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

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Running Title:

M₁ and M₂ Muscarinic Receptor Subtypes

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Document Statistics:

Abstract: 236
Introduction: 749
Discussion: 1497
References: 76
Figures: 7
Tables: 3

Non-Standard Abbreviations:

AMPA: α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; NMDA: N-methyl-D-aspartate; SCH226206: 2-amino-3-methyl-phenyl-[4-[4-[[4-(3 chlorophenyl)sulfonylphenyl]methyl]-1-piperidyl]-1-piperidyl]methanone; TRD: treatment-resistant depression; VU0255035: N-[3-oxo-3-[4-(4-pyridinyl)-1-piper azinyl]propyl]-2,1,3-benzothiadiazole-4-sulfonamide
Abstract

Scopolamine produces rapid and significant symptom improvement in patients with depression, and most notably in patients that do not respond to current antidepressant treatments. Scopolamine is a non-selective muscarinic acetylcholine receptor antagonist and it is not known which one or more of the 5 receptor subtypes in the muscarinic family are mediating these therapeutic effects. We utilized 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 in order to define the receptor subtypes are relevant. Only the M₁ and M₂ knockout 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 5 muscarinic receptors. Muscarinic antagonists, biperiden, pirenzepine, and VU0255035 with selectivity for M₁ over M₂ receptors, also demonstrated activity in the forced-swim test which was attenuated in M₁ but not M₂ receptor knockout mice. An antagonist with selectivity of M₂ over M₁ receptors (SCH226206) was also active in the forced-swim assay and effects were deleted in M₂⁻/⁻ mice. Brain exposure and locomotor activity in the knockout 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 over our understanding of MDD and its therapeutic moderation (Iversen, 2005; Millan, 2009). Generally safe and efficacious, these antidepressants don’t relieve all symptoms of depression and 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 and equally troubling, in another subset of MDD patients, 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 treatment options but after all is tried, there still remain about 30% of patients for which response to any treatment including electroconvulsive therapy (ECT) is not achieved.

A renewed hope for TRD patients was generated when a replication of earlier findings by Berman et al (2000) confirmed that the NMDA receptor channel blocker, ketamine, produced rapid improvement in mood in depressed patients that had not otherwise responded to either standard of care antidepressant medications or in some cases to ECT (Zarate et al., 2006). This clinical finding has now been systematically replicated and disclosed in the literature in multiple clinical reports (Zarate et al., 2013) and confirms the NMDA blockade hypothesis of depression first posited by and Trullas and Skolnick over twenty years ago (Trullas and Skolnick, 1990; Pilc 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 disclosed (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 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 lab (Gillin et al., 1991) reporting rapidly-emerging antidepressant effects of scopolamine. In their 2006 report, scopolamine produced a response after the first dose and generated a large effect size (2.2-3.4 as assessed by the MADRS and the HARS). 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 pharmacological interventions for TRD patients (Witkin, 2011). Importantly, these data suggested that new generation 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 a key to finding biological mechanisms against which improved medications can be discovered (Witkin, 2011). There have been advances since 2006 in our understanding of how NMDA receptor blockers might impact the biological substrates underlying depression (Li et al., 2010; Autry et al., 2011; Ota and Duman 2012; 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 the 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 pharmacological tools including selective muscarinic antagonists and mice in which each muscarinic receptor subtype had been deleted. The forced-swim assay that detects antidepressant effects in vivo (Cryan et al., 2002) was utilized along with these research tools.

We found that mice without M₁ receptors or without M₂ receptors had a blunted response to scopolamine but not to the non-muscarinic antidepressant imipramine in the forced-swim assay. Additional studies confirmed these findings with compounds with improved M₁ or M₂ subtype selectivity and documented overlap in the pharmacological actions of scopolamine and ketamine. Combined with the appreciation of M₂ receptor involvement in cardiovascular function (Krejci and Tucek, 2002), it is 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, German Town, NY), used in tail-suspension assays, weighing 24-35g were housed in plastic cages with a maximum of 6 mice/cage in a vivarium at least 7 days before the experiments. Male, wild-type (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, Germantown, NY); these mice were used in forced-swim studies. Age-matched C57BL/6 line were used as the wild-type comparator to the KO mice since the mice had been previously bred to a congenic status using the C57BL/6 genetic background. Thus, all mice were bred homozygous x homozygous for this study.

