does impair memory after 24 h, whereas blockade of muscarinic receptors impairs memory after 20 min, but not after 24 h.
Coapplication of the nicotinic antagonist MLA and a muscarinic antagonist resulted in impairment at a delay of 24 h, but not at 20 min.

**Effect of nicotinic antagonism on object recognition memory**

The results demonstrate that when given so as to be active during acquisition, MLA impairs object recognition memory after 24 h, but not after 20 min. MLA has a well-documented action antagonizing neuronal α-bungarotoxin binding sites (Ward et al. 1990), which are known to reside primarily on α7 receptors (Davies et al. 1999; Whiteaker et al. 1999). However, there is evidence that MLA may also act at α3β2 and α4β2 nicotinic binding sites (Mogg et al. 2002). Accordingly, contributions to the effects produced by MLA on recognition memory by actions at α4β2 containing nicotinic receptors cannot be excluded. Effects from action at the α3β2 site are less likely, as α3β2 subunits are not among the main nicotinic receptor subunits in rodent cortex (Gotti et al. 2006). The profile of long-term (24-h) but not short-term (20-min) memory impairment produced by MLA is the same as that found with NMDA receptor antagonism (Barker et al. 2006b), metabotropic glutamate receptor antagonism (Barker et al. 2006a), L-type voltage-dependent calcium channel blockade (Seoane et al. 2009), and calcium calmodulin-dependent kinase II (CAMKII) antagonism with KN-62 or autoantiglute-2 related inhibitory peptide (AfIP) (Tinsley et al. 2009). A previous study (Pichat et al. 2007) found no effect of MLA on object recognition memory at a 1-h delay, though MLA administered intracerebroventricularly in rats has been reported to impair social recognition memory at a 24-h delay (Van Kampen et al. 2004). Further, the α7 receptor agonist, N-[(3R)-1-azabicyclo[2.2.2]oct-3-yl]-7-[2-(methoxy)phenyl]-1-benzofuran-2-carboxamide (ABBF), enhances social recognition memory after a 24-h delay, this enhancement being blocked by MLA (Boes et al. 2007). As the effects of infusing MLA after acquisition were not tested in the present study, it is possible that the memory deficit at a 24-h delay is due to consolidation rather than acquisition mechanisms. Scopolamine given after acquisition or so as to be active during retrieval does not produce an impairment in object recognition memory at a 20-min delay (Warburton et al. 2003); hence, this memory impairment can be ascribed to antagonism of muscarinic receptors during acquisition, rather than actions on retrieval or consolidation mechanisms.

**Effect of muscarinic antagonism on object recognition memory**

Muscarinic antagonism impaired object recognition memory after a 20-min delay, but not after a 24-h delay. This same unusual pattern of impairment was found for scopolamine, AFDX-384, and pirenzepine. Pirenzepine produced a parallel pattern of impairment to scopolamine (see Supplemental Material), suggesting that the dependency of the deficit is dependent upon M1 rather than other subtypes of muscarinic receptors. The same pattern of deficit was found for both systemic and local perirhinal infusions of scopolamine, in both Dark Agouti and Lister Hooded rat strains (see Supplemental Material). Such a counterintuitive pattern of impairment with amnesia being followed by remembrance has also been described when perirhinal kainate receptors are antagonized (Barker et al. 2006b). AFDX-384 given systemically (1 mg/kg—lower than the dose used in the current experiments) has previously been reported to have no effect on object recognition memory with a 1-h delay (Vannucchi et al. 1997). Impairments of object recognition memory by scopolamine have been reported for delays of 20 min (Warburton et al. 2003), 1 h (Vannucchi et al. 1997), 3 h (Dodart et al. 1997), and 24 h (Winters et al. 2006). In unpublished experiments in Bristol (Fontaine-Palmer 2008) scopolamine was found to impair object recognition memory after a delay of 1 h (in agreement with Vannucchi et al. 1997) or 3 h (Dodart et al. 1997), but not after a delay of 6 h. We have found no reports of the effects of scopolamine on recognition memory at long (>3 h) delays in either monkeys or humans, but there are reports of impairment in monkeys at short delays (Aignier et al. 1991; Tang et al. 1997; Turchi et al. 2008; but, see Browning et al. 2010).

