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Modulation of prepulse inhibition through both M_1 and M_4 muscarinic receptors in mice

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Abstract

Rationale—Muscarinic cholinergic M_1 and M_4 receptors may participate in schizophrenia's etiology, and have been proposed as targets for antipsychotic medications.

Objective—Here we investigated the involvement of these receptors in behavioral measures pertinent to schizophrenia using knockout mice lacking M_1 receptors (M_1−/−), M_4 receptors (M_4−/−) or both (M_1−/−M_4−/−).

Methods—We measured prepulse inhibition of startle (PPI) without drugs, and after treatment with scopolamine (0.32–1.8 mg/kg), xanomeline (3.2 mg/kg) oxotremorine (0.032–0.1 mg/kg), clozapine (1.0–5.6 mg/kg), or haloperidol (0.32–3.2 mg/kg).

Results—In female (but not male) mice, combined deletion of both M_1 and M_4 receptors decreased PPI relative to wild-type mice, while knockout of either receptor alone had no significant effect. Scopolamine disrupted PPI in wild-type and M_4−/− mice, but not in female M_1−/−M_4−/− or female M_1−/− mice. When administered before scopolamine, xanomeline restored PPI in wild-type mice and M_1−/− mice, but not in M_4−/− mice. In contrast, pretreatment with oxotremorine increased PPI regardless of genotype. Effects of clozapine and haloperidol on PPI were not hindered by either mutation.

Conclusions—Deletion of both M_1 and M_4 receptors can disrupt PPI, suggesting that (at least partially redundant) M_1 and M_4 receptor-dependent functions are involved in sensorimotor gating mechanisms. PPI-disrupting effects of muscarinic antagonists appeared dependent upon M_1 receptor blockade. Our data also suggest that xanomeline exerts antipsychotic-like effects mainly through M_4 receptor stimulation, while stimulation of non-M_1/M_4 subtypes may also have antipsychotic potential. Finally, our results do not support a role of M_1/M_4 receptors in mediating antipsychotic-like effects of clozapine.

Keywords

prepulse inhibition; muscarinic; acetylcholine receptor; antipsychotic; knockout mice; M_1; M_4

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Introduction

Despite an abundance of available antipsychotic drugs, there is still a need for highly efficacious schizophrenia medications with favorable side-effect profiles. In particular, cognitive deficits are often debilitating symptoms of schizophrenia that are typically poorly improved by current medications (Buchanan et al. 2007). Evidence suggests cholinergic dysregulation is part of schizophrenia's etiology, including abnormal densities and/or function of the muscarinic M_1 and M_4 receptor subtypes, which have therefore been proposed as novel antipsychotic drug targets (Bymaster et al. 2002, 2003; Sarter et al. 2005; Vakalopoulos 2006; Raedler et al. 2007). These cholinergic hypotheses likely extend, rather than contradict, the “dopamine hypothesis” of schizophrenia, as the two systems are intricately interconnected. Muscarinic antagonists induce striatal dopamine efflux in rodents and humans, can produce psychotic-like effects similar to dopamine agonists and psychostimulants, and worsen positive symptoms in schizophrenic patients (Tandon et al. 1991; Dewey et al. 1993; Chapman et al. 1997; Perry and Perry 1995; Halpern 2004). Conversely, muscarinic agonists have been shown to produce functional dopamine antagonism, which may account, at least in part, for their antipsychotic-like effects in various preclinical assays (Bymaster et al. 1998; Fink-Jensen et al. 1998; Shannon et al. 1999,2000; Stanhope et al. 2001; Andersen et al. 2003; Jones et al. 2005).

Muscarinic systems are also critically involved in memory and cognition (Power et al. 2003; Hasselmo 2006). Muscarinic antagonists disrupt memory and cognitive performance in laboratory animals as well as in healthy individuals, and worsen cognitive impairment in schizophrenic patients (Sitaram et al. 1978; Johnstone et al. 1983; Minzenber et al. 2004; Ellis et al. 2006). Conversely, muscarinic agonists can improve cognitive performance in laboratory animals and humans (Davis et al. 1978; Sitaram et al. 1978; Bartus 1979; Harries et al. 1998; Hodges et al. 1999). Muscarinic agonists such as the M_1/M_4-preferring xanomeline are being evaluated and shown some promise as cognitive enhancers in schizophrenia as well as other disorders, e.g., Alzheimer's disease (Bodick et al. 1997; Friedman 2004; Langmead et al. 2008b; Shekhar et al. 2008).

There are five cloned muscarinic receptor subtypes (M_1–M_5), and little is known about which of these mediate potential therapeutic effects, and whether different subtypes mediate undesirable effects. While the usefulness of nonselective muscarinic agonists and acetylcholinesterase inhibitors are limited by side effects, the recent emergence of highly subtype-selective allosteric ligands has intensified the interest in effects mediated through specific subtypes (Chan et al. 2008; Jones et al. 2008; Langmead et al. 2008a; Shirey et al. 2008; Conn et al. 2009). M_1 and M_4 receptors are expressed in striatal areas and in areas relevant to cognitive function, e.g., cingulate cortex, prefrontal cortex and hippocampus (Levey et al. 1991). In contrast, the M_2 and M_3 receptors are sparser in the brain but represent the major subtypes mediating (undesirable) peripheral effects such as alterations in heart rate, gastrointestinal contractility, and exocrine gland secretion, causing symptoms like nausea, diarrhea, and salivation (Bymaster et al. 2002; Wess 2004). M_1 and M_4 are the most abundant muscarinic receptor subtypes on striatal neurons, with M_4 receptors and D_1 receptors exerting directly opposing effects on cyclic AMP synthesis, while M_1 receptors oppose the effects of D_2 receptors (Di Chiara et al. 1994; McGinty 1999; Onali and Olianas 2002). Functional dopamine antagonism in striatal pathways therefore seems most likely mediated through M_1 and/or M_4 receptors. Accordingly the M_1 and M_4 receptors are generally thought to mediate cognition-enhancing and antipsychotic-like effects of muscarinic agonists (Bymaster et al. 2002; Langmead et al. 2008b; Conn et al. 2009).

One of the most validated preclinical assays for predicting antipsychotic drug action in humans is prepulse inhibition (PPI) of the startle response, in which the reflex elicited by a

startling stimulus, such as a loud noise, is reduced when it is immediately preceded by a low-intensity "prepulse" stimulus (Geyer et al. 2001,2002; Swerdlow et al. 2008). PPI is on average reduced in the schizophrenic population, and is believed to reflect sensorimotor gating, or the ability of the brain to "filter out" irrelevant stimuli (Braff et al. 2001; Swerdlow et al. 2008). PPI can be disrupted pharmacologically in both rodents and healthy humans by drugs that can induce psychotic-like symptoms – including muscarinic receptor antagonists (Wu et al. 1993; Jones and Shannon 2000; Geyer et al. 2001; Kumari et al. 2001). Such disruptions in PPI can be reversed by administration of dopamine receptor antagonists or antipsychotic drugs (Geyer et al. 2001; Swerdlow et al. 2008). In the present study, we tested the hypothesis that the M_1 and/or M_4 receptors play a role in sensorimotor gating by evaluating PPI in knockout mice lacking either or both receptor subtypes. We also tested the hypothesis that M_1 and/or M_4 receptors are involved in the responsiveness to muscarinic ligands or antipsychotic drugs. Specifically, we evaluated PPI in $M_1{-}/{-}$, $M_4{-}/{-}$, $M_1{-}/{-}M_4{-}/{-}$ and wild-type mice after treatment with the non-selective muscarinic antagonist scopolamine, with and without xanomeline (M_1/M_4-preferring agonist) or oxotremorine (non-selective muscarinic agonist) pretreatments. We also evaluated the effects of the atypical antipsychotic clozapine, which has measurable affinity for muscarinic receptors including M_1 and M_4 subtypes, and the typical antipsychotic haloperidol, which does not (Olianas et al. 1999; Weiner et al. 2004; Davies et al. 2005).