Male, Sprague-Dawley rats (175-200g, from Harlan Sprague-Dawley) were received 7 days prior to testing. They were housed 3 per cage and weighed about 225g when they were ready to be tested. Water and rodent chow were available freely except during the test procedure. The vivarium was illuminated from 06:00 to 18:00 and experiments were conducted between the hours of 12:00 and 16:00. Animals were removed from the vivarium to the testing area in their home cages and allowed to adapt to the new environment for at least one hour before testing. All experiments were conducted according to the NIH 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 radiolabeled N-methylscopolamine ([3H]-NMS, 82 Ci/mmol; Perkin-Elmer; Waltham, MA) and membranes from recombinant CHO cells expressing one of the receptors (Perkin-Elmer). Briefly, compounds were serially diluted in DMSO (final concn, 1%) and added to 96-well plates (Corning) containing assay buffer (20 mM NaH2PO4, 2 mM MgCl2, pH 7.4), 1nM 3H-NMS, 3.6 U/ml of membrane, and 5 mg/ml of WGA SPA beads (Perkin Elmer). Final reaction volume was 250 µl per well. Non-specific binding as determined using 50 µM of atropine. Covered plates were shaken for 20-30 sec and then incubated for at least 2 hrs at room temp. Radioactivity was quantified using a scintillation counter (Wallac Microbeta). Data were calculated as Ki values using a four-parameter logistic curve fitting program (ActivityBase v5.3.1.22).

Note: human cloned M1-M5 receptors were utilized 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 wild-type and receptor knockout mice and sacrificed by decapitation at 30 min post dosing, and plasma and whole brain was harvested and submitted for bioanalysis of drug levels. Other compounds studied were also evaluated for plasma and brain exposures in wild-type mice and were dosed as follows: SCH226206 (30 mg/kg, i.p.), VU0255035 (60 mg/kg, i.p.), pirenzepine (3 and
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10 mg/kg, i.p.), biperiden (0.3 mg/kg, i.p.), and AZD8055 (10 mg/kg, p.o.). All compounds were dosed 30 min prior to decapitation except AZD8055 (60 min). Brain samples were weighed and a 3-fold volume of water/methanol (4:1, v/v) was added prior to homogenization with an ultrasonic tissue disrupter. Control (naïve) 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 control plasma or control brain homogenate to produce calibration standards with concentrations ranging from 1 to 5000 ng/mL. Aliquots of each study sample, calibration standard, and control samples 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 LC-MS/MS analysis using an Applied Biosystems/MDS Sciex API 4000 (Foster City, CA) equipped with a TurboIonSpray interface, and operated in positive ion mode. The analytes were chromatographically separated with a gradient LC system and detected and quantified with Selected Reaction Monitoring (SRM) (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 433.1>164.1; Pirenzepine m/z 352.2>113.2; Biperiden m/z 312.2>128.2; AZD8055 m/z 466.2> 40.82 ).

The mass spectrometer quadrapoles were tuned to achieve unit resolution (0.7 DA at 50% FWHM) and data were acquired and processed with Applied Biosystems/MDS Sciex Analyst software (version 1.4.2).
**Mouse forced-swim test.** This test was performed using the original method described by Porsolt (Porsolt et al. 1977). Briefly, mice were placed individually in clear plastic cylinders (diameter 10 cm; height: 25 cm) filled 6 cm high with water (22-25 °C). The duration of immobility was recorded during the last 4 min of a six-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.

