M₁ and M₂ Muscarinic Receptor Subtypes Regulate Antidepressant-Like Effects of the Rapidly Acting Antidepressant Scopolamine

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Received May 21, 2014; accepted July 25, 2014

ABSTRACT
Scopolamine produces rapid and significant symptom improvement in patients with depression, and most notably in patients who do not respond to current antidepressant treatments. Scopolamine is a nonselective muscarinic acetylcholine receptor antagonist, and it is not known which one or more of the five receptor subtypes in the muscarinic family are mediating these therapeutic effects. We used the mouse forced-swim test, an antidepressant detecting assay, in wild-type and transgenic mice in which each muscarinic receptor subtype had been genetically deleted to define the relevant receptor subtypes. Only the M₁ and M₂ knockout (KO) mice had a blunted response to scopolamine in the forced-swim assay. In contrast, the effects of the tricyclic antidepressant imipramine were not significantly altered by gene deletion of any of the five muscarinic receptors. The muscarinic antagonists biperiden, pirenzepine, and VU0255035 ([N-[3-oxo-3-[4-(4-pyridinyl)-1-piperazinyl]propyl]-2,1,3-benzothiadiazole-4-sulfonamide] with selectivity for M₁ over M₂ receptors also demonstrated activity in the forced-swim test, which was attenuated in M₁ but not M₂ receptor KO mice. An antagonist with selectivity of M₂ over M₁ receptors (SCH226206 [(2-amino-3-methyl-phenyl)-[4-[4-[[4-(3 chlorophenyl)sulfonyl-phenyl]methyl]-1-piperidyl]-1-piperidyl][methyl]-1-piperidyl][methyl]-1-piperidyl[methyl]-1-piperidyl[methanone]) was also active in the forced-swim assay, and the effects were deleted in M₂−/− mice. Brain exposure and locomotor activity in the KO mice demonstrated that these behavioral effects of scopolamine are pharmacodynamic in nature. These data establish muscarinic M₁ and M₂ receptors as sufficient to generate behavioral effects consistent with an antidepressant phenotype and therefore as potential targets in the antidepressant effects of scopolamine.

Introduction
The treatment of major depressive disorder (MDD) has evolved dramatically over just a few decades from the use of amphetamines and opiates to the pervasive therapeutic use of multiple types of monoamine uptake inhibitors. Thus, the monoamine hypothesis of depression continues to provide a strong guide in our understanding of MDD and its therapeutic modulation (Iversen, 2005; Millan, 2009). Generally safe and efficacious, these antidepressants do not relieve all symptoms of depression, and they require several weeks of dosing for full treatment efficacy to emerge (Katz et al., 2004). Further, standard-of-care agents do not lead to remission in all patients; equally troubling, in another subset of MDD patients, the response to antidepressant treatment is minimal or absent altogether (Trivedi et al., 2006; Trivedi and Daly, 2008). Such treatment-resistant depression (TRD) leaves clinicians with a limited range of options; after all has been tried, there still remain about 30% of patients in whom no response to treatment, including electroconvulsive therapy, can be achieved.

Renewed hope for TRD patients has come from replication of earlier findings by Berman et al. (2000) that confirmed that the N-methyl-D-aspartate (NMDA) receptor channel blocker ketamine produced rapid improvement in the mood of depressed patients who had not otherwise responded to either standard-of-care antidepressant medications or, in some cases, electroconvulsive therapy (Zarate et al., 2006). This clinical finding has now been systematically replicated; multiple clinical reports (Zarate et al., 2013) confirm the NMDA blockade hypothesis of depression first posited by and Zarate et al., 2013). Rapid follow up to these clinical results has led to other NMDA receptor antagonists being put into clinical trials for MDD and TRD with initial positive results (Pilc et al., 2013; Zarate et al., 2013).

In the same year that the double-blind, placebo-controlled study of ketamine was reported, Furey and Drevets (2006)
published the results of a double-blind, placebo-controlled trial with another older drug, the antimuscarinic scopolamine. This work provided an elegant replication of earlier work from Janowsky’s laboratory (Gillin et al., 1991) reporting on the rapidly emerging antidepressant effects of scopolamine. In Furey and Drevets’ 2006 report, scopolamine produced a response after the first dose and generated a large effect size (2.2–3.4 as assessed by the Montgomery-Asberg Depression Rating Scale and the Hamilton Anxiety Rating Scale). Subsequently, this research group disclosed multiple reports of efficacy with scopolamine in MDD patients (see Jaffe et al., 2013; Zarate et al., 2013). Findings by Furey and Drevets (2006), like the work of Zarate and colleagues with ketamine, revived a path to mechanisms that might extend the range of pharmacologic interventions for TRD patients (Witkin, 2011). Importantly, these data suggested that a new generation of antidepressants might be engineered to provide rapid, large effect, and somewhat persistent efficacy for TRD patients.

