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Decreased Amphetamine-Induced Locomotion and Improved Latent Inhibition in Mice Mutant for the M5 Muscarinic Receptor Gene Found in the Human 15q Schizophrenia Region

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M5 muscarinic receptors are coexpressed with D2 dopamine receptors in the ventral tegmentum and striatum, and are important for reward in rodents. Previously, we reported that disruption of the M5 receptor gene in mice reduced dopamine release in the nucleus accumbens. In this study, we established a polymerase chain reaction (PCR) genotyping method for M5 mutant mice, and, using RT-PCR, found that M5 mRNA expression was highest in the ventral tegmentum, striatum, and thalamus in wild-type mice. In the M5 mutant mice, D2 mRNA expression was increased in several brain structures, including the striatum. Genome mapping studies showed the M5 gene is localized to chromosome 2E4 in mice, and to 15q13 in humans in the region that has been linked to schizophrenia. Amphetamine-induced locomotion, but not baseline locomotion or motor functions, decreased in M5 mutant mice, consistent with lower accumbal dopamine release. Previous reports found latent inhibition improvement in rats following nucleus accumbens lesions, or blockade of dopamine D2 receptors with neuroleptic drugs. Here, latent inhibition was significantly increased in M5 mutant mice as compared with controls, consistent with reduced dopamine function in the nucleus accumbens. In summary, our results showed that M5 gene disruption in mice decreased amphetamine-induced locomotion and increased latent inhibition, suggesting that increased M5 mesolimbic function may be relevant to schizophrenia.
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INTRODUCTION

The effects of amphetamine and neuroleptic drugs in humans indicate that high dopamine function is responsible for positive symptoms of schizophrenia (Carlsson, 2001). Genetic factors on several chromosomes are believed to influence schizophenia, but most of these genes are not yet clearly identified (Kennedy *et al*, 2003).

Amphetamine-induced locomotion and latent inhibition reduction in rats are caused by high dopamine function in the nucleus accumbens. For example, 6-hydroxydopamine lesions of dopamine terminals in nucleus accumbens block locomotion induced by systemic amphetamine, and injections of amphetamine into nucleus accumbens induce locomotion at low doses (Joyce and Koob, 1981; Ikemoto, 2002). Nucleus accumbens lesions improve latent inhibition, and block the effects of amphetamine or D2 dopamine receptor blockers (Joseph *et al*, 2000). In rats, latent inhibition can be attenuated by increasing dopamine release with amphetamine (Russig *et al*, 2002; Weiner and Feldon, 1987), or facilitated by blocking dopamine receptors with neuroleptics (Moser *et al*, 2000).

Latent inhibition occurs when the association between paired stimuli is reduced by pre-exposure to the conditioned stimulus (CS) without the unconditioned stimulus (US), allowing subjects to ignore stimuli that previously did not predict US occurrence. In nonmedicated, acute schizophrenia, latent inhibition is reduced (Lubow and Gewirtz, 1995; Williams *et al*, 1998; Gray *et al*, 1995). Therefore, latent inhibition deficits provide an animal model of positive symptoms of schizophrenia (Gray, 1998; Weiner,

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2003). The brainstem sources of dopamine activation relevant to latent inhibition, and the genes critical for dopamine-dependent changes in latent inhibition have not been studied.

Mesolimbic input to the nucleus accumbens originates in dopamine neurons of the ventral tegmental area (VTA), where M5 muscarinic receptors activate dopaminergic functions (Yeomans *et al*, 2001). M5 mRNA is colocalized with mRNA for D2 dopamine receptors near VTA and substantia nigra dopamine neurons, and M5 mRNA is lost after 6-hydroxydopamine lesions (Weiner *et al*, 1990; Vilaro *et al*, 1990). Both M5 and D2 receptors strongly influence dopamine release from striatal terminals (Forster *et al*, 2001; 2003; Zhang *et al*, 2002; Schmitz *et al*, 2002). Generation of knockout mice for each of the 5 muscarinic genes has allowed analysis of their functions (Bonner *et al*, 1987; Hamilton *et al*, 1997; Gomeza *et al*, 1999). Reduction of M5 receptors reduced brain-stimulation reward in rats (Yeomans *et al*, 2000) and opiate reward was reduced in M5 mutant mice (Basile *et al*, 2002).

Mesopontine cholinergic neurons activate mesolimbic dopamine neurons (Bolam *et al*, 1991; Lacey *et al*, 1991). Stimulation of mesopontine cholinergic cell groups in rats increases striatal dopamine release in two VTA-dependent phases: a nicotinic phase lasting 3 min and a muscarinic phase lasting from 10–60 min (Forster and Blaha, 2000, 2003). In M5 mutant mice, the muscarinic phase was lost, indicating that M5 receptors are needed for prolonged accumbal dopamine release (Forster *et al*, 2001).