Materials and methods

Animals and housing

$M_1{-}/{-}$ and $M_4{-}/{-}$ mice were generated as described previously (Gomeza et al. 1999; Miyakawa et al. 2001) and backcrossed for 11 generations to C57BL/6NTac females. Double knockout mice were bred by intercrossing the single knockout lines and then maintained as a separate line, due to the low yield of $M_1{-}/{-}M_4{-}/{-}$ mice if bred by heterozygous intermating. Age- and sex-matched C57BL/6NTac were therefore used as wild-type controls. All mice were bred at Taconic Farms (Germantown, NY). Male and female double knockout mice were tested first, together with wild-type mice, in two sequential batches of N = 5–7/sex/genotype. A second batch was tested because initial results indicated an effect of sex, therefore additional mice were obtained both to duplicate the findings, and to increase sample size to allow for the detection of sex effects. Results obtained from both batches were comparable, and all data are therefore reported as both batches combined. Subsequently to the double knockout mice, female $M_1{-}/{-}$ mice and female $M_4{-}/{-}$ mice (each N=12) were tested simultaneously with an additional group of female wild-type mice (N=9), in a single batch.

Animals were kept in a 12-h light/dark cycle (lights on at 07:00) at 22°C and 55% humidity. Tap water and standard rodent chow (rodent diet 5001, PMI Feeds, Inc., St. Louis, MO) were accessible ad libitum. For enrichment purposes rodent "treats" were given once or twice weekly, and nesting material (cotton), hiding/nesting devices and exercise devices were provided. Fresh litter (pine wood shavings) was provided twice weekly. The air in each cage was actively circulated. Animals were group housed up to 4 per cage, and were left to acclimate to the housing facilities at least 7 days before experiments began. All testing was conducted in animals at least 8 weeks of age and during the light phase of the circadian cycle (the latter, to match relevant previous behavioral studies). Animal health was evaluated daily. All procedures were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the Principles of Laboratory Animal Care.

Drugs

Scopolamine hydrobromide, oxotremorine sesquifumarate and xanomeline were dissolved in 0.9% saline. Clozapine and haloperidol were dissolved in a small volume of sterile water acidified to pH≈1 with HCl, then diluted to the desired concentrations with saline and water, respectively. Final pH was adjusted to 5–5.5 with NaOH. For clozapine and haloperidol vehicles, equivalent amounts of HCl and NaOH were added to saline or water (for comparable pH). Xanomeline was synthesized at the McLean Hospital following published methods (Sauerberg et al. 1992; Kane et al. 2008). Other drugs were purchased from Sigma-Aldrich (St Louis, MO). Doses refer to weights of the drug salts.

Startle and prepulse inhibition of startle

Apparatus and session—Startle reactivity was assessed using four startle chambers (SR-LAB, San Diego Instruments, San Diego, CA). The apparatus and procedures have been described in detail elsewhere (Thomsen et al., 2007). Each 20-min session consisted of startle trials ("*pulse alone*"; a 40 msec 120 dB broadband pulse), prepulse trials ("*prepulse +pulse*"; a 20 ms prepulse followed by a 100 ms interstimulus interval and a 40 msec 120 dB pulse), and no-stimulus trials ("*nostim*"; background noise alone, 65 dB). Prepulse intensities were 6, 12 and 16 dB above background (i.e., 71, 77 and 81 dB). A session consisted of a 5 min acclimation period with background noise, five startle (*pulse alone*) trials, then 10 presentations of each trial type (*pulse alone*, *prepulse+pulse* of each prepulse intensity and *nostim*) in pseudorandom order, then five startle trials. Intertrial intervals averaged 15 sec (range: 12–18 sec). Each animal was assigned one apparatus, balanced for sex and genotype. Chambers were cleaned with a paper towel and water after each session. The amount of PPI for each prepulse intensity was calculated as %PPI = 100 – [[(ASR for *prepulse+pulse*)/(ASR for *pulse alone*)] × 100], using the average of the ten presentations of each trial type. Startle amplitude (ASR for *pulse alone*) was calculated as the average of the 10 mid-session *pulse alone* trials.

Spontaneous and drug-modulated PPI—Drug- and experimentally naïve mice were used. Dose-effect functions were assessed for saline and scopolamine (0.32, 1.0 and 1.8 mg/kg), and the saline data were analyzed for baseline startle and PPI differences. Following scopolamine experiments, female mice only were studied further. The M_1/M_4 preferring agonist xanomeline (3.2 mg/kg) was tested as a pretreatment to 1.0 mg/kg scopolamine. The non-selective muscarinic agonist oxotremorine was tested as pretreatment to 1.0 mg/kg scopolamine (0.032, 0.1 mg/kg) and alone (0.01, 0.032, 0.1 mg/kg). Clozapine was tested at doses of 1.0, 1.8, 3.2 and 5.6 mg/kg, and in the first batch of double knockout mice only, haloperidol at doses of 0.3, 1.0 and 3.2 mg/kg. Drug doses plus vehicle were tested according to a Latin square design within-subjects, with the exception of 5.6 mg/kg clozapine which was tested last. Animals were allowed at least three days between tests, and at least one week between each drug. Scopolamine, clozapine and haloperidol were injected i.p. (10 ml/kg), oxotremorine and xanomeline s.c. (10 ml/kg). Scopolamine was injected immediately before placing the animal in the test chamber (i.e., 5 min before the first startle stimulus), clozapine and haloperidol 30 min before placing the animal in the chamber, oxotremorine and xanomeline 15 min before placing the animal in the chamber. Drug doses were chosen based on previous studies (Thomsen et al., 2007) and pilot experiments in wild-type mice (scopolamine, xanomeline, oxotremorine).

Statistical analysis

Statistical comparisons were performed by mixed model ANOVA with genotype and sex as between-subjects factors and drug dose as a within-subjects factor (repeated measures), unless otherwise indicated. Significant effects of dose were analyzed post-hoc by Dunnett's

multiple comparisons test vs. vehicle, and significant effects of genotype by Bonferroni-corrected two-sided unpaired t-test vs. wild-type. Genotype comparisons were designed to examine effects of each mutation relative to their respective group of wild-type controls, and no comparisons were made either between the various knockout lines or between the two groups of wild-type controls. The level of significance was set at $p \leq 0.05$. All t-tests reported are two-sided. For PPI, prepulse intensity was always included as a factor in an initial analysis, and was always highly significant ($p < 0.0001$). For brevity, %PPI was collapsed across prepulse intensity for graphic representations of the data and for analyses (with the exception of baseline PPI). In cases where drug effects were detected for startle amplitude, possible correlations between startle and PPI in individual mice were investigated (in each genotype) by linear regression.

Results

Baseline effects

Baseline PPI—PPI was assessed in male and female $M_1-/-M_4-/-$ and wild-type mice, then in female $M_1-/-$, $M_4-/-$ and wild-type mice. An ANOVA using genotype and sex as between-subjects factors and prepulse intensity as a within-subjects factor showed a significant effect of genotype [$F_{1,43} = 6.10$, $p < 0.05$], prepulse intensity [$F_{2,86} = 77.09$, $p < 0.0001$], and a sex by prepulse intensity interaction [$F_{2,86} = 3.31$, $p < 0.05$]. Because of the significant interaction, the data were re-analyzed for female and male mice separately. As expected, %PPI increased with prepulse intensity in both female mice [$F_{2,44} = 20.28$, $p < 0.0001$] and male mice [$F_{2,42} = 75.21$, $p < 0.0001$]. There was a significant effect of genotype only in the female mice [$F_{1,22} = 8.96$, $p < 0.01$]. Figure 1A–D shows %PPI as a function of prepulse intensity in male (**A**) and female (**B**) $M_1-/-M_4-/-$ and wild-type mice. It can be seen that PPI was reduced in the female $M_1-/-M_4-/-$ mice relative to wild-type at all prepulse intensities ($p < 0.05$ to $p < 0.01$), while there was no effect of genotype in the male mice.