Given the rapid and dramatic restitution of mood in MDD and TRD patients, an answer to the question of the mechanisms of action of ketamine and scopolamine might be key to identifying the biologic mechanisms against which improved medications can be targeted (Witkin, 2011). There have been advances since 2006 in our understanding of how NMDA receptor blockers might impact the biologic substrates of depression (Li et al., 2010; Autry et al., 2011; Ota and Duman 2013; Pilc et al., 2013). However, there has been little progress in understanding the mechanisms of action of scopolamine in depressed patients.

Scopolamine is a muscarinic receptor antagonist that acts at all multiple muscarinic receptor subtypes (M1–M5) with relatively equal potency. One major question addressed by our present study was to identify the receptor subtype(s) against which scopolamine might be engendering its fast-acting and large effect size. The approach we took was to make use of a combination of genetic and pharmacologic tools, including selective muscarinic antagonists and mice in which each muscarinic receptor subtype had been deleted. The forced-swim assay, which detects antidepressant effects in vivo (Cryan et al., 2002), was used along with these research tools. We found that mice without M1 or M2 receptors had a blunted response to scopolamine, but not to the nonmuscarinic antidepressant imipramine in the forced-swim assay. Additional studies confirmed these findings with compounds with improved M1 or M2 subtype selectivity. Combined with the appreciation of M2 receptor involvement in cardiovascular function (Krejci and Tucek, 2002), we have concluded that M1 receptors might be sufficient as a new protein target for the design of novel agents for TRD therapeutics.

Materials and Methods

Animals. Male CD1 mice (purchased from Taconic Farms, Germantown, NY) used in tail-suspension assays, weighing 24–35 g, were housed with a maximum of 6 mice/cage in plastic cages in a vivarium at least 7 days before the experiments. Male, wild-type (WT) C57BL/6 and muscarinic receptor M1–M5 null mice were generated as previously described for the M4 null mouse (Gomeza et al., 1999) and bred at Taconic Farms (Transgenic Models and Services Division); these mice were used in forced-swim studies. Age-matched C57BL/6 line was used as the WT comparator to the knockout (KO) mice as the mice had been previously bred to a congenic status using the C57BL/6 genetic background. Thus, all mice were bred homozygous × homozygous for this study.

Male Sprague-Dawley rats (175–200 g; Harlan Sprague-Dawley, Indianapolis, IN) were received 7 days before testing. They were housed three per cage and weighed about 225 g when they were ready to be tested. Water and rodent chow were available freely except during the test procedure. The vivarium was illuminated from 6:00 AM to 6:00 PM, and experiments were conducted between the hours of 12:00 Noon and 4:00 PM. Animals were removed from the vivarium to the testing area in their home cages and were allowed to adapt to the new environment for at least 1 hour before testing. All experiments were conducted according to the National Institutes of Health Guidelines for Care and Use of Laboratory Animals under protocols approved by the Institutional Animal Care and Use Committee. All animals were experimentally and drug naïve at the time of testing and were used for only one experiment.

Muscarinic Receptor Affinities and Selectivities. Affinities of the ligands studied in the present experiment for M1–M5 muscarinic receptors were evaluated in competition binding assays using radio-labeled N-methylscopolamine ([3H]NMS; 82 Ci/mmol; PerkinElmer Life and Analytical Sciences, Waltham, MA) and membranes from recombinant Chinese hamster ovary cells expressing one of the receptors (PerkinElmer). Briefly, compounds were serially diluted in dimethylsulfoxide (final concentration 1%) and added to 96-well plates (Corning Life Sciences, Tewksbury, MA) containing assay buffer: 20 mM NaH2PO4, 2 mM MgCl2, pH 7.4; 1 nM [3H]NMS, 3.6 U/ml of membrane, and 5 mg/ml of wheat germ agglutinin scintillation proximity assay beads (PerkinElmer). The final reaction volume was 250 μl per well. Nonspecific binding was determined using 50 μM of atropine.

Covered plates were shaken for 20–30 seconds and then incubated for at least 2 hours at room temperature. Radioactivity was quantified using a scintillation counter (Wallac MicroBeta/PerkinElmer, Gaithersburg, MD). Data were calculated as Ki values using a four-parameter logistic curve fitting program (ActivityBase v5.3.1.22; IBS, Alameda, CA). Note that human cloned M1–M5 receptors were used to define the affinities of ligands in the present study. The high structural homology between human and rodent muscarinic receptors (Bonner et al., 1987) enabled affinity comparisons with respect to brain exposure levels in rodents.

