Supplemental Material

Materials and Methods

Animals

Eighteen male Dark Agouti rats (200-230g; Bantin and Kingman, Hull, UK) and eight male Lister Hooded (230-270g; Harlan, UK) rats were used for object recognition experiments. All the animals were housed under a 12 h light/dark cycle (light phase, 18.00 to 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. The same surgical techniques and experimental techniques were used as outlined in the paper.

Infusions and intraperitoneal injections

Experiment 1S A: Dark Agouti rats were administered scopolamine hydrobromide systemically by at a dose of 0.05mg/kg (Y-maze). B: Lister Hooded rats were administered scopolamine hydrobromide systemically at a dose of 2mg/kg (arena). The higher dose was not used with Dark Agouti rats because of their greater susceptibility to the drug.

Experiment 2S B: Dark Agouti rats were administered the non-selective muscarinic antagonist pirenzepine at a dose of 0.07ng/µl [infusate=165nM] intracerebrally. A dose of pirenzepine was required to only blockade M1 muscarinic receptors (Massey et al 2001). For pirenzepine the chosen concentration was ~25 times higher than the Ki with the aim of selectively targeting just M1 receptors.

Ki values previously reported for pirenzepine (M1-M5): ~6nM-220nM (Dorje et al 1991). Half-lives for the pirenzepine = 10.2h (Lee et al 1986). 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 and pirenzepine) and an object recognition experiment was performed.
Results

In Experiment 1S the effects of scopolamine were tested first using a Y-maze and then using Lister Hooded rats as that apparatus and that strain were used by Winters et al. (2006). Systemic infusions were used as these produced the same effects as local perirhinal infusions in Experiment 2.

Experiment 1S: Object recognition memory following administration of scopolamine tested in the Y maze and in Lister Hooded rats

A: Systemic injection of scopolamine in Dark Agouti rats tested in the Y maze

To determine whether the unusual pattern of impairment of object recognition memory produced by muscarinic antagonism was specific to the testing apparatus, further experiments were performed using a Y-maze (as used by Winters et al. 2006) rather than an arena. Fig 1S A shows the results when rats were injected with scopolamine (0.05mg/kg, i.p.) or vehicle and tested at delays of 20min and 24h.

An overall analysis by two factor ANOVA (delay [20min or 24h]; treatment [vehicle or drug]) showed a significant interaction of treatment by delay (F_{1,14} = 10.0, p = 0.01). Post hoc analysis with ANOVA for the 20min delay showed significant impairment for the scopolamine-treated compared to control condition (ANOVA: F_{1,7} = 8.34, p = 0.02); when scopolamine-treated the animals did not show significant discrimination whereas when given vehicle they did (DR > 0: t-test: p = 0.2 and p = 0.01, respectively). Post hoc analysis with ANOVA for the 24h delay showed no significant impairment for the scopolamine-treated animals compared to controls (ANOVA: F_{1,7} = 1.82, p = 0.2); whether given vehicle or drug the animals showed significant discrimination (DR > 0: t-tests, p < 0.002).

Thus changing the testing apparatus did not change the pattern of scopolamine-induced impairment. As Winters et al. (2006) used Lister Hooded rather than Dark Agouti rats, the effects of scopolamine were tried in that strain.

B: Systemic injection of scopolamine Lister Hooded rats tested in the arena
To determine whether the differences in results with Winters et al. (2006) were due to a strain difference, the effects of scopolamine were tested using Lister-hooded rats in the arena. This environment was chosen so that the experiment only differed in one variable from that described in Experiment 2. Fig. 1S B shows the results when rats were injected with scopolamine (2mg/kg, i.p.) or vehicle and tested at delays of 20min and 24h.

An overall analysis by two factor ANOVA (delay [20min or 24h]; treatment [vehicle or drug]) showed a significant interaction of treatment by delay (F\(\text{1,14} = 6.83, p = 0.02\)). Post hoc analysis with ANOVA for the **20min delay** showed significant impairment for the scopolamine-treatment compared to control (ANOVA: F\(\text{1,7} = 29.91, p = 0.001\)); with scopolamine treatment the animals did not show significant discrimination (DR = 0: t-test: p > 0.1). Post hoc analysis with ANOVA for the **24h delay** showed no significant impairment for the scopolamine treatment compared to control (ANOVA: F\(\text{1,7} = <1, p=0.4\)); animals showed significant discrimination after either drug or vehicle administration (DR > 0: t-test, p < 0.002).

Thus the same pattern of scopolamine-induced impairment was found for Lister-hooded as for Dark Agouti rats. Even though a slightly higher dose was used in the Lister-hooded than the Dark Agouti rats no impairment was found at 24h. The pattern of impairment was not strain-dependent.

These results confirm the findings of Experiment 2 that broad spectrum muscarinic antagonism with scopolamine produces recognition memory impairment at a 20 min but not at a 24 h delay.

**2S: Intraperirhinal infusion of pirenzepine**

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 infused locally into perirhinal cortex at a dose chosen to target only M1 receptors (0.07ng/µl) (Massey et al 2001, Dorje et al 1991).

Fig 2S shows pirenzepine produced a parallel pattern of memory deficit to that of scopolamine and AFDX-384: impairment at a 20min but not a 24h delay (Fig.5B). An analysis by two factor ANOVA (delay [20min or 24h], treatment [vehicle or drug]) revealed a significant effect of treatment (F\(\text{1,18} = 5.16, p = 0.04\)) and a not quite significant treatment by delay
interaction ($F_{(1,18)} = 4.16, p = 0.10$). Although the interaction of treatment by delay was not significant for pirenzepine treatment, the objective of the experiment was to investigate if the effects of scopolamine and pirenzepine were similar for the long and short delays; these effects were therefore further analysed at the two delays as designed comparisons.