**Rat forced-swim test.** Male Sprague-Dawley rats (250-275g, from Harlan Sprague-Dawley) were received 7 days prior to testing. They were housed 4 rats/cage. Animals weigh about 300g when they are ready to be tested. Animals were always brought to the testing room at least 1 hr prior to testing. 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 min (the immobility of the first 5 min 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 4 pairs of photo-detectors (RLC Products, Rockville, MD) for 5 min. Total beam breaks, clockwise rotations, and counterclockwise rotations were recorded. The duration of locomotion measured was within the same time frame utilized 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 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 Chemical Co., St. Louis, MO), pirenzipine dihydrochloride (Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT), biperiden HCl (Knoll Pharmaceutical Co., Whippany, NJ), NBQX (Tocris Bioscience, Ellisville, MO), citalopram HBr, SCH226206, AZD8055, and VU0255035 (synthesized by Eli Lilly and Co.). All compounds except NBQX, SCH226206, AZD8055, and VU0255035 were prepared in water vehicles. NBQX was dissolved in water and titrated to solution with dilute NaHO. VU0255035 and AZD8055 were dissolved in 20% captisol in PBS buffer (pH=2-3). SCH226206 was suspended in hydroxyl-ethyl cellulose (1%), Tween 80 (0.25%), and Dow antifoam (0.05%). All compounds and vehicle were administered in a volume of 10 ml/kg, 30 min prior to testing and were dosed as the forms listed. The route of administration for all drugs was i.p. except for NBQX that was given s.c.

**Data analysis.** Dose-effect curves and comparisons of genetically-modified mice were analyzed by ANOVA followed by post-hoc Dunnett’s tests. 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 endpoint of % inhibition, an ANOVA was applied to test the coefficient of the interaction term in a 2x2 full factorial model of the two compounds. Data analyses were conducted using Graphpad Prism software (v. 6) and with SAS/JMP software (v. 9). Results were considered significantly
different than comparison standards when p < 0.05.
Results

**Rat forced-swim assay.** In depressed patients, scopolamine was given 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 wild-type (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 hr prior to testing, scopolamine was not active at 24 hr post dosing; doses of scopolamine of 0.1-1 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 sec). Although the brain concentration of scopolamine is above the \( K_i \) for \( M_1-M_5 \) receptors at 1 mg/kg (Tables 1 and 2), we extended the doses of scopolamine to 10 mg/kg to inquire if an effect in the forced-swim assay could be detected at 24 hr post dosing. In this study, scopolamine produced decreases in immobility at 24 hr post dosing of 3.8% (at 1 mg/kg), 7.0% (at 3 mg/kg), and 10.7% (at 10 mg/kg); none of these effects were statistically different than vehicle control levels.

We made further attempts to lower the effective dose of scopolamine by systematic replication of the work of Voleti et al. (2013). In these studies we increased immobility time under basal conditions by conducting two forced-swim sessions (196 ±
11 vs 222 ± 12 under the two day method) in which scopolamine was dosed after the swim session on day one and before the swim session on day 2. Scopolamine at 0.03-0.3 mg/kg was studied under these conditions. As with the one session forced-swim method, wild-type mice did not show significant decreases in immobility at doses below 1 mg/kg scopolamine (213 ± 10; 221 ± 13; 211 ± 11 after 0.03, 0.1, and 0.3 mg/kg scopolamine, respectively).

The basal levels of immobility of WT and the M1-M5 -/- mice differed significantly from one another (F5,47 = 25, p < 0.0001). This effect of genotype was accounted for by significant differences between WT and M1 and WT and M3 mice (p <0.01) (Fig. 3). Effects of scopolamine in this assay were dose-dependent in WT and did not differ significantly from WT mice in the M3, M4, and M5 receptor -/- 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, non-selectively interacts with all five muscarinic subtypes. Likewise, biperiden interacts with M1-M3 receptor subtypes but with lower affinity for the M2 binding sites. The other 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 vs. M2 receptors. In contrast, SCH226206 is selective for M2 receptors as previously
reported (Wang et al., 2002). Selective molecules for M1 and M2 receptors were utilized subsequently to further substantiate a role for M1 and M2 receptors in the antidepressant-like effects of scopolamine (below).

**Brain and plasma exposures.** In order to determine whether any differences in the behavioral effects of scopolamine or imipramine across mouse strains was dependent upon drug exposure differences, the plasma and whole brain levels of drug were determined 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 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 to their WT controls. M1 receptor -/- mice displayed higher levels of scopolamine in the plasma (~2-fold; p < 0.05) and showed a trend toward higher brain levels when compared to 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 of WT and muscarinic receptor -/- mice was made to determine if differences in behavioral effects across strains and across drugs might be influenced by intrinsic differences in locomotion. These studies revealed no differences across mouse strains when drug vehicle was administered (Fig. 4). However, the M4 and M5 -/- mice also showed a significantly greater increase in
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locomotion when dosed with 1 mg/kg scopolamine than WT controls (Fig. 4). Statistical comparisons shown are for Vehicle vs. Drug separately for each KO strain.