Because there was an effect of the double mutation in female mice, female single knockout mice lacking either M_1 or M_4 receptors were subsequently tested, with an additional cohort of female wild-type mice. Figure 1C–D shows %PPI in the $M_1-/-$ mice and $M_4-/-$ mice, which demonstrated only a small and non-significant decrease in PPI relative to wild-type mice. Separate ANOVAs were performed comparing each knockout to wild-type, with genotype as between-subjects factor and prepulse intensity as within-subjects factor. For the $M_1-/-$ mice, %PPI was related to prepulse intensity [$F_{2,38} = 52.84$, $p < 0.0001$], but there was no significant effect of M_1 genotype or genotype by intensity interaction (Fig. 1C). Similarly for the $M_4-/-$ mice, %PPI was related to prepulse intensity [$F_{2,38} = 20.99$, $p < 0.0001$], with no significant effect of M_4 genotype or interaction (Fig. 1D). It is noteworthy that PPI levels were very similar between cohorts of wild-type mice.

Baseline startle reactivity—Baseline startle amplitudes (i.e., following saline administration) are shown in Figure 1E–H. Startle amplitudes did not differ significantly between wild-type and $M_1-/-M_4-/-$ mice, but were higher in male mice than in female mice [$F_{1,44} = 7.55$, $p < 0.01$]. There was no significant genotype by sex interaction. In the single knockout experiment, baseline startle reactivity was not significantly altered by the M_1 mutation, but was increased in the $M_4-/-$ mice ($p < 0.05$; Fig. 1H).

Scopolamine effects

Effect of scopolamine on PPI—Figure 2A–D shows PPI dose-effect functions for scopolamine in double and single knockout mice. For the double knockout experiment, because of the differential effect of the mutation on baseline PPI between sexes, the data

were analyzed separately in male and female mice. In male mice, scopolamine decreased PPI dose-dependently [$F_{3,66} = 9.13$, $p < 0.0001$], but there was no effect of genotype or dose by scopolamine interaction (see Fig. 2A for the significance of specific scopolamine doses). In female mice, PPI was related to scopolamine dose [$F_{3,66} = 9.25$, $p < 0.0001$] with a genotype by scopolamine interaction [$F_{3,66} = 3.84$, $p < 0.05$], but no main effect of genotype. Further analysis showed the effect of scopolamine was significant only in wild-type mice ($p < 0.0001$; Fig. 2B). In contrast, scopolamine did not affect PPI significantly in the female $M_1-/-M_4-/-$ mice. Also, PPI was significantly lower in female $M_1-/-M_4-/-$ mice relative to wild-type in the saline condition only ($p < 0.05$).

In the $M_1-/-$ single knockout experiment, PPI was related to scopolamine dose [$F_{3,57} = 9.18$, $p < 0.0001$], with a significant genotype by scopolamine interaction [$F_{3,57} = 3.69$, $p < 0.05$], but no main effect of genotype. Further analysis showed scopolamine decreased PPI in wild-type mice [$F_{3,24} = 8.09$, $p < 0.001$], but had no significant effect in $M_1-/-$ mice (Fig. 2C). In the $M_4-/-$ single knockout experiment, PPI was related to scopolamine dose [$F_{3,57} = 14.53$, $p < 0.0001$], with a trend for genotype [$F_{1,19} = 3.81$, $p = 0.07$], with no significant interaction. Scopolamine decreased PPI in both wild-type mice and $M_4-/-$ mice (both $p < 0.001$; Fig. 2D). Thus with respect to scopolamine's effects, the deletion of M_1 receptors alone produced a phenotype comparable to the double mutation while the deletion of M_4 receptors alone had little or no effect.

Effect of scopolamine on startle amplitude—Figure 2E–H shows the effect of scopolamine on startle amplitude. In the double knockout experiment, ANOVA showed a significant effect of genotype [$F_{1,43} = 9.11$, $p < 0.01$], sex [$F_{1,43} = 12.20$, $p < 0.01$], and scopolamine dose [$F_{3,129} = 39.89$, $p < 0.0001$], with no significant interactions (Fig. 2E–F). Data were analyzed in each sex, showing significant effects of genotype in both male mice [$F_{1,21} = 4.47$, $p < 0.05$] and female mice [$F_{1,22} = 4.85$, $p < 0.05$], significant effects of scopolamine dose in both male mice [$F_{3,63} = 16.67$, $p < 0.0001$] and female mice [$F_{3,66} = 30.28$, $p < 0.0001$], and a genotype by scopolamine interaction in the female mice only [$F_{3,66} = 5.03$, $p < 0.01$]. Further analysis in each genotype confirmed that scopolamine increased startle reactivity in all four groups (all $p < 0.001$). There was no significant correlation between scopolamine's effect on startle and PPI in any group. The $M_1-/-M_4-/-$ mice appeared more sensitive to scopolamine-induced increases in startle, although all three doses of scopolamine reached significance in all groups. In the female mice, startle amplitudes were significantly higher in $M_1-/-M_4-/-$ mice than in wild-type mice at the two highest scopolamine doses ($p < 0.05$). No doses reached significance in the male mice.

In the single knockout experiment, for the M_1 mutation, startle amplitude was increased by scopolamine [$F_{3,57} = 14.65$, $p < 0.0001$], with no significant effect of M_1 genotype or interaction (Fig. 2G). In contrast for the M_4 mutation, startle amplitude was related to genotype [$F_{1,19} = 29.21$, $p < 0.0001$] and scopolamine dose [$F_{3,57} = 9.87$, $p < 0.0001$], with no significant interaction. Startle amplitude and PPI were negatively correlated in the $M_4-/-$ mice ($r^2 = 0.19$, $p < 0.01$), with no correlation in the other genotypes. While scopolamine increased startle reactivity in both $M_4-/-$ mice [$F_{3,33} = 7.29$, $p < 0.001$] and wild-type mice [$F_{3,24} = 6.45$, $p < 0.01$], only the lowest dose scopolamine reached significance relative to saline in the wild-type mice, while all three doses did so in the $M_4-/-$ mice, and startle was significantly higher in $M_4-/-$ mice relative to wild-type at the two highest doses ($p < 0.01$; Fig. 2H). Thus, in contrast to the PPI modulation effects, the effect of the double mutation on startle reactivity was mimicked by the single M_4 knockout, but not the M_1 knockout.

Xanomeline effects

Effect of xanomeline on PPI—Following baseline and scopolamine tests, only the female mice were selected for further evaluation in the double knockout experiment. Figure 3A–C shows the effect of xanomeline pretreatment (3.2 mg/kg) in female double and single knockout mice treated with 1.0 mg/kg scopolamine. Vehicle and scopolamine-alone data are the same as in Figure 2, shown to aid visual comparisons. Pre-planned t-tests were done to evaluate the effect of xanomeline pretreatment vs. scopolamine alone in each genotype, as well as between-genotype comparisons in the xanomeline-treated state. In wild-type mice from both the double and single knockout experiments, the scopolamine-induced decrease in PPI was completely reversed by xanomeline ($p < 0.001$, $p < 0.05$). In contrast, female $M_1-/-M_4-/-$ mice showed no significant effect of either scopolamine or xanomeline. Similar to the vehicle condition, the $M_1-/-M_4-/-$ mice had lower PPI than wild-type mice after xanomeline + scopolamine treatment ($p < 0.01$).