Brain and Plasma Drug Exposures. Scopolamine (1 mg/kg i.p.) and imipramine (15 mg/kg i.p.) were administered to WT and receptor KO mice, which were sacrificed by decapitation at 30 minutes after dosing. Plasma and whole brain were harvested and submitted for bioanalysis of drug levels. Other compounds studied were also evaluated for plasma and brain exposures in WT mice and were dosed as follows: SCH226206 ([2-amino-3-methyl-phenyl]-[4-[4-[4-[3-chlorophenyl]sulfonylphenyl]-methyl]-1-piperidyl]-1-piperidyl)methanone (30 mg/kg i.p.), VU0255035 ([N-[3-oxo-3-[4-(4-pyridinyl)-1-piperazinyl]propyl]-2,1,3-benzothiadiazole-4-sulfonamide) (60 mg/kg i.p.),pirezepine (3 and 10 mg/kg i.p.), biperiden (0.3 mg/kg i.p.), and AZD8055 (5-[2-bis[3(S)-3-methylmorpholin-4-yl]pyrido[2,3-d]pyrimidin-7-yl]-2-methoxyphenyl) methanol) (10 mg/kg p.o.). All compounds were dosed 30 minutes before decapitation except for AZD8055 (60 minutes).

Brain samples were weighed, and a 3-fold volume of water/methanol (4:1, v/v) was added before homogenization with an ultrasonic tissue disrupter. Control (naive) brain tissue was also homogenized to generate control homogenate for preparation of calibration standards. Stock solutions containing 1 mg/ml of each of the analytes were diluted to produce working solutions which were then used to fortify plasma samples for preparation of calibration standards with concentrations ranging from 1 to 5000 ng/ml. Aliquots of each study sample, calibration standard, and control sample were then transferred to 96-well plates, mixed with acetonitrile/methanol (1:1, v/v) containing an internal standard to precipitate sample proteins, and centrifuged to pellet insoluble material. The resulting supernatants were subjected to quantification by liquid chromatography with tandem mass spectrometry analysis using an Applied Biosystems/MSD Sciex.
API 4000 (Foster City, CA) equipped with a TurbolonSpray interface, and operated in positive ion mode. The analytes were chromatographically separated with a gradient liquid chromatography system and detected and quantified with selected reaction monitoring (M+H)^+ transitions specific to each compound: imipramine m/z 281.1 > 86.1; scopolamine m/z 304.2 > 138.2; SCJ226206, m/z 566.2 > 134.2; VU0255035 m/z 453.1 > 164.1; pirenzepine m/z 322.2 > 113.2; biperiden m/z 312.2 > 128.2; AZD8055 m/z 466.2 > 40.82.

The mass spectrometer quadrupoles were tuned to achieve unit resolution (0.7 Da at half height), and data were acquired and processed with Applied Biosystems/MDS Sciex Analyst software (version 1.4.2).

**Mouse Forced-Swim Test.** The forced-swim test was performed using the original method described by Porsolt et al. (1977). Briefly, mice were placed individually in clear plastic cylinders (diameter 10 cm; height 25 cm) filled with water (22–25°C). The duration of immobility was recorded during the first 4 minutes of a 6-minute trial during which the mouse was in the swim tank. A mouse was regarded by a trained observer as immobile when floating motionless or making only those movements necessary to keep its head above the water. Reliability of the scoring was established by independent blinded-observer correlations (r = 0.94, P < 0.05). Mice were used in this test only once and only at one dose level.

**Tail Suspension Test.** The tail suspension test was performed using a computerized device (mouse Tail Suspension Package, MED-SOF-820) developed by Med Associates Inc. (St. Albans, VT). Briefly, male CD1 mice (purchased from Charles River, Chicago, IL), weighing 24–35 g, housed 4 mice/cage, and left in the vivarium at least 7 days prior to testing, were suspended by the tail using adhesive scotch tape. The tape was then attached to a hook that connected to a strain gauge that picked up all the movements and transmitted them to a central unit that collected and analyzed the movement of each mouse for a 5-minute period. The time of immobility during the 5-minute period was determined after establishing a threshold level for each mouse that was set precisely at the activity level that could exclude all movements and only encompass immobility. Time below the threshold indicated immobility.

**Rat Forced-Swim Test.** Male Sprague-Dawley rats (250–275 g; Harlan Sprague-Dawley) were received 7 days before testing. They were housed four rats per cage. The animals weighed about 300 g when they were ready to be tested, and they were always brought to the testing room at least 1 hour before testing. The rats were placed in clear plastic cylinders (diameter 18 cm; height 40 cm) filled with water (22–25°C) to a depth of 16 cm for 15 minutes (the immobility of the first 5 minutes was recorded). A rat was regarded as immobile by a trained observer when floating motionless or making only those movements necessary to keep its head above the water. Reliability of the scoring was established by independent blinded-observer correlations (r = 0.92, P < 0.05). Rats were used in this test only once and only at one dose level.

**Locomotor Activity.** Mice were tested for locomotor activity in a circular maze with four pairs of photodetectors (RLC Products, Rockville, MD) for 5 minutes. Total beam breaks, clockwise rotations, and counterclockwise rotations were recorded. The duration of locomotion measured was within the same time frame used within the forced-swim test. Mice were used in this test only once and only at one dose level. After the test, mice were sacrificed, and their plasma and brain were collected and frozen at −80°C until samples were assayed for drug levels.