**Selective muscarinic receptor antagonists.** Additional molecules were studied in WT and muscarinic receptor null mice for two reasons. Since the antidepressant-like effects of scopolamine were not observed in the M1 or the M2 receptor -/- 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 -/- mice.

In comparison to 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) like that of scopolamine (Fig. 1) when tested in WT mice. Like scopolamine, effects of biperiden and pirenzepine were significantly attenuated when studied in M1 -/- mice (Fig. 5). Since M2 receptors were also identified as a potential target for the effects of scopolamine (Fig. 3) and since biperiden has affinity (100 nM) for M2 receptors and achieves brain levels above the Ki for M2 receptors (Table 3), we evaluated the contribution of this drug in WT vs. M2 receptor KO mice to evaluate the contribution of M2 receptors. In this study, 0.3 mg/kg biperiden produced a 68% decrease in immobility in WT mice (p < 0.05) but did not significantly alter immobility time at 0.3 mg/kg (11.3% decrease) but was able to achieve a small but significant decrease at 1 log-dose higher (26.6% decrease at 3 mg/kg, p < 0.05).
SCH226206 demonstrated selectivity for M2 over M1 receptors (40-fold) as previously reported (Wang et al., 2002). However, this compound shows limited selectivity over other muscarinic receptor subtype targets. Nonetheless, since the effects of scopolamine were attenuated in M2−/− mice, we utilized this pharmacological tool to further explore the potential involvement of this receptor subtype in the antidepressant-related phenotype of scopolamine. In these studies, SCH226206 dose-dependently decreased immobility time in the forced swim assay in WT mice, an effect that was completely prevented in M2−/− mice (Fig. 5).

VU0255035 was 69-fold selective for M1 over M2 receptors. Moreover, VU0255035 also displayed selectivity over other muscarinic receptor subtypes as previously reported (Sheffler et al., 2009). The effects of VU0255035 were evaluated at doses of 10, 30 and 60 mg/kg, i.p. Significant, albeit small, antidepressant-like effects were also observed with this selective M1 receptor antagonist at 60 mg/kg (Fig. 6). VU0255035 was also studied after i.v. dosing in the rat forced-swim assay where 60 mg/kg significantly decreased immobility time (Fig. 6).

Since VU0255035, SCH226206, and pirenzepine are non-lipophilic molecules which could restrict their access to the central nervous system, we collected plasma and whole brain samples mice at 35 min post dosing to correspond to exposures in the mice at the time of testing in the forced-swim assay (Table 3). These data indicate that all compounds entered the central nervous system, achieved brain levels at or above the Ki for their target receptor (Table 1) and produced behavioral effects.

The effects of the relatively selective M1 and M2 receptor antagonists were also evaluated for their effects on locomotor activity. As with the locomotor activity data
with scopolamine, compounds were dosed as in the forced-swim assay and tested at 30 min post dosing for 5 min, the time post dosing they were tested for antidepressant-related activity, and for the approximate duration of that 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 non-significant 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.** In order 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 in our hands are relatively resistant to effects of standard of care antidepressant agents that we tested (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 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) that achieves significant effects in the forced-swim assay.
Discussion

The adrenergic–cholinergic balance hypothesis of depression proposed by Janowsky and colleagues (1972) was based primarily on findings that cholinesterase inhibitors could mimic symptoms of depression and reverse manic symptomatology (Dilsaver et al., 1986; Janowsky et al., 1972, 1983). It was the pivotal data of Furey and Drevets that provided remarkable proof and concept of this hypothesis (Furey and Drevets, 2006). 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 re-energizing of both the cholinergic hypothesis of MDD and the pressing need for improved antidepressant agents, the present series of studies was undertaken.

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, pharmacological classes, and different mechanisms of action (Porsolt et al., 1977; Willner, 1984; Cryan et al., 2002). Indeed, forced-swim is the only assay used to detection antidepressant agents with significant correlation between anti-immobility potencies and clinical dosage (Willner, 1984). Interestingly, since the inception, anticholinergics were considered false negatives in forced-swim (Willner, 1984; Borsini and Meli, 1988). We used this assay in conjunction with M1-M5–receptor deficient mice and pharmacological tools to discriminate the
muscarinic receptor subtype(s) responsible for the antidepressant effects of scopolamine in humans.