In the $M_1-/-$ single knockout mice, xanomeline increased PPI ($p = 0.050$). As in the vehicle condition, PPI levels were comparable between $M_1-/-$ and wild-type mice after xanomeline + scopolamine treatment. In contrast, in the $M_4-/-$ single knockout mice, xanomeline failed to reverse the effect of scopolamine on PPI. PPI levels after xanomeline + scopolamine treatment thus appeared reduced in the $M_4-/-$ mice relative to the wild-type mice, an effect which approached significance ($p = 0.051$). In summary, the ability of xanomeline to block a scopolamine-induced PPI deficit was absent in the $M_1-/-M_4-/-$ mice and in the $M_4-/-$ mice, but appeared intact in the $M_1-/-$ mice.

Effect of xanomeline on startle amplitude—As described above, 1.0 mg/kg scopolamine increased startle reactivity in all genotypes (Fig. 3D–F). Pre-planned t-tests were done as for PPI Xanomeline pretreatment did not significantly alter startle amplitudes relative to scopolamine alone in any genotype. The exaggerated effect of scopolamine in the female $M_1-/-M_4-/-$ mice relative to wild-type mice was not significant after xanomeline pretreatment. Comparable to the baseline and scopolamine-alone tests, no difference was found between $M_1-/-$ mice and wild-type mice in startle reactivity, while startle was increased in $M_4-/-$ mice relative to wild-type regardless of xanomeline treatment (Fig. 3F).

Oxotremorine effects

Effect of oxotremorine on scopolamine-modulated PPI—The ability of the non-selective muscarinic agonist oxotremorine to reverse scopolamine-induced PPI deficits was then evaluated. Figure 4A–C shows the effect of 1.0 mg/kg scopolamine alone and after pretreatment with 0.032 or 0.1 mg/kg oxotremorine in female double and single knockout mice. Vehicle and scopolamine-alone data are the same as in Figure 2, shown to aid visual comparisons. ANOVAs were performed for each mutation with genotype as between-subjects factor and dose oxotremorine as within-subjects factor (relative to scopolamine alone). In the double-knockout experiment, oxotremorine increased PPI [$F_{2,40} = 31.02$, $p < 0.0001$], with no significant effect of genotype and no interaction. In both genotypes, oxotremorine increased PPI at both doses ($p < 0.01$ vs. scopolamine). Similarly for both the M_1 and the M_4 single knockout mutations, there was a main effect of oxotremorine dose [$F_{2,38} = 9.26$, $p < 0.001$; $F_{2,38} = 10.95$, $p < 0.001$], but no effect of genotype and no interaction. In all three genotypes, oxotremorine increased PPI significantly from the scopolamine level for at least one dose ($p < 0.05$ or $p < 0.01$).

Effect of oxotremorine on scopolamine-modulated startle amplitude—As described above, 1.0 mg/kg scopolamine increased startle reactivity in all genotypes (Fig. 4 D–F). ANOVAs were performed for startle amplitude the same way as for PPI. In the double-knockout experiment, startle was higher in $M_1-/-M_4-/-$ mice than wild-type [$F_{1,40}$

= 9.15, $p < 0.01$], significant for scopolamine alone ($p < 0.05$, Fig. 4D). There was a main effect of oxotremorine [$F_{2,40} = 6.35$, $p < 0.01$], which was significant only in the wild-type mice [$F_{2,22} = 3.49$, $p < 0.05$], but neither dose reached significance vs. scopolamine post-hoc. There was also a main effect of oxotremorine dose for both the M_1 and the M_4 single knockout comparisons [$F_{2,38} = 7.46$, $p < 0.01$; $F_{2,38} = 8.05$, $p < 0.01$], which was significant in M_1−/− mice [$F_{1,22} = 7.95$, $p < 0.01$] and M_4−/− mice [$F_{1,22} = 9.00$, $p < 0.01$], but not wild-type mice. Oxotremorine 0.1 mg/kg reached significance post-hoc in the M_4−/− mice only ($p < 0.01$); in the other genotypes, no dose oxotremorine reached significance vs. scopolamine. There was an effect of the M_4 genotype [$F_{1,38} = 24.81$, $p < 0.0001$] but not M_1, consistent with the scopolamine alone data. There was also a M_4 genotype by oxotremorine interaction [$F_{2,38} = 3.13$, $p < 0.05$]. Startle was significantly higher in the M_4−/− mice relative to wild-type for scopolamine alone and scopolamine+0.032 mg/kg oxotremorine ($p < 0.01$, $p < 0.001$, Fig 4F). Thus there was a general trend for oxotremorine to reverse scopolamine's effects on startle. Effects on startle and PPI were not correlated in any genotype.

Effect of oxotremorine alone on PPI and startle amplitude—Oxotremorine alone was also tested, in M_1−/−M_4−/− mice and wild-type mice (Fig. 5A–B). PPI was related to oxotremorine dose [$F_{3,60} = 7.71$, $p < 0.001$], with a genotype by oxotremorine interaction [$F_{3,60} = 16.57$, $p < 0.0001$], but no main effect of genotype. When analyzed in each genotype, the oxotremorine effect was significant only in wild-type mice [$F_{3,30} = 30.69$, $p < 0.0001$], in which 0.1 mg/kg oxotremorine produced a decrease in PPI ($p < 0.01$ vs. vehicle). Genotype effects were explored due to the interaction, again showing lower PPI in M_1−/−M_4−/− mice than wild-type after vehicle administration ($p < 0.05$). In contrast PPI was higher in the M_1−/−M_4−/− mice relative to wild-type at the highest oxotremorine dose ($p < 0.0001$). Oxotremorine also decreased startle amplitude [$F_{3,60} = 34.37$, $p < 0.001$], with a significant effect of genotype [$F_{1,60} = 9.90$, $p < 0.01$] but no interaction. Oxotremorine decreased startle amplitude in both M_1−/−M_4−/− mice [$F_{3,30} = 2.26$, $p < 0.05$] and wild-type mice [$F_{3,30} = 4.16$, $p < 0.01$], at doses of 0.032 and/or 0.1 mg/kg ($p < 0.05$ to $p < 0.01$, see Fig. 5B). Startle amplitude and PPI were positively correlated in the wild-type mice ($r^2 = 0.55$, $p < 0.0001$), but not correlated in the M_1−/−M_4−/− mice. Further analysis of the genotype effect showed that startle amplitude was higher in M_1−/−M_4−/− mice than in wild-type mice under vehicle conditions ($p < 0.05$, in contrast to previous evaluations), and at the highest dose oxotremorine ($p < 0.01$).

Clozapine and haloperidol effects

Effect of clozapine on PPI—Figure 6A–C shows the effect of clozapine on PPI in female M_1−/−M_4−/− mice, M_1−/− mice, M_4−/− mice, and their wild-type controls. In the double knockout experiment, PPI was related to clozapine dose [$F_{4,88} = 5.10$, $p = 0.001$], with a genotype by clozapine interaction [$F_{4,88} = 7.77$, $p < 0.0001$], while the overall effect of genotype was not significant. Further analysis showed a significant effect of clozapine in both M_1−/−M_4−/− mice [$F_{4,44} = 3.51$, $p < 0.05$] and wild-type mice [$F_{4,44} = 7.58$, $p < 0.0001$]. Clozapine increased PPI above vehicle level at 1.8, 3.2 and 5.6 mg/kg ($p < 0.05$, $p < 0.01$) in the M_1−/−M_4−/− mice, while increases in the wild-type mice did not reach significance. At the highest dose clozapine decreased PPI in the wild-type mice ($p < 0.01$), making PPI significantly higher in the M_1−/−M_4−/− mice relative to wild-type ($p < 0.05$) - likely related to a profound suppression of startle amplitude (see Fig. 6D and next section of results).