**Compounds.** The following compounds were used: scopolamine HBr, ( )-scopolamine methyl nitrate, and imipramine HCl (Sigma-Aldrich, St. Louis, MO); pirenzepine dihydrochloride (Boehringer-Ingelheim Pharmaceuticals, Ridgefield, CT); biperiden HCl (Knoll Pharmaceutical, Whippany, NJ); NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzof[quinoline-2,3-dione) (Tocris Bioscience, Ellisville, MO); and citalopram HBr, SCH226206, AZD8055, and VU0255035 (synthesized by Eli Lilly and Co.). All compounds except NBQX, SCH226206, AZD8055, and VU0255035 were prepared in water vehicle. NBQX was dissolved in water and titrated to solution with dilute NaOH. VU0255035 and AZD8055 were dissolved in 20% Captisol in phosphate-buffered saline buffer (pH 2.0–3.0). SCH226206 was suspended in hydroxyl-ethyl cellulose (1%), Tween 80 (0.25%), and Dow antifoul (0.05%). All compounds and vehicle were administered in a volume of 10 ml/kg, 30 minutes before testing, and were dosed as the forms listed. The route of administration for all drugs was intraperitoneal except for NBQX, which were administered subcutaneously.

**Data Analysis.** Dose-effect curves and comparisons of genetically modified mice were analyzed by analysis of variance followed by post-hoc Dunnett’s test. For studies evaluating drug synergy, a synergy analysis was conducted using the method of Bliss independence (Greco et al., 1995; Fitzgerald et al., 2006); using the end point of the percentage of inhibition, analysis of variance was applied to test the coefficient of the interaction term in a 2 × 2 full factorial model of the two compounds. Data analyses were conducted using GraphPad Prism software (v.6; GraphPad Software, San Diego, CA) and with SAS/JMP software (v.9; SAS Institute, Cary, NC). P < 0.05 was considered statistically significant.

**Results**

**Rat Forced-Swim Assay.** In depressed patients, scopolamine was administered by intravenous infusion (c.f., Furey and Drevets, 2006). In rats, intravenous doses of scopolamine HBr produced a dose-dependent decrease in immobility time in the forced-swim assay with a minimal effective dose of 0.03 mg/kg and a maximal effect of 66% at 1 mg/kg (Fig. 1).

**Mouse Forced-Swim Assay.** In WT C57BL/6 mice, both scopolamine and imipramine produced dose-dependent decreases in immobility time in the forced-swim assay (Fig. 2). In contrast, methyl-scopolamine, an analog with markedly reduced brain penetrability, was without effect when given up to 3 mg/kg, a dose 3 times higher than the minimal effective dose for scopolamine (Fig. 2). In contrast to these findings obtained when scopolamine was dosed 0.5 hours before testing, scopolamine was not active at 24 hours after dosing; doses of scopolamine of 0.1–1.0 mg/kg produced no significant decrease in immobility times (vehicle: 218 ± 10; 0.1 mg/kg: 207 ± 14; 0.3 mg/kg: 213 ± 12; 1 mg/kg: 212 ± 14 seconds). Although the...
Effects of scopolamine in this assay were dose dependent in WT mice and did not differ significantly in the M3, M4, and M5 receptor−/− mice compared with the WT mice. In these mouse strains, the minimal effective dose for engendering antidepressant-like activity was 1 mg/kg i.p. (Fig. 3). In contrast, M1 and M2 receptor null mice did not display significant effects of scopolamine up to 3 mg/kg. The tricyclic antidepressant imipramine also had antidepressant-like effects in this assay, but these effects were not significantly altered in any of the M1−M5−/− mouse strains (Fig. 3).

**Muscarinic Receptor Affinities and Selectivities.** The affinities of the muscarinic antagonists studied are shown in Table 1. Scopolamine, as reported by others, nonselectively interacts with all five muscarinic subtypes. Likewise, biperiden interacts with M1−M2 receptor subtypes but with lower affinity for the M3 binding sites. The compounds pirenzepine and VU0255035 are relatively selective for M1 relative to M2−M5 receptor subtypes and, as with biperiden, show a relatively high selectivity for M1 versus M2 receptors. In contrast, SCH228206 is selective for M2 receptors as previously reported (Wang et al., 2002). Selective molecules for M1 and M2 receptors were used subsequently to further substantiate a role for M1 and M2 receptors in the antidepressant-like effects of scopolamine.