Using M1-M5 receptor -/- mice, we found anti-immobility efficacy of scopolamine not observed in M1 and M2 -/- mice. Analysis of scopolamine levels in plasma and brain of WT and muscarinic receptor knockout 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 M1 and M4 null mice in previous studies. The M1 -/- mice are modestly spontaneously hyperactive (Miyakawa et al., 2001), a result not observed in our studies. M4 -/- mice are also modestly spontaneously hyperactive with even greater increases in hyperlocomotion observed in the presence of a D1 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 M4 and M5 -/- mice. However, activity was not enhanced by scopolamine in the M3 -/- mice who retained the antidepressant-phenotype with scopolamine like that of the M3, M4 or M5 -/- mice. Therefore, the presence or absence of locomotor stimulation was not a likely contributor to the anti-immobility effects of scopolamine. Nonetheless, work with these mice in other models that detect antidepressants will help to better understand the implications of these motor effects.

Additionally, we made use of compounds with greater selectivity of M1 over M2 receptors to decipher the behavioral effects of scopolamine. Three molecules with enhanced selectivity profiles (biperiden, pirenzepine, and VU0255035) demonstrated antidepressant-like effects. Although pirenzepine has a restricted 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, 2001) as did the analog, telenzepine (Voleti et al., 2013). Behavioral effects of pirenzepine, like those of scopolamine, were absent in M<sub>1</sub> -/- receptor mice in the present study. The exposure of VU0255035 was not greatly above its K<sub>i</sub> value and might account for its modest behavioral effects reported here. Importantly, it was shown that effects of biperiden, while significantly and fully attenuated in M<sub>1</sub> -/- mice were not markedly affected in the M<sub>2</sub> receptor knockout mouse. The M<sub>2</sub> receptor selective antagonist, SCH226206, demonstrated M2 receptor-mediated effects in the present study. Although both M<sub>1</sub> and M<sub>2</sub> receptors appear necessary for the anti-immobility effects of scopolamine, independent M<sub>1</sub> or M<sub>2</sub> 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 post 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 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<sub>1</sub> receptor interactions have also been documented from
studies of long-term depression where activation of M₁ receptors engenders AMPA receptor endocytosis (Dickenson et al., 2009).

The antidepressant effects of scopolamine in this study does have some limitations. First, only one antidepressant-relevant assay was employed. Secondly, 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 the present study were higher than the Kᵢ values for all muscarinic receptor subtypes. Finally, we were unable to study effects of scopolamine at 24 hrs post 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 swim are consistent with the doses (1 mg/kg and higher) and time parameters (30 min post dosing) we utilized in this study. Scopolamine was also active in the tail suspension test, an assay we found to be relatively insensitive to SSRI mechanism based antidepressants (Witkin 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 utilizing forced-swim 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 better translation to the clinical data. Specifically, other measures of antidepressant-related biological activity at multiple levels will be critical to increasing translational understanding of these findings into potential 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 support 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 postmortem frontal cortex from 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 M₂ 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 NO/sGC/PKG-dependent signaling processes.

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 M₁ receptors on dendrites 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 were 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 pharmacological tools, we have identified both M1 and M2 receptors in the antidepressant-like effects of scopolamine. As blockade of M2 receptors have 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 for use in TRD patients.
Acknowledgement

We thank Ron Duman for prior comments on an earlier version of this manuscript and for helpful discussion of the data.
Author Contributions

JM Witkin, C Overshiner, X Li, J Catlow, GN Wishart, A Nikolayev, and VV Tolstikov designed, conducted, and analyzed data from experiments and wrote the manuscript.

DA Schober, M-S Kuo, and CC Felder helped with the design of the studies and wrote the manuscript.

WH Anderson and RE Higgs provided analysis of data and wrote the manuscript.

There are no conflicts of interest with any author.
References


JPET/2014/216804


Footnotes

1We dedicate this paper to our 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 us.