Analysis of effects at the two delays indicated that the effects of the two muscarinic antagonists were indeed similar. For the 24h delay test, one factor ANOVA (dose, treatment) showed no significant effect of treatment ($F_{(1,9)} = 0.26, p = 0.6$). Moreover, the pirenzepine group showed a high mean discrimination ratio which was significantly greater than 0 (t-test: $p = 0.001$). In contrast, for the 20min delay test, there was a significant effect of treatment (ANOVA, $F_{1,9} = 5.43, p = 0.05$) and the discrimination ratio for the pirenzepine group did not differ significantly from zero (t-test: $p = 0.08$).

Taken together, the results for muscarinic antagonists (scopolamine, AFDX-384 and pirenzepine) show that they impaired object recognition memory after a 20min delay but did not significantly impair such memory after a 24h delay. The same pattern of impairment was found for all muscarinic antagonists tested: there was no significant interaction between drug and effect at the two delays (3 factor ANOVA [treatment, drug-type, delay]: $F_{2,55} < 1, p = 0.8$). An analysis comparing the results with MLA with those of pirenzepine revealed a significant interaction of drug by treatment by delay (3 factor ANOVA [treatment, drug-type, delay]: $F_{1,60} = 6.80, p = 0.01$), in replication of the dissociation found for scopolamine.

Experiments 1, 2 and 2S establish a double dissociation in the timing of the memory impairments produced by MLA and either scopolamine or pirenzepine.

References


### Tables

<table>
<thead>
<tr>
<th>Infusate type</th>
<th>trial</th>
<th>delay</th>
<th>Experiment 1SA scop (Y-maze)</th>
<th>Experiment 1SB scop (LH rats)</th>
<th>Experiment 2S pirenzepine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>Acquisition</td>
<td>20min</td>
<td>23(1)</td>
<td>38(4)</td>
<td>20(2)</td>
</tr>
<tr>
<td>Vehicle</td>
<td>Acquisition</td>
<td>24h</td>
<td>23(1)</td>
<td>36(1)</td>
<td>18(1)</td>
</tr>
<tr>
<td>Vehicle</td>
<td>Choice</td>
<td>20min</td>
<td>9(1)</td>
<td>30(3)</td>
<td>11(1)</td>
</tr>
<tr>
<td>Vehicle</td>
<td>Choice</td>
<td>24h</td>
<td>10(1)</td>
<td>29(5)</td>
<td>10(1)</td>
</tr>
<tr>
<td>Drug</td>
<td>Acquisition</td>
<td>20min</td>
<td>22(2)</td>
<td>35(2)</td>
<td>21(2)</td>
</tr>
<tr>
<td>Drug</td>
<td>Acquisition</td>
<td>24h</td>
<td>23(2)</td>
<td>36(2)</td>
<td>21(2)</td>
</tr>
<tr>
<td>Drug</td>
<td>Choice</td>
<td>20min</td>
<td>10(1)</td>
<td>33(3)</td>
<td>10(1)</td>
</tr>
<tr>
<td>Drug</td>
<td>Choice</td>
<td>24h</td>
<td>10(1)</td>
<td>30(3)</td>
<td>13(2)</td>
</tr>
</tbody>
</table>

**Table 1S.** Exploration of animals in the acquisition and choice phases. Values shown are mean time exploring both objects in seconds (S.E.M.) for vehicle or drug at each of the time-points investigated. For acquisition trials, 3 factor ANOVA (treatment, route/dose, delay) for Experiments 1S or 2 factor ANOVA for Experiments 2S (treatment, delay) revealed no significant effects across any of the experimental groupings. Similarly for choice trials, 3 factor ANOVA and 2 factor ANOVA revealed no significant effects across any of the experimental groupings.
<table>
<thead>
<tr>
<th>trial</th>
<th>delay</th>
<th>Experiment 1SA</th>
<th>Experiment 1SB</th>
<th>Experiment 2B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>scop (Y-maze)</td>
<td>scop (LH rats)</td>
<td>pirenzepine</td>
<td></td>
</tr>
<tr>
<td>Acquisition</td>
<td>20min</td>
<td>S3(8)</td>
<td>S4(8)</td>
<td>C2(10)</td>
</tr>
<tr>
<td></td>
<td>24h</td>
<td>S3(8)</td>
<td>S4(8)</td>
<td>C2(10)</td>
</tr>
</tbody>
</table>

Labels for rat groupings:
S3 - systemic group 3 - Dark Agouti rats
S4 - systemic group 4 - Lister Hooded rats
C2 - cannulated group 2 : Dark Agouti rats

**Table 2S.** Table showing animal groups used in both of the supplementary experiments. Label denotes rat group and the number of animals in each group is shown in brackets.
Figures

**Fig. 1S.** Effects of scopolamine on object recognition memory at 20min and 24h delays tested in the Y-maze and in Lister Hooded rats. **A,** Effect of systemic injection of scopolamine (0.05mg/kg, i.p.) in Dark Agouti rats prior to object recognition memory testing in the Y-maze. Scopolamine impaired memory after a 20min (A1) but not a 24h (A2) delay. **B,** Effect of systemic injection of scopolamine (2mg/kg, i.p.) in Lister Hooded rats prior to object recognition memory testing performed in a square arena. Scopolamine impaired memory after a 20min (B1) but not 24h (B2) delay. Asterisk: p<0.05.
Fig. 2S. Effect of intraperirolinal infusion of pirenzepine (0.7ng/ul) prior to object recognition memory testing. Pirenzepine impaired memory after a 20min (B1) but not a 24h (B2) delay. Asterisk: p<0.05.