**Brain and Plasma Exposures.** To determine whether any differences in the behavioral effects of scopolamine or imipramine across mouse strains depended on drug exposure differences, we determined the plasma and whole brain levels of the drug at the time at which behavioral testing was conducted. Both scopolamine and imipramine produced high plasma and brain levels in WT C57BL/6 mice and in each of the M1−M5−/− mouse strains (Table 2). The concentrations of scopolamine in brain were above the Kᵢ values for all muscarinic receptor subtypes (Table 1). However, for either scopolamine or imipramine, the drug levels in either body compartment in general did not differ remarkably across mouse strains. The M2−/− mice had substantially lower plasma and brain imipramine but not scopolamine levels compared with their WT controls. M1 receptor−/− mice displayed higher levels of scopolamine in plasma (∼2-fold; P < 0.05) and showed a trend toward higher brain levels when compared with WT control mice. Although there was a tendency for decreases in plasma levels of scopolamine to be reduced in M2 and M5−/− mice, these effects did not reach statistical significance.

**Locomotor Activity.** Assessment of the locomotor activity in WT and muscarinic receptor−/− mice was made to determine whether differences in behavioral effects across strains and across drugs might be influenced by intrinsic differences in locomotion. These studies revealed no differences across mouse

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**TABLE 1**

<table>
<thead>
<tr>
<th>Compound</th>
<th>hM1 (S.E.M.)</th>
<th>hM2 (S.E.M.)</th>
<th>hM3 (S.E.M.)</th>
<th>hM4 (S.E.M.)</th>
<th>hM5 (S.E.M.)</th>
<th>M2/M1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scopolamine</td>
<td>0.83 (0.05)</td>
<td>5.3 (1.4)</td>
<td>0.34 (0.96)</td>
<td>0.38 (0.07)</td>
<td>0.34 (0.11)</td>
<td>6.4</td>
</tr>
<tr>
<td>Pirenzepine</td>
<td>2.2 (0.23)</td>
<td>102 (24)</td>
<td>5.3 (1.3)</td>
<td>3.1 (0.8)</td>
<td>4.4 (1.4)</td>
<td>46</td>
</tr>
<tr>
<td>VU0255035</td>
<td>43 (14)</td>
<td>4200 (1370)</td>
<td>468 (172)</td>
<td>148 (53)</td>
<td>237 (122)</td>
<td>98</td>
</tr>
<tr>
<td>SCH228206</td>
<td>1240 (255)</td>
<td>30 (11)</td>
<td>529 (194)</td>
<td>76 (20)</td>
<td>128 (14)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Potencies of the antimuscarinic ligands used in the present study assessed against human cloned muscarinic receptor subtypes

Human cloned receptors were expressed in Chinese hamster ovary cell membranes. Binding was measured as competition with [3H]N-methyl-scopolamine. Data represent the mean of three to six experiments ± S.E.M. expressed as Kᵢ in nanomolar.
Selective Muscarinic Receptor Antagonists. Additional molecules were studied in WT and muscarinic receptor null mice for two reasons. Because the antidepressant-like effects of scopolamine were not observed in the M1 or the M2 receptor KO mice, both or either of these protein targets could be responsible for the anti-immobility effects of scopolamine. Therefore, compounds with increased selectivity of M1 over M2 receptors were evaluated. Additionally, we felt it important to add to the structural diversity and subtype selectivity profile of compounds studied in WT and in muscarinic receptor null mice. In comparison with scopolamine (6-fold selective), biperiden was 46-fold and pirenzepine was 98-fold selective for M1 over M2 receptors (Table 1). Biperiden and pirenzepine produced antidepressant-like signatures (Fig. 5) similar to that of scopolamine (Fig. 1) when tested in WT mice. Like scopolamine, the effects of biperiden and pirenzepine were significantly attenuated when studied in M1 KO mice (Fig. 5). Because M2 receptors were also identified as a potential target for the effects of scopolamine (Fig. 3) and because biperiden has affinity (100 nM) for M2 receptors and achieves brain levels above the concentrations that was completely prevented in M2 KO mice (Fig. 5).

Imipramine was dosed as the HCl at 15 mg/kg i.p., 30 minutes before testing. Scopolamine was dosed as the HBr at 1 mg/kg i.p., 30 minutes before testing. Values are mean ± S.E.M. of responses from at least eight mice. *P < 0.05 by post-hoc Dunnett's test compared with vehicle (veh) control.

### Table 2

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Plasma</th>
<th>Brain</th>
<th>Plasma</th>
<th>Brain</th>
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<tr>
<td></td>
<td>nM</td>
<td></td>
<td>nM</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>1496 ± 139.0</td>
<td>21,333 ± 3013</td>
<td>33.3 ± 7.9</td>
<td>185.7 ± 18.0</td>
</tr>
<tr>
<td>M1</td>
<td>1023 ± 192.6</td>
<td>14,202 ± 9408</td>
<td>70.6 ± 2.1*</td>
<td>284.3 ± 50.0</td>
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<tr>
<td>M2</td>
<td>662.7 ± 113.6*</td>
<td>8407 ± 3080*</td>
<td>19.4 ± 12.9</td>
<td>155.1 ± 13.5</td>
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<tr>
<td>M3</td>
<td>1060 ± 198.8</td>
<td>18,638 ± 3803</td>
<td>7.65 ± 2.9*</td>
<td>120.6 ± 24.7</td>
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<tr>
<td>M4</td>
<td>1190 ± 583.8</td>
<td>20,513 ± 3475</td>
<td>30.3 ± 11.8</td>
<td>195.7 ± 38.3</td>
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<tr>
<td>M5</td>
<td>817.4 ± 101.0</td>
<td>15,366 ± 2181</td>
<td>8.3 ± 0.88</td>
<td>156.8 ± 7.6</td>
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</table>