2All authors are employees of Eli Lilly and Company where the research was conducted and funded. There are no conflicts of interest of disclosures for any author.
Figure Legends

Figure 1. Intravenous scopolamine produces dose-dependent antidepressant-like effects in the forced-swim assay in rats. Scopolamine HBr was given i.v., 30 min prior to testing; imipramine at 30 mg/kg, i.p. (imi) was given 30 min prior. Each point represents the mean ± SEM of responses from 6-7 male, Sprague-Dawley rats. * p < 0.05 by post-hoc Dunnett’s test compared to vehicle (veh) control values.

Figure 2. Scopolamine HBr and imipramine HCl but not scopolamine methylbromide were active in the forced-swim assay in mice. Scopolamine (○), imipramine (□), or scopolamine methyl nitrate (●) were given i.p., 30 min prior to testing. Each point represents the mean ± SEM of responses from 8 wild-type C57BL/6 mice.* p < 0.05 by post-hoc Dunnett’s test compared to vehicle (veh) control values for each dose response curve.

Figure 3. Antidepressant-like effects of scopolamine but not imipramine are significantly attenuated by deletion of M₁ or M₂ receptors. Scopolamine or imipramine were given i.p., 30 min prior to testing. Each point represents the mean ± SEM of responses from at least 8 muscarinic receptor null mice or C57BL/6 wild-type (WT) mice. Imipramine (imi) was given as a positive control for each dose-effect function (15 mg/kg, i.p.)* p < 0.05 by post-hoc Dunnett’s test compared to vehicle (veh) control values for each dose response curve.
Figure 4. Locomotor activity of wild-type and M₁ to M₅ receptor knockout mice.
Locomotion was accessed for 5 min at 30 min post dosing, comparable to mice studied in the forced-swim assay (6 min; 30 min prior). Each bar represents the mean ± SEM of responses from 6-12 C57BL/6 wild-type (WT) or muscarinic receptor knockout mice. Statistical comparisons shown are for Vehicle vs. Drug separately for each KO strain. * p < 0.05 by post-hoc Dunnett’s test compared to comparable genotype given vehicle.

Figure 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 wild-type (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 i.p., 30 min prior to testing. Each point represents the mean ± SEM of responses from 8 mice. * p < 0.05 by post-hoc Dunnett’s test compared to vehicle (veh) control values for each dose response curve.

Figure 6. Anti-immobility efficacy of the selective M₁ receptor antagonist VU0255035 in rats dosed intravenously and mice dosed intraperitoneally.
VU0255035 was tested 30 min post 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 ± SEM of responses from 6-7 male, Sprague-Dawley rats (A).
or 8 wild-type C57BL/6 mice (B). * p < 0.05 by post-hoc Dunnett’s test compared to vehicle (veh) control values for each dose response curve.

Figure 7. Scopolamine is active in the tail-suspension test in CD1 mice whereas citalopram is not. Each point represents the mean ± SEM of 7-10 CD1 mice.
Scopolamine HBr (●) or citalopram (○) were given i.p., 30 min prior to testing. * p<0.05 compared to vehicle control (veh) by post-hoc Dunnett’s test.
Table 1. Potencies of the antimuscarinic ligands used in the present study assessed against human cloned muscarinic receptor subtypes\(^1\).

<table>
<thead>
<tr>
<th>Compound</th>
<th>hM(_1)</th>
<th>hM(_2)</th>
<th>hM(_3)</th>
<th>hM(_4)</th>
<th>hM(_5)</th>
<th>(M_2/M_1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scopolamine</td>
<td>0.83 (0.06)</td>
<td>5.3 (1.4)</td>
<td>0.34 (0.06)</td>
<td>0.38 (0.07)</td>
<td>0.34 (0.11)</td>
<td>6.4</td>
</tr>
<tr>
<td>Biperiden</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>Pirenzepine</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>VU0255035</td>
<td>124 (62)</td>
<td>8530 (4000)</td>
<td>2000 (628)</td>
<td>2430 (854)</td>
<td>4970 (1390)</td>
<td>69</td>
</tr>
<tr>
<td>SCH226206</td>
<td>1240 (255)</td>
<td>30 (11)</td>
<td>528 (194)</td>
<td>76 (20)</td>
<td>128 (14)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

\(^1\)Human cloned receptors were expressed in CHO cell membranes. Binding was measured as competition with \([^3]H\)-N-methyl-scopolamine. Data represent the mean of three to six experiments ± (SEM) expressed as Ki in nM.
Table 2. Plasma and brain concentrations of imipramine and of scopolamine in wildtype (WT) and muscarinic receptor deficient mice (M1-M5)\textsuperscript{1}.