For the single M_1 mutation, PPI was related to clozapine dose [$F_{4,79} = 35.21$, $p < 0.0001$], with no genotype effect or interaction. PPI was decreased at 5.6 mg/kg clozapine in both genotypes ($p < 0.01$), likely related to the profound reduction in startle amplitude (see Fig.

6E and next section). In contrast, for the single M_4 mutation, there was an effect of genotype [$F_{1,19} = 13.61$, $p < 0.01$] and clozapine dose [$F_{4,76} = 13.97$, $p < 0.0001$], with a significant interaction [$F_{4,76} = 7.78$, $p < 0.0001$]. Further analysis showed a significant effect of clozapine in the wild-type mice [$F_{4,32} = 14.24$, $p < 0.0001$], but not in the M_4−/− mice. Because clozapine decreased PPI in the wild-type mice, PPI was significantly higher in the M_4−/− mice relative to wild-type at 3.2 and 5.6 mg/kg ($p < 0.01$, $p < 0.05$).

Effect of clozapine on startle amplitude—Figure 6D–F shows the effect of clozapine on startle amplitude in female M_1−/−M_4−/− mice, M_1−/− mice, M_4−/− mice, and wild-type controls. In the double knockout experiment, startle was related to genotype [$F_{1,22} = 16.86$, $p < 0.001$] and clozapine dose [$F_{4,88} = 11.58$, $p < 0.0001$], with a significant interaction [$F_{4,88} = 4.51$, $p < 0.01$]. Further exploration of the interaction showed a significant effect of clozapine in both M_1−/−M_4−/− mice [$F_{4,44} = 2.81$, $p < 0.05$] and wild-type mice [$F_{4,44} = 20.92$, $p < 0.0001$]. Startle amplitude and PPI were positively correlated in the wild-type mice ($r^2 = 0.38$, $p < 0.0001$), but were not correlated in the M_1−/−M_4−/− mice. Clozapine *increased* startle amplitude above vehicle level at 1.8 mg/kg ($p < 0.05$) in the M_1−/−M_4−/− mice, and *decreased* startle amplitude at 3.2 and 5.6 mg/kg in the wild-type mice ($p < 0.01$).

In the single M_1 mutation, startle amplitude was related to clozapine dose [$F_{4,76} = 53.07$, $p < 0.0001$], with no effect of M_1 genotype and no interaction. For both genotypes 3.2 and 5.6 mg/kg clozapine significantly suppressed startle ($p < 0.01$ vs. vehicle). Startle amplitude and PPI were positively correlated in both genotypes ($r^2 = 0.48$ and $r^2 = 0.37$, both $p < 0.0001$). For the single M_4 mutation, startle was related to genotype [$F_{1,19} = 10.86$, $p < 0.01$] and clozapine dose [$F_{4,76} = 21.06$, $p < 0.0001$], with a significant interaction [$F_{4,76} = 2.57$, $p < 0.05$]. There was an effect of clozapine in both M_4−/− mice [$F_{4,44} = 5.86$, $p < 0.001$] and wild-type mice [$F_{4,32} = 23.72$, $p < 0.0001$], with significant decreases in startle amplitude at 5.6 mg/kg in the M_4−/− mice ($p < 0.05$) and at 3.2 and 5.6 mg/kg in the wild-type mice ($p < 0.01$). Startle amplitude and PPI were not correlated in the M_4−/− mice.

Effect of haloperidol on PPI and startle amplitude—Haloperidol was tested in the first batch of female M_1−/−M_4−/− mice and their wild-type controls. Haloperidol increased PPI in both genotypes (Fig. 7A). PPI was related to genotype [$F_{1,10} = 9.20$, $p < 0.05$] and haloperidol dose [$F_{3,30} = 4.50$, $p < 0.05$], with no significant interaction. Because of the small sample size and the lack of interaction, dose effects are reported for both genotypes combined. Figure 7B shows startle amplitudes, which were not significantly related to genotype or haloperidol dose, and with no interaction. Because the effect of haloperidol did not differ between genotypes, it was not tested further.

Discussion

We evaluated PPI and pharmacological modulation of PPI in mice constitutively lacking muscarinic cholinergic M_1 or M_4 receptors or both receptor subtypes. PPI was decreased in female mice lacking both M_1 and M_4 receptors, while the deletion of either subtype alone had no significant effect on baseline PPI. Thus a lack of M_1 and M_4 receptors can generate a phenotype indicative of reduced sensorimotor gating. While a constitutive inactivation of a gene in mice cannot “model” a complex human brain disorder, this finding supports a possible involvement of muscarinic M_1 and M_4 receptors in the etiology of schizophrenia, suggested by numerous studies reporting decreased densities of muscarinic M_1 and/or M_4 receptors in the brains of schizophrenic patients (Crook et al. 1999; Dean et al. 2002; Mancama et al. 2003; Raedler et al. 2003; Zavitsanou et al. 2004; Deng and Huang 2005; Newell et al. 2007, Scarr et al. 2007; and see Vakalopoulos 2006). In an intriguing article Borda et al. (2002) demonstrated the presence of anti-M_1 antibodies in the serum of schizophrenic patients, but not of healthy controls, and suggested an autoimmune etiology to

schizophrenia (see also Stefansson et al. 2009). Our results further suggest overlapping functions of M_1 and M_4 receptors, at least to the extent that constitutive deletion of either subtype can be compensated for in mice to maintain largely normal function. Similar to our findings, a previous study reported unaltered baseline PPI in M_1−/− mice, although only male mice were tested (Miyakawa et al. 2001). But M_1−/− mice have also shown some phenotypes reminiscent of schizophrenia-like symptoms, such as striatal hyperdopaminergia, hyperactivity and impaired performance in some cognitive tests (Gerber et al. 2001; Miyakawa et al. 2001; Anagnostaras et al. 2003). M_4−/− mice showed normal passive avoidance and only inconsistent locomotor hyperactivity at baseline, but did show striatal hyperdopaminergia and exaggerated responses to direct and indirect dopamine agonists or phencyclidine (Gomeza et al. 1999; Felder et al. 2001; Tzavara et al. 2003, 2004). Electrophysiological recordings in striatal slices suggested altered synaptic plasticity in M_4−/− mice (Bonsi et al. 2008). Thus deletion of either M_1 or M_4 receptors may cause less overt impairments which when combined produce a phenotype including reduced PPI. It should be noted however that in earlier studies sexes were combined, unspecified, or male only, while we detected significant effects of the M_1/M_4 deletion only in female mice.

In the initial experiments of this investigation we tested both male and female mice. We found that in female mice the double mutation affected both baseline PPI and responses to scopolamine, while this phenotype was not observed in male mice. The only statistically significant difference between wild-type and M_1−/−M_4−/− male mice was increased startle reactivity after scopolamine administration, similarly observed in the female mice. As previous studies in M_1−/− or M_4−/− mice typically used only male mice or reported results as sexes combined, we cannot draw parallels to potential sex effects in those investigations. More generally, in rodents most cholinergic neurons express estrogen receptors and cholinergic systems show many sex differences, including rates of maturation and aging, cell size and enzyme activity, and sensitivity to pharmacological manipulations or lesions (Fragkouli et al. 2006). Sex differences in brain muscarinic binding sites were also reported in wild-type mice (Fragkouli et al. 2006). Thus it may be that background sex differences in cholinergic systems resulted in male and female mice developing compensatory mechanisms differently, the male mice being perhaps better able to surmount effects of the mutation as measured in this investigation. There are numerous examples of sex differences in the expression of gene deletions in mice, including of muscarinic receptors. We previously saw PPI deficits in M_5−/− mice that were more pronounced in female than in male mice (Thomsen et al. 2007). In other studies, female M_5−/− mice were protected from the reduction in cerebral blood flow seen in male mice, and ovariectomy uncovered this phenotype in the female mice (Kitamura et al. 2009). Male and female M_1−/− or M_2−/− mice showed differential phenotypes in stress responses, with the clearest phenotypes in female versus male M_1−/− mice, but the converse in M_2−/− mice (Rhodes et al. 2005, 2008). In humans, reports of reduced muscarinic receptor levels in schizophrenic patients typically reported sexes combined, and were also observed in an all-male sample (Deng and Huang 2005). Thus the differences between sexes we observed may be more related to general sex differences in mouse cholinergic systems development, rather than being predictive of related sex differences in human schizophrenic patients, although the latter cannot be excluded. While sex differences in human cholinergic systems are not well-characterized, recent evidence that estrogen therapy in post-menopausal women protected against age-related loss of brain M_1/M_4 receptor density suggests some interaction between brain muscarinic systems and sex hormones in humans (Norbury et al. 2007). Further studies, e.g., using overiectomized mice, are warranted to explore interactions between sex, muscarinic receptor-related phenotypes, and drug effects.