The lack of impairment found here for muscarinic antagonism at the 24-h delay contrasts with the previously reported scopolamine-induced impairment at that delay (Winters et al. 2006). However, in that study (Winters et al. 2006), the scopolamine-induced impairment was partial and the scopolamine-treated animals’ discrimination was similar to that of other control groups in the study, albeit lower than that of its particular matched control group. There are several procedural differences between the two studies, including rat strain, testing arena shape, and precise details of cannulae implantation. Neither rat strain nor testing arena shape appear able to explain the pattern of impairment: in the present experiments, using systemic administration, no impairment for a 24-h delay was found for Dark Agouti rats in a square arena or in a Y-maze such as used by Winters et al. (2006) and neither was impairment found for Lister Hooded rats, such as used by Winters et al. (2006), when tested in a square arena.
The effect of confusing α7 nicotinic and muscarinic antagonists on object recognition memory

In the current experiments, when muscarinic antagonists were coapplied with the nicotinic receptor antagonist MLA, memory was blocked after a 24-h delay, but memory after a 20-min delay was unimpaired. In each instance, the dose of the muscarinic antagonist given alone was sufficient to produce amnesia at the shorter (20-min) delay. Hence, unexpectedly, for memory after a 20-min delay, addition of a potentially amnesic dose of a muscarinic antagonist to a potentially amnesic dose of a nicotinic antagonist removed the impairment produced by the single dose of the muscarinic antagonist. Accordingly, the effects of giving a muscarinic and an α7 nicotinic antagonist together were not additive: The anticipated memory impairment at both 20 min and 24 h was not seen. Titrating dosages of antagonists against each other was not an objective of the current experiments, so it remains to be discovered whether there are doses of a nicotinic plus a muscarinic antagonist that would result in impairment at both 20-min and 24-h delays. (However, coinfusion of MLA with a very high dose of scopolamine [10 μg/μL] left an impairment at the 20-min delay, but did not produce impairment at the 24-h delay [data not included].) These findings were unexpected and provide a challenge to simple ideas about the action of cholinergic receptors in recognition memory: Clearly, the effects of muscarinic and nicotinic antagonism are neither independent nor additive. We have not found reports that scopolamine has agonistic/antagonistic effects on nicotine receptors or that MLA has effects on muscarinic receptors. However, there are multiple opportunities for interactions involving the two types of antagonism, for example, involving presynaptic autoreceptors or alterations in the balance of excitation and inhibition in local circuits. Indeed, there is evidence of interactions involving the two types of receptors: The muscarinic receptor antagonist atropine can either inhibit or potentiate the responses of nicotinic receptors (Zwart and Vijverberg 1997; Parker et al. 2003; Gonzalez-Rubio et al. 2006).

Materials and Methods

Animals

Forty-seven male Dark Agouti rats (200–230 g; Bantin and Kingman, Hull, UK) were used for object recognition experiments. All of the animals were housed under a 12:12-h light/dark cycle (light phase, 18.00–06.00 h). Experiments were conducted during the dark phase of the cycle. All animal procedures were performed in accordance with the United Kingdom Animals Scientific Procedures Act (1986) and University of Bristol Ethical Review Group.

Object recognition following administration of cholinergic antagonists

Surgery

Prior to testing in the novel object preference test (NOP) test, 25 rats underwent surgical procedures to implant guide cannulae into the perirhinal cortex. Rats were anesthetized with isoflurane (Merial, Harlow, UK) and placed in a stereotaxic frame with the incisor bar set so as to achieve a horizontal skull. Cranietomies were made 5.6-mm posterior and 4.5-mm lateral to bregma, and 10-mm length, 26-gauge stainless-steel guide cannulae (Plastics One) were inserted 6.7 mm below the surface of the skull in the coronal plane at an angle of 20° to the vertical. The cannulae were attached to the surface of the skull by constructing an implant made from bone cement (DePuy, UK) and attached to the skull with stainless-steel screws (Plastics One). After surgery, rats were allowed a 2-wk recovery period, during which they were housed singly. Post-recovery, cannulated rats were housed in pairs in large cages. Obdurators (Plastics One) were used to keep the cannulae patent between intusions.