*P < 0.05 by analysis of variance followed by Dunnett’s test compared with WT.
activity. As with the locomotor activity data with scopolamine, the compounds were dosed as in the forced-swim assay and tested at 30 minutes after dosing for 5 minutes, the time after dosing they were tested for antidepressant-related activity, and for the approximate duration of the latter behavioral evaluation. No statistically significant alterations in locomotion were observed: SCH226206 (30 mg/kg i.p.), VU0255035 (60 mg/kg i.p.), pirenzepine (mg/kg i.p.), and biperiden (0.3 mg/kg i.p.) showed nonsignificant alterations of vehicle-control levels of locomotion of 86.3, 82.2, 67.8, and 76.8% of control, respectively (*P < 0.05).

Tail-Suspension Test. To determine whether an assay other than the forced-swim assay would detect effects of scopolamine, we performed tail-suspension studies. In the tail-suspension test, CD1 mice that we tested have been relatively resistant to the effects of standard-of-care antidepressant agents (e.g., imipramine, fluoxetine, bupropion) and yet are responsive to the TRD agent ketamine (Witkin et al., 2011). We made use of this test to establish whether scopolamine would produce ketamine-like efficacy after acute dosing. Scopolamine produced clear and significant efficacy in this test (Fig. 7). In contrast, the selective serotonin reuptake inhibitor (SSRI) citalopram, used as a negative control, was not active in these mice even at a dose as high as 10 mg/kg (Fig. 7) which achieves significant effects in the forced-swim assay.

Discussion

The adrenergic–cholinergic balance hypothesis of depression proposed by Janowsky et al. (1972) was based primarily on findings that cholinesterase inhibitors could mimic symptoms of depression and reverse manic symptomatology (Dilsaver, 1986; Janowsky et al., 1972, 1983). The pivotal data of Furey and Drevets (2006) provided the remarkable proof of concept for this hypothesis. Clinical findings have been replicated and extended to other antimuscarinics and treatment populations (Howland, 2009; Dagytė et al., 2011; Janowsky, 2011; Drevets et al., 2013; Jaffe et al., 2013). It is noteworthy that amitriptyline, a tricyclic antidepressant with greater efficacy than SSRIs (Anderson, 1998; Deisenhammer et al., 2000), has one of the richest of antimuscarinic profiles (Rathbun and Slater, 1963). Given the reenergizing of both the cholinergic hypothesis of MDD and the pressing need for improved antidepressant agents, we undertook the present series of studies.

The forced-swim assay was brought forward as a model of behavioral despair (Porsolt et al., 1977). Not a model of depression per se, it detects standard-of-care antidepressants with diverse chemical structures, pharmacologic classes, and different mechanisms of action (Porsolt et al., 1977; Willner, 1984). Indeed, the forced swim is the only assay used to detect antidepressant agents that has a significant correlation between anti-immobility potencies and clinical dosage (Willner, 1984). Interestingly, since their inception, anticholinergics were considered false negatives in this assay (Willner, 1984; Borsini and Meli, 1988). We used this assay in conjunction with M1–M5 receptor-deficient mice and

Fig. 4. Locomotor activity of WT and M1 to M5 receptor KO mice. Locomotion was accessed for 5 minutes at 30 minutes after dosing, comparable to mice studied in the forced-swim assay (6 minutes; 30 minutes before testing). Each bar represents the mean ± S.E.M. of responses from 6–12 C57BL/6 WT or muscarinic receptor KO mice. Statistical comparisons shown are for vehicle versus drug separately for each KO strain. *P < 0.05 by post-hoc Dunnett’s test compared with comparable genotype given vehicle.

Fig. 5. Production of an antidepressant-related behavioral response by the M1 selective antagonists biperiden and pirenzepine and the M2 selective antagonist SCH226206 and their modification by genetic deletion. (A) Effects of both biperiden (●) and pirenzepine (■) observed in WT C57BL/6 mice were significantly attenuated in M1 receptor−/− mice (open symbols). (B) Effects of SCH226206 in WT mice were significantly attenuated in M2 receptor−/− mice (open symbol at 10 mg/kg). Imipramine (imi) (15 mg/kg i.p.) was studied as a positive control in WT mice. Compounds were given intraperitoneally 30 minutes before testing. Each point represents the mean ± S.E.M. of responses from 8 mice. *P < 0.05 by post-hoc Dunnett’s test compared with vehicle (veh) control values for each dose response curve.
pharmacologic tools to discriminate the muscarinic receptor subtype(s) responsible for the antidepressant effects of scopolamine in humans.