In addition to affecting baseline PPI, the M_1 and M_4 receptor mutations affected the pharmacological modulation of startle and PPI. The non-subtype selective muscarinic

antagonist scopolamine decreased PPI in male and female wild-type mice, consistent with previous reports in rats and mice (Wu et al. 1993; Jones and Shannon 2000; Sipos et al. 2001; Stanhope et al. 2001; Ukai et al. 2004; Jones et al. 2005). We found that in female mice lacking M_1 receptors alone or both M_1 and M_4 receptors, scopolamine failed to significantly suppress PPI. Mice lacking M_4 receptors alone showed decreases in PPI comparable to wild-type mice. While it cannot be excluded that the lack of effect in the $M_1-/-M_4-/-$ mice reflects a "floor effect" due to the low baseline PPI, this is unlikely to be the case for the $M_1-/-$ mice, which showed baseline PPI comparable to wild-type mice. Based on pharmacological studies, some authors have suggested an involvement of M_3 or M_4 receptors in muscarinic antagonist-induced PPI disruption (Ukai et al. 2004). Others demonstrated the difficulty in obtaining conclusive evidence to implicate one receptor subtype based on available, moderately selective, muscarinic antagonists (Jones and Shannon 2000). While not excluding the possible contribution of other subtypes, our results support a role of M_1, but not M_4 receptors in the disruption of PPI by scopolamine. Scopolamine also increased startle reactivity in all genotypes, in agreement with a previous study in rats (Sipos et al. 2001), although in many investigations scopolamine did not increase startle reactivity significantly (Jones and Shannon 2000; Ouagazzal et al. 2001; Stanhope et al. 2001). In contrast to PPI, the deletion of M_4 receptors or of both M_1 and M_4 receptors seemed to exacerbate the effect of scopolamine on startle but also resulted in increased baseline startle reactivity, while the M_1 mutation had little effect. This dissociation between startle and PPI, along with a general lack of correlation between startle and PPI, makes it unlikely that scopolamine-induced decreases in PPI were attributable to direct effects on startle reactivity. In summary our data are consistent with a role of both M_1 and M_4 receptors in scopolamine's behavioral effects in mice, but most clearly supports a role of M_1 receptors in its PPI-disrupting effect.

The M_1/M_4-preferring agonist xanomeline has been suggested as a novel antipsychotic agent, showing antipsychotic-like effects in both preclinical and clinical studies (Shannon et al. 2000; Stanhope et al. 2001; Andersen et al. 2003; Bodick et al. 1997; Shekhar et al. 2008). In the present study xanomeline pretreatment reversed scopolamine-induced disruption of PPI in wild-type mice, in agreement with previous studies in rats showing amelioration of apomorphine- or scopolamine-induced PPI disruption (Stanhope et al. 2001; Jones et al. 2005). Xanomeline has moderate functional selectivity for M_1/M_4 receptors over other muscarinic subtypes, and may also elicit effects via non-muscarinic receptors, such as 5-HT receptors (Shannon et al. 1994; Watson et al. 1998; Langmead et al. 2008a). It is not well established which receptors mediate antipsychotic-like effects of xanomeline. Here we found that the PPI-ameliorating effect of xanomeline was absent in the $M_4-/-$ and $M_1-/-M_4-/-$ mice, but was largely intact in the $M_1-/-$ mice, suggesting a critical role of M_4 but not M_1 receptors in xanomeline's antipsychotic efficacy. This finding is consistent with the hypothesis advanced by Bymaster and coworkers, who proposed that M_4 agonists may have antipsychotic effects while M_1 agonists may be useful primarily as cognitive enhancers (Bymaster et al. 2002). Similarly a recent report indicated that xanomeline's ability to reverse amphetamine-induced hyperactivity was absent in $M_4-/-$ mice, with a trend for attenuation in $M_1-/-$ mice (Woolley et al. 2009). Also, recently developed M_4-selective positive allosteric modulators showed antipsychotic-like effects in preclinical assays in rats, including PPI (Brady et al. 2008; Chan et al. 2008). Taken together these observations suggest xanomeline exerts antipsychotic-like effects primarily through M_4 receptor stimulation.

We further assessed whether a non-selective muscarinic agonist would reverse scopolamine-induced PPI disruption and whether those effects would similarly be lacking in the $M_1-/-M_4-/-$ and $M_4-/-$ mice. We found that oxotremorine ameliorated PPI regardless of genotype. The same doses of oxotremorine *per se* failed to increase PPI, with an apparent

but non-significant increase in the M_1−/−M_4−/− mice only. The highest doses decreased startle amplitude dramatically, ruling out that too low doses were tested to observe an effect. Previous investigations similarly showed oxotremorine ameliorated PPI disrupted by apomorphine, methamphetamine or ketamine in rats, and increased PPI only in a mouse strain with low baseline PPI (Jones et al. 2005; Maehara et al. 2008). Therefore the lack of increase in PPI (without scopolamine challenge) in the present study likely reflects a "ceiling effect" rather than a scopolamine/oxotremorine interaction. Because M_1 and M_4 receptor deletions did not diminish oxotremorine's effects in the present investigation, our findings indicate that M_2, M_3 or M_5 receptor stimulation is sufficient to ameliorate PPI, at least in the knockout mice. The extent to which compensatory changes in the knockout mice may have altered contributions of other receptors cannot be fully ascertained. However the targets that were investigated revealed no changes: no changes in muscarinic M_2, dopamine $D_{1\text{-like}}$ or $D_{2\text{-like}}$ receptor expression were detected in M_4−/− mice, and no changes in striatal M_2-M_5 receptor expression were detected in M_1−/− mice (Gomeza et al. 1999; Miyakawa et al. 2001). We previously reported decreased PPI in M_5−/− mice, suggesting a role of M_5 receptors (Thomsen et al. 2007). While M_2−/− mice showed normal PPI, they have shown profound deficits in neuronal plasticity as well as impaired performance in cognitive tests (Felder et al. 2001; Bymaster et al. 2002; Tzavara et al. 2003; Seeger et al. 2004). In addition the M_2/M_4 partial agonist M_1 antagonist BuTAC reversed apomorphine-suppressed PPI in rats (Jones et al. 2005). Thus M_2 and M_5 receptor stimulation both appear plausible additional mechanisms of PPI amelioration, at least in mice lacking M_1 and/or M_4 receptors.