Novel object preference test (NOP) test

In a pretraining period, rats were habituated to an arena (length: 100 cm, depth: 100 cm, arena height: 40 cm) surrounded by curtains, (height from arena base: 40–160 cm) in one 5-min period each day for 4 d. For the novel object preference test (NOP) test,
rats underwent an acquisition trial and choice trial separated by a delay (20 min or 24 h). During the acquisition trial, each rat was initially exposed to two identical copies of an object (Object A) until it had explored the objects for >40 sec or had spent a maximum of 4 min in the arena. Objects were constructed from Duplo (Lego UK, Slough, UK) (length: ~16 cm, depth: ~16 cm, height: ~12 cm). After a delay period of 20 min or 24 h, each rat was exposed to another copy of Object A and a novel object (Object B) in a choice trial lasting 3 min. In one preferential object recognition memory experiment rats were placed in a Y-maze with the objects positioned in two of the arms (see Supplemental Material; Winters et al. 2006).

**Infusions and intraperitoneal injections**

**Drugs**

In each experiment, drug administration was either by a systemic injection or by bilateral intracerebral infusion via guide cannulae directed at the perirhinal cortex. Systemic administration was via an intraperitoneal injection at a volume of 1 mL/kg given 30 min prior to the acquisition trial. Rats received bilateral infusions into the perirhinal cortex using 33-gauge infusion cannula (Plastics One) inserted into the implanted guide cannula and attached to a 25-µL Hamilton syringe by polyethylene tubing. An infusion pump (Harvard Bioscience) was used to inject a volume of 1 µL to each hemisphere during a 2-min period. Infusion cannulae were kept in place for a further 5 min following the infusion. Intracerebral drug infusions were given 15 min prior to acquisition trial, thereby allowing time for the systemic injections to be effective, as in previous studies (Warburton et al. 2003; Winters et al. 2006). The receptor antagonists used were methyllylcaconitine (MLA), scopolamine hydrobromide, AFDX-384, and pirenzipine, all supplied by Tocris Biosciences (Bristol, UK). Previous work with intracerebral infusions indicates that drugs may persist at effective concentrations for ~1 h post-infusion (Day et al. 2003).

**Experiment 1.** Dark Agouti rats were administered the nicotinic antagonist methyllylcaconitine (MLA) either systemically at a dose of 87.5 µg/kg or via an intracerebral injection of 0.0875 ng/µL (infusate = 100 nM).

**Experiment 2. A:** Dark Agouti rats were administered the muscarinic agonist scopolamine hydrobromide systemically at a dose of 0.05 ng/µL (arena) (infusate = 130 nM).

**Experiment 3.** Dark Agouti rats were administered the nonselective muscarinic antagonist AFDX-384 at a dose of 12 ng/µL (infusate = 25 µM) intracerebrally.

**Experiment 4. A:** Dark Agouti rats were administered both MLA and 0.05 ng/µL of scopolamine. B: Dark Agouti rats were administered scopolamine hydrobromide via an intracerebral infusion (0.05 ng/µL) (arena) (infusate = 130 nM).