Using M_{1}\text{--}M_{5} receptor^{-/-} mice, we found that the anti-immobility efficacy of scopolamine was not observed in M_{1} and M_{2}^{-/-} mice. Analysis of scopolamine levels in the plasma and brain of WT and muscarinic receptor KO mice documented that changes in pharmacokinetics could not account for the behavioral effects of scopolamine. Neither were basal motoric levels. Significant basal level locomotor disturbances have only been observed in the M_{1} and M_{4} null mice in previous studies. The M_{1}^{-/-} mice are modestly spontaneously hyperactive (Miyakawa et al., 2001), a result not observed in our studies. M_{4}^{-/-} mice are also modestly spontaneously hyperactive with even greater increases in hyperlocomotion observed in the presence of a D_{1} agonist (Gomeza et al., 1999). In our study, scopolamine, at 1 mg/kg i.p., produced small but significant increases in locomotor activity in only the M_{4} and M_{2}^{-/-} mice. However, activity was not enhanced by scopolamine in the M_{2}^{-/-} mice, who retained the antidepressant phenotype with scopolamine in humans.

The M_{2} receptor selective antagonist SCH226206 demonstrated M_{2} receptor–mediated effects in our study. Although both M_{1} and M_{2} receptors appear necessary for the anti-immobility effects of scopolamine, independent M_{1} or M_{2} receptor blockade appears to be sufficient.

Like scopolamine, ketamine has also been shown to have a rapid onset, large effect size in MDD and TRD patients (Furey and Drevets, 2006; Zarate et al., 2006). Convergent data point to a cascade of events after ketamine or scopolamine administration that follows the course set by glutamate-induced amplification of neuronal signaling. Increases in extracellular glutamate levels have been observed after both ketamine and scopolamine administration (Voleti et al., 2013). The impact of glutamate upon \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and enhanced excitatory current through AMPA receptors is thought to be a primary driver of antidepressant efficacy (Alt et al., 2006). These findings are also consistent with previous reports on the AMPA-sensitive locomotor-stimulant (Willins et al., 1992) and cognitive-disrupting effects (Malá et al., 2012) of scopolamine. AMPA receptor/M_{4} receptor interactions have also been documented from studies of long-term depression where activation of M_{1} receptors engenders AMPA receptor endocytosis (Dickinson et al., 2009).

This study of the antidepressant effects of scopolamine does have some limitations. First, only one antidepressant-relevant assay was employed. Second, the doses required to produce efficacy were higher than previously reported by Voleti et al. (2013). It is important to note that the brain exposures of scopolamine in our study were higher than the \(K_i\) values for all access to the central nervous system upon systemic dosing, higher doses have proven to gain functional access central muscarinic receptors (Witkin et al., 1988) (Table 3). Indeed, pirenzepine administered directly into the nucleus accumbens was shown to engender an antidepressant-like phenotype in rats in studies by Hoebel’s group (Chau et al., 1999, 2011) as did the analog telenzepine (Voleti et al., 2013). Behavioral effects of pirenzepine, like those of scopolamine, were absent in M_{1}^{-/-} receptor mice in this study. The exposure of VU0255035 was not greatly above its \(K_i\) value and might account for its modest behavioral effects reported here. Importantly, it was shown that the effects of biperiden, significantly and fully attenuated in M_{1}^{-/-} mice, were not markedly affected in the M_{2} receptor KO mouse. The M_{2} receptor selective antagonist SCH226206 demonstrated M_{2} receptor–mediated effects in our study. Although both M_{1} and M_{2} receptors appear necessary for the anti-immobility effects of scopolamine, independent M_{1} or M_{2} receptor blockade appears to be sufficient.

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TABLE 3
Plasma and brain concentrations of other ligands studied
All compounds were given intraperitoneally 30 minutes before. Values are mean ± S.E.M. of three mice.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose Plasma</th>
<th>nM</th>
<th>Brain</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCH226206</td>
<td>30</td>
<td>3340 ± 767</td>
<td>1863 ± 441</td>
</tr>
<tr>
<td>VU0255035</td>
<td>3</td>
<td>699.9 ± 73.2</td>
<td>52.1 ± 0.47</td>
</tr>
<tr>
<td>Pirenzepine</td>
<td>10</td>
<td>1639 ± 109.1</td>
<td>144.6 ± 14.6</td>
</tr>
<tr>
<td>Biperiden</td>
<td>0.3</td>
<td>3.56 ± 0.2</td>
<td>379.1 ± 5.5</td>
</tr>
</tbody>
</table>