Finally we evaluated the ability of the atypical antipsychotic clozapine and of the typical antipsychotic haloperidol to ameliorate the PPI deficit seen in the female M_1−/−M_4−/− mice. Clozapine and its metabolites have high affinity for muscarinic receptors, and M_1 stimulation has been speculated to contribute to clozapine's antipsychotic effects (Olianas et al. 1999; Sur et al. 2003; Weiner et al. 2004; Davies et al. 2005). We found that clozapine increased PPI in the female M_1−/−M_4−/− mice, reversing the PPI deficit to wild-type levels. Thus neither M_1 nor M_4 receptors likely played a significant role in the antipsychotic-like effects of clozapine in the present experiments. Our results are consistent with a previous report showing comparable attenuation of locomotor activity by clozapine in wild-type and M_1−/− mice (Gerber et al. 2001). Clozapine appears to be only a weak partial agonist at M_1 receptors in vivo, and behavioral data are consistent with general muscarinic antagonist, rather than agonist, actions of clozapine (Bymaster et al. 2003; Prus et al. 2004). Taken together these observations do not support a significant role of M_1 (or M_4) receptor stimulation in mediating antipsychotic-like effects of clozapine. At high doses, we found that clozapine strongly suppressed startle reactivity and produced parallel decreases in PPI in wild-type mice. This is in agreement with similar effects on startle in rats and mice (Ouagazzal et al. 2001; Maehara et al. 2008). In contrast to PPI this effect of clozapine was absent in the M_1−/−M_4−/− mice and dramatically reduced in the M_4−/− mice, but intact in the M_1−/− mice. Thus it is possible that M_4 receptors play a role in adverse effects of clozapine, although the doses at which these effects were overt in the present study may not be clinically relevant. Finally, haloperidol also increased PPI in the M_1−/−M_4−/− mice, comparable to its effect in wild-type mice. Thus the PPI deficit seen in M_1−/−M_4−/− mice could be reverted by clozapine or haloperidol administration, with no indication that M_1 or M_4 receptors were essential to the antipsychotic-like effects of either drug.

In conclusion, we evaluated PPI and pharmacological modulation of PPI in mice lacking muscarinic cholinergic M_1 or M_4 receptors or both receptor subtypes. We found that constitutive deletion of either subtype did not affect baseline PPI but that combined deletion of both subtypes decreased PPI in female mice, lending support to the theory that muscarinic dysfunction with decreased M_1/M_4 receptor densities is involved in the etiology of schizophrenia. The reason why the phenotype was apparent only in female animals remains

unclear and deserves further investigation. Our data also suggested that suppression of PPI by the muscarinic antagonist scopolamine was dependent upon M_1 receptors and that amelioration of PPI by the potential antipsychotic drug xanomeline was dependent upon M_4 receptors. The latter finding supports the concept that the M_4 receptor, rather than the M_1 receptor, represents a key target for developing potential novel antipsychotic drugs. Finally, clozapine and haloperidol both ameliorated PPI in the double knockout mice, failing to support the notion that M_1 stimulation is critical to clozapine's antipsychotic efficacy.

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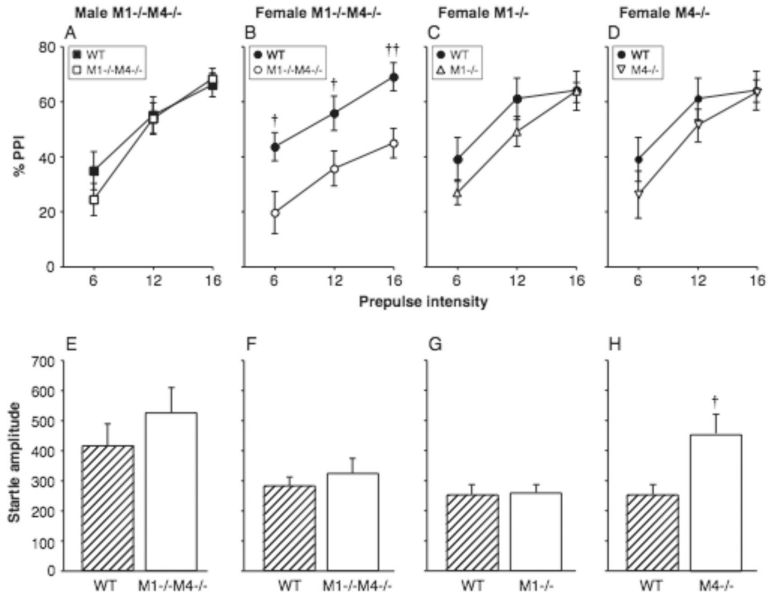

Figure 1. PPI was decreased in female mice lacking both M_1 and M_4 receptors

Abscissae: prepulse intensity (dB above background noise; A–D), genotype (E–H). Ordinates: %PPI (A–D), startle amplitude (arbitrary units; E–H). N=9–13. Data are group means, bars represent one s.e.m. †p<0.05, ††p<0.01 vs. wild-type, unpaired-sample t-test.

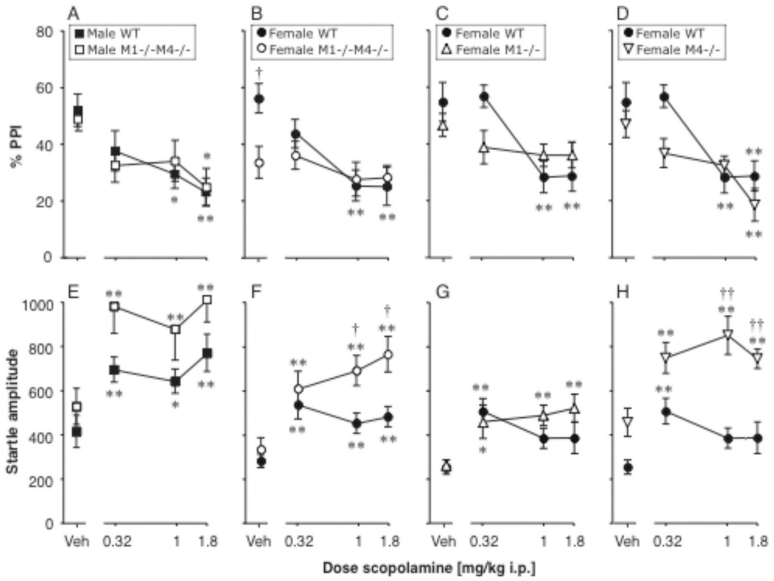

Figure 2. The PPI-suppressing effect of scopolamine was absent in female mice lacking M_1 receptors or both M_1 and M_4 receptors

In contrast, scopolamine increased startle reactivity in all genotypes. Abscissae: scopolamine dose [mg/kg]. Ordinates: Total %PPI (A–D), startle amplitude (E–H). N=9–13. Data are group means, bars represent one s.e.m. *p<0.05, **p<0.01 vs. vehicle, Dunnett's multiple comparisons test; †p<0.05, ††p<0.01 vs. wild-type, Bonferroni-corrected unpaired-sample t-test.

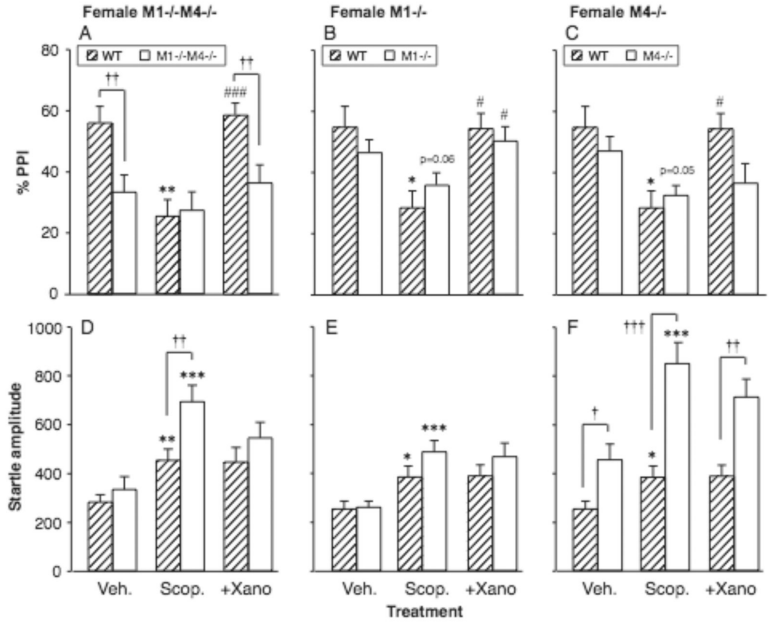

Figure 3.