The dose for MLA was selected so as not to produce deficits in locomotion (Chilton et al. 2004). A high dose of AFDX-384 was required with the aim of blocking all muscarinic subtypes, as occurs with scopolamine (Dorje et al. 1991; Collison et al. 2000). Concentrations of infusate (or systemic doses) were chosen to be 50–100 times that of the designated Ki value for the target receptor to ensure maximal inhibition, but limit nonspecific effects. Ki values previously reported for the compounds used are MLA(α): ~1 nM (Ivy Carroll et al. 2007), scopolamine (M1–M5): ~0.3–2 nM (Huang et al. 2001), and AFDX-384 (M1–M5): ~6 nM–530 nM (Dorje et al. 1991). Half-lives for the drugs used were: MLA = 18 min (Steigemeier et al. 2003), scopolamine = 3.7 h (Ebert et al. 1998), and AFDX-384 = 40 min (Mickala et al. 1996).

Each experiment was run in two parts in a cross-over design. In the first part, rats were randomly assigned an infusion/injection of drug or vehicle (0.9% saline for MLA, scopolamine; 0.2% dimethyl sulfoxide (DMSO) in 0.9% saline for the AFDX-384) and an object recognition experiment was performed. In the second part, each rat was given the opposite infusate/injectate and a second object recognition experiment was performed using new objects. There was a minimum separation of 48 h between the two parts of the experiment.

**Data analysis**

The time spent exploring each object was scored using a computer program with the experimenter blind to the treatment. Exploratory behavior was defined as the rat directing its nose toward the object at a distance of <2 cm; other behavior, such as looking around while sitting on the object, was not considered exploration. A discrimination ratio (DR) was used to measure memory and was calculated by dividing the difference in time exploring the novel and the familiar object by the time taken exploring both objects. Rats that failed to complete a minimum of a 10-sec exploration in the acquisition phase or a minimum of 5 sec for the choice trial, were excluded from the analysis. Over the course of the experiments, occasionally rats had to be excluded from the analysis due to cannula failures.

Table 2 includes a summary of the rat groups included in the statistical analysis of the drug experiments. Where rats were used in more than one experiment, the experiments were performed in the following order. Group C1: (1) MLA, 20 min, (2) MLA, 24 h, (3) AFDX-384, 20 min, (4) AFDX-384, 24 h, (5) AFDX-384 & MLA, 20 min, and (6) AFDX-384 & MLA, 24 h. Group C2: (1) scopolamine, 20 min, (2) scopolamine and MLA, 24 h, and (3) scopolamine, 24 h. Discrimination ratios were analyzed using repeated measures ANOVA with factors treatment (drug/vehicle) and infusion time. The significance level was P = 0.05, two-tailed.

**Verification of cannulae positions**

Rats were anesthetized with Euthatal (Rhône Mérieux, Toulouse, France) and transcardially perfused with 0.1 M phosphate buffer containing formal saline, pH 7.4. Coronal sections (40 µm) were cut on a freezing microtome and the sections were stained with cresyl violet. Cannula locations were compared with a stereotaxic atlas (Paxinos and Watson 1998), and histological examination confirmed that the tips of the cannulae were within the perirhinal cortex (Shi and Cassell 1999), see Figure 1A. Our cannula-tip

<table>
<thead>
<tr>
<th>Table 2.</th>
<th>Table showing animal groups used in each of the four experiments. Label denotes rat group and the number of animals in each group is shown in brackets</th>
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<tbody>
<tr>
<td>Trial</td>
<td>Delay</td>
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<tr>
<td>MLA (pp)</td>
<td>MLA (cannula)</td>
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Rat groupings: (S1) systemic group 1: Dark Agouti rats; (S2) systemic group 2: Dark Agouti rats; (C1) cannulated group 1: Dark Agouti rats; (C2) cannulated group 2: Dark Agouti rats.
locations matched the caudal region of the perirhinal cortex (~5.8 with respect to bregma) known to correlate with object recognition memory deficits (Albasser et al. 2009). Data from other laboratories (Martin 1991; Izzaro et al. 2000a; Attwell et al. 2001) and our data (Seoane et al. 2011) indicate that the infused tissue extended to an ~0.5–1 mm radius of the cannula tip. This volume includes the majority of perirhinal cortex (Shi and Cassell 1999), but only minor parts of neighboring entorhinal cortex or area Te2.

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**References**


Cholinergic antagonism and recognition memory


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