Fig. 6. Anti-immobility efficacy of the selective M_{1} receptor antagonist VU0255035 in rats dosed intravenously and mice dosed intraperitoneally. VU0255035 was tested 30 minutes after dosing in the forced-swim assay. Imipramine (imi) was studied as a positive control (30 mg/kg i.p. for rats; 15 mg/kg i.p. for mice). Each point represents the mean ± S.E.M. of responses from six to seven male Sprague-Dawley rats (A) or eight WT C57BL/6 mice (B). *P < 0.05 by post-hoc Dunnett’s test compared with vehicle (veh) control values for each dose response curve.
muscarinic receptor subtypes. Finally, we were unable to study the effects of scopolamine at 24 hours after dosing (Voleti et al., 2013) as we did not achieve any significant behavioral changes. However, other literature values for both rat (Mancinelli et al., 1988) and mouse (Su et al., 2013) forced swims are consistent with the doses (1 mg/kg and higher) and time parameters (30 minutes after dosing) we used in this study. Scopolamine was also active in the tail-suspension assay, an assay we found to be relatively insensitive to SSRI mechanism-based antidepressants (Withkin et al., 2011). However, it is important to comment that variations in this assay can achieve efficacy with SSRI agents (Vaugeois et al., 1997).

Taken as a whole, we argue that these data using forced swimming provide an appropriate metric to elucidate the muscarinic receptor(s) contributing to these anti-immobility effects. However, further experimentation with these genetically altered mouse strains in a broader range of behavioral, neurochemical, and electrophysiological studies is warranted to refine the present conclusions and enable a better translation to clinical data. Specifically, other measures of antidepressant-related biologic activity at multiple levels will be critical to increasing translational understanding of these findings into a potential treatment for depressed patients. Until we have such data in hand, it thus remains possible that other aspects of the antidepressant-related efficacy of scopolamine might be controlled by receptor subtypes distinct from those uncovered here.

Nevertheless, additional evidence that supports our current research regarding the role of muscarinic receptor subtypes in antidepressant-like behavioral exists. Using radiolabels for M₁–M₄, Gibbons et al. (2009) evaluated receptor density changes in the postmortem frontal cortex of patients with MDD or bipolar disorder (BD). Both M₂ and M₃ binding sites were downregulated in MDD but only M₂ in BD. M₁ and M₃ RNA are downregulated after electroconvulsive therapy in rats (Mingo et al., 1998). M₂ receptor binding was decreased in the anterior cingulate cortex of patients with BD compared with MDD and healthy controls (Cannon et al., 2006). An involvement of M₄ receptors in MDD is also suggested from its potential role in regulating glutamatergic transmission via the hippocampus to the medial prefrontal cortex (Wang and Yuan, 2009). However, evidence also exists for control by M₁ receptors. For example, Huang and Hsu (2010) showed that M₁-triggered long-term depression involves presynaptic nitric oxide–dependent signaling.

In rodent studies, swim stress induced long-lasting increases in acetylcholine efflux in the nucleus accumbens. The perikarya of these neurons are densely localized in the shell of this nucleus (Meredith et al., 1989) and form excitatory synapses via M₃ receptors on dentrites and cell bodies of medium spiny GABA output neurons (Phelps and Vaughn, 1986). Corresponding to the increases in acetylcholine, decreases in M₁ receptor and receptor gene expression have been observed (Rada et al., 2006). Local injection of pirenzepine into the shell of the nucleus accumbens also has antidepressant-like effects in rats (Chau et al., 1999). In addition, acetylcholine efflux in this brain area has been implicated in the antidepressant effects of fluoxetine (Chau et al., 1999, 2011). Taken together, these data implicate this dopamine projection area in the M₁ receptor regulation of mood and the behavioral symptoms of despair.

In conclusion, using combined studies with transgenic mice and pharmacologic tools, we have identified both M₁ and M₂ receptors in the antidepressant-like effects of scopolamine. As blockade of M₂ receptors has implications for cardiac side effects, these data establish the muscarinic M₁ receptor as a potential protein target for the discovery of novel rapidly acting antidepressants. The overall goal of such work would be to uncover a molecule with a reduced side-effect profile but comparable efficacy to that of scopolamine or ketamine in use in TRD patients.

Acknowledgments

The authors thank Ron Duman for prior comments on an earlier version of this manuscript and for helpful discussion of the data. The authors dedicate this paper to their friend and colleague, Conchi Pedregal, who died too early. Her contributions to the treatment of depression and other central nervous system disorders will be remembered. Her whole-hearted dedication to the alleviation of suffering and to helping those in need will always be with them.

Authorship Contributions

- **Participated in research design:** Witkin, Overshiner, Li, Catlow, Wishart, Schober, Nikolayev, Tolstikov, Kuo, Felder.
- **Conducted experiments:** Witkin, Overshiner, Li, Catlow, Wishart, Tolstikov, Nikolayev.
- **Performed data analysis:** Witkin, Overshiner, Li, Catlow, Wishart, Nikolayev, Tolstikov, Anderson, Higgs.
- **Wrote or contributed to the writing of the manuscript:** Witkin, Overshiner, Li, Catlow, Wishart, Schober, Nikolayev, Tolstikov, Anderson, Higgs, Kuo, Felder.

References