Xanomeline increased scopolamine-suppressed PPI in wild-type mice and in mice lacking M_1 receptors, but not in mice lacking M_4 or both receptors. Abscissae: treatment, Veh. = vehicle, Scop. = scopolamine 1.0 mg/kg, +Xano = scopolamine 1.0 mg/kg with xanomeline 3.2 mg/kg pretreatment. Ordinates: Total %PPI (A–D), startle amplitude (E–H). N=9–12. Data are group means, bars represent one s.e.m. *p<0.05, **p<0.01, ***p<0.001 vs. vehicle; #p≤0.05, ###p<0.001 vs. scopolamine alone, paired-sample t-test; †p<0.05, ††p<0.01, †††p<0.001vs. wild-type, unpaired-sample t-test.

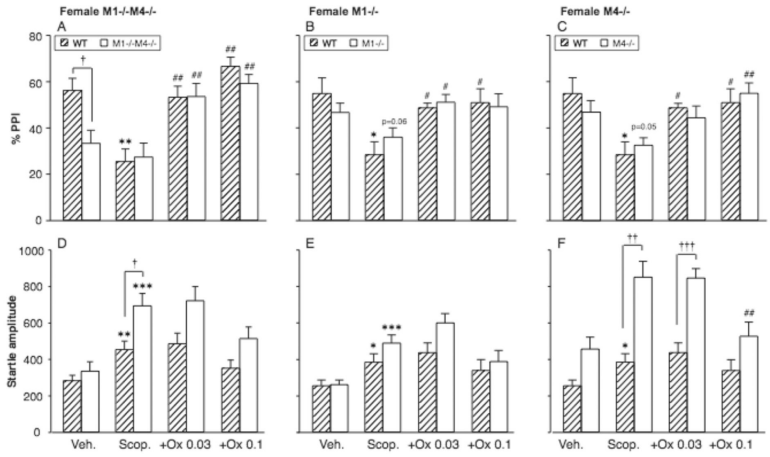

Figure 4. Oxotremorine increased scopolamine-suppressed PPI regardless of M_1 or M_4 genotype

Abscissae: treatment, Veh. = vehicle, Scop = scopolamine 1.0 mg/kg, +Ox = scopolamine 1.0 mg/kg with oxotremorine pretreatment, 0.032 and 0.1 mg/kg. Ordinates: Total %PPI (A–D), startle amplitude (E–H). N=9–12. Data are group means, bars represent one s.e.m. *p<0.05, **p<0.01. ***p<0.001 vs. vehicle, paired-sample t-test; †p<0.05, ††p<0.01, †††p<0.001 vs. wild-type, unpaired-sample t-test; #p<0.05, ##p<0.01 vs. scopolamine alone, Dunnett's multiple comparisons test.

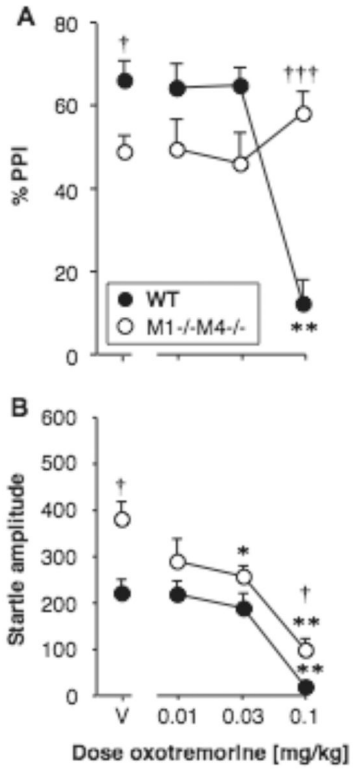

Figure 5. Oxotremorine alone did not increase PPI

Abscissae: dose oxotremorine [mg/kg]. Ordinates: Total %PPI (A), startle amplitude (B). N=11. Data are group means, bars represent one s.e.m. *p<0.05, **p<0.01 vs. vehicle, Dunnett's multiple comparisons test; †p<0.05, †††p<0.001 vs. wild-type, Bonferroni-corrected unpaired-sample t-test.

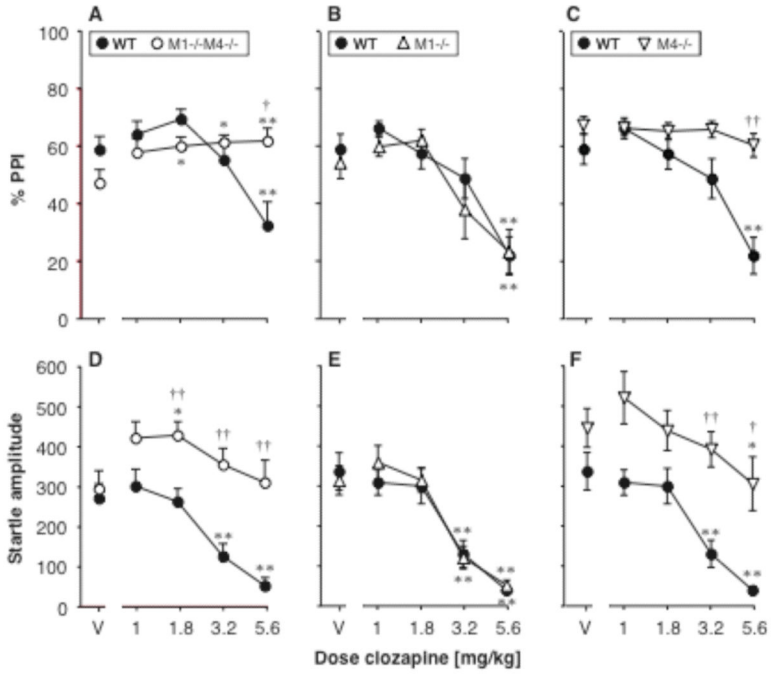

Figure 6. Clozapine ameliorated the PPI deficit in the female mice lacking M_1 and M_4 receptors, while startle-suppressing effects of high doses were absent in mice lacking M_4 or both receptors

Abscissae: dose clozapine [mg/kg]. Ordinates: Total %PPI (A–C), startle amplitude (D–F). N=9–12. Data are group means, bars represent one s.e.m. *p<0.05, **p<0.01 vs. vehicle, Dunnett's multiple comparisons test; †p<0.05, ††p<0.01 vs. wild-type, Bonferroni-corrected unpaired-sample t-test.

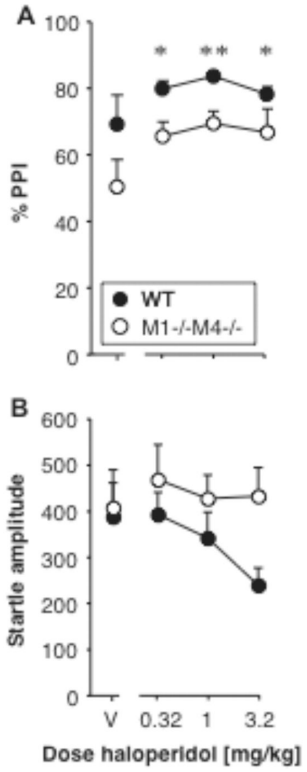

Figure 7. Haloperidol increased PPI regardless of M_1/M_4 genotype

Abscissae: dose haloperidol [mg/kg]. Ordinates: Total %PPI (A), startle amplitude (B). N=6. Data are group means, bars represent one s.e.m. *p<0.05, **p<0.01 vs. vehicle, Dunnett's multiple comparisons test (genotypes combined).