<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>WT</td>
<td>1496 ± 139.0</td>
<td>21333 ± 3013</td>
<td>33.3 ± 7.9</td>
<td>185.7 ± 18.0</td>
</tr>
<tr>
<td>M\textsubscript{1}</td>
<td>1023 ± 192.6</td>
<td>14302 ± 9408</td>
<td>70.6 ± 2.1*</td>
<td>284.3 ± 50.0</td>
</tr>
<tr>
<td>M\textsubscript{2}</td>
<td>662.7 ± 113.6*</td>
<td>8407 ± 3080*</td>
<td>19.4 ± 12.9</td>
<td>155.1 ± 13.5</td>
</tr>
<tr>
<td>M\textsubscript{3}</td>
<td>1060 ± 198.8</td>
<td>18638 ± 3803</td>
<td>7.65 ± 2.9*</td>
<td>120.6 ± 24.7</td>
</tr>
<tr>
<td>M\textsubscript{4}</td>
<td>1190 ± 583.8</td>
<td>20513 ± 3475</td>
<td>30.3 ± 11.8</td>
<td>195.7 ± 38.3</td>
</tr>
<tr>
<td>M\textsubscript{5}</td>
<td>817.4 ± 101.0</td>
<td>15366 ± 3181</td>
<td>8.83 ± 0.88</td>
<td>156.8 ± 7.6</td>
</tr>
</tbody>
</table>

\textsuperscript{1}Imipramine was dosed as the HCl at 15 mg/kg, i.p., 30 min prior. Scopolamine was dosed as the HBr at 1 mg/kg, i.p., 30 min prior. Values are means ± SD. * p<0.05 by ANOVA followed by Dunnett’s test.
Table 3.  Plasma and brain concentrations of other ligands studied$^1$.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose (mg/kg)</th>
<th>[Plasma] (nM)</th>
<th>[Brain] (nM)</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>60</td>
<td>13.9 ± 8.1</td>
<td>206.2 ± 31.2</td>
</tr>
<tr>
<td>Pirenzepine</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>

$^1$Values are means ± SEM of 3 mice. All compounds were given 30 min prior by i.p.
Figure 2

Graph showing the effect of dose on immobility time for three substances:
- **Scopolamine**
- **Methyl-scopolamine**
- **Imipramine**

The x-axis represents dose (mg/kg) with values: 0.1, 0.3, 1.0, 3.0, 5.0, 10, 15, 30.

The y-axis represents immobility time (s) with values: 0, 50, 100, 150, 200, 250.

- Scopolamine shows a significant increase in immobility time as the dose increases.
- Methyl-scopolamine has a similar trend, with some points marked with an asterisk (*) indicating statistical significance.
- Imipramine also shows an increase in immobility time with dose, with asterisks indicating significance.

The graph also includes a note that this article has not been copyedited and formatted. The final version may differ from this version.
Figure 3

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Figure 4

The bar graph shows the locomotor counts per 5 minutes for different treatments: Vehicle, Scopolamine, and Imipramine. The treatments are compared across different strains: WT, M1, M2, M3, M4, and M5. The graph indicates a significant increase in locomotor counts for the Scopolamine treatment compared to Vehicle, with a notable peak at the M4 strain. The Imipramine treatment also shows a trend, but with less pronounced differences compared to Scopolamine.
**Figure 5**

(A) Effect of biperiden and pirenzepine on immobility in the tail-suspension test. The mice were administered various doses of biperiden or pirenzepine, and the immobility time was recorded. The data are represented as mean ± SEM. *p < 0.05 compared to the veh group.

(B) Effect of SCH226206 on immobility in the tail-suspension test. The mice were administered various doses of SCH226206, and the immobility time was recorded. The data are represented as mean ± SEM. *p < 0.05 compared to the veh group.
Figure 6

A

Immobility (s)

VU0255035 (mg/kg)

B

Immobility (s)

VU0255035 (mg/kg)
Figure 7

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JPET Fast Forward. Published on September 3, 2014 as DOI: 10.1124/jpet.114.216804

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