Muscarinic receptors are known to contribute to schizophrenia (Singh and Kay, 1985; Tandon and Greden, 1989; Yeomans, 1995; Perry and Perry, 1995; Bymaster *et al*, 1999). The importance of M5 receptors to mesolimbic dopamine functions suggested a role for these receptors in schizophrenia (Yeomans, 1995). Therefore, we studied changes in amphetamine-induced locomotion, latent inhibition, and M5 and D2 mRNA expression in M5 mutant and wild-type mice. The chromosomal locations of the M5 gene in mice and human genomes were identified, and compared with genetic linkage studies of schizophrenia involving chromosome 15q12–15.

MATERIALS AND METHODS

Animals

M5 mutant mice were created by recombinant methods at the University of Toronto by Takeuchi *et al* (2002). The M5 muscarinic gene is a single exon with 1599 bases in the coding regions in both mouse (accession number AF 264051) and human (Bonner *et al*, 1991; Liao *et al*, 1989). The mouse, and human sequences are identical except for 188 of these bases, with a resulting change in less than 10.7% of amino acids, 85% of which are in the long ic3 loop (Bonner *et al*, 1988; Bonner, 1989; Wess *et al*, 1996). A deletion of 503 b in the ic3 loop of the M5 muscarinic receptor gene was previously identified by a 0.5 kb shift in genomic DNA Southern blot bands.

The mice resulting from this mutation were healthy in homozygous, heterozygous, and wild-type genotypes (CD1 X 129SvJ strain). All experimental animals were housed at the Zoology animal facility of the University of Toronto and maintained in a light-controlled room (12 h light/dark cycle) under standard animal housing conditions. Mice were group-caged, and food and water were available *ad libitum* except during behavioral testing. All experimental protocols using animals were performed in accordance with the *Guide to the Care and Use of Experimental Animals* (Canada), and approved by the Animal Care Committee of the University of Toronto.

Genotypes of four mice randomly selected from each of the three groups were confirmed by a fast polymerase chain reaction (PCR) method newly developed here. Two pairs of primers were used: one pair for specific amplification of a fragment covering the deleted part of the mouse M5 gene; the other pair for the neomycin-resistance gene (neoR), a marker of the mutation.

For latent inhibition studies, male and female mice of wild-type and homozygous mutant genotypes were matched for age (2–4 months) and mass (25–35 g), and housed in separate cages throughout pretraining, training, and testing. The mean litter sizes of these two groups of mice were not found to differ. For motor studies, only male mice were used, but the age (2–4 months), mass (25–35 g), and separate housing conditions were the same as above.

Reagents and Kits

The drugs used in the present study were obtained from Sigma Canada (Oakville, ON, Canada) unless otherwise indicated. The kit for reverse transcription (RT) was from Applied Biosystems (Foster City, CA, USA), and the kit for PCR were from Qiagen Inc. (Mississauga, ON, Canada).

Primers used: P1 (sense primer), neoR U: 5′-agg atc tcc tgt cat ctc acc ttg ct-3′; P2 (antisense primer), neoR D: 5′-aag aac tcg tca aga agg cga tag aa-3′. The product of 492 bp was used for amplification of neoR. P3 (sense primer), MR-M5-U4: 5′-ata ctc tac tgc cgg atc tac cgg ga-3′; P4 (antisense primer) MR-M5-D4: 5′-tgt gat gat gaa ggc cag gag aat gg-3′. The product of 732 bp was used for amplification of the mouse M5 muscarinic receptor gene, covering the internal deleted part and partial sequences outside the deleted sequence. P5 (sense primer) M-M5SA-UA-U1: 5′-aag aag aga aaa ccg gct cac agg-3′; P6 (antisense primer) M-M5SA-UA-D1: 5′-ctc ctg ggt ccc gtc agc ttt tac-3′. The product of 436 bp was used for amplification of the deleted part of the mouse M5 receptor gene. P7 (sense primer), HM-A-Tub-U: 5′-att ggc aag gag atc att gac-3′ (376–396, NM_011653); P8 HM-A-Tub-D (antisense primer): 5′-caa cct cct cat aat cct tct-3′ (1234–1254, NM_011653). The product of 929 bp was used for specific amplification of the mouse α-tubulin gene. P9 HM-D2R-U (sense primer): 5′-gat ctg gag agg cag aac tg-3′ (163–182, NM_010077); P10 HM-D2R-D (antisense primer): 5′-aga tgt cac agt gaa tcc tg-3′ (438–457, NM_010077). The product of 295 bp was used for specific amplification of the mouse dopamine D2 receptor gene.

Genomic DNA PCR and PCR Southern Blot Analyses for Genotyping of Wild-Type, Heterozygous, and Mutant Mice

For PCR, genomic DNA was isolated from mouse tails following the methods of Strauss (1999). Briefly, 8–10 mm of the tip of mouse tails (cut under anesthesia) were digested

with lysis buffer (Tris-HCl 100 mM, EDTA 5 mM, SDS 0.2%, NaCl 200 mM, proteinase K 0.2 mg/ml) at 50°C for 18 h. These was treated with equal volumes of phenol/chloroform for 20 min and centrifuged at 1700*g*, 4°C for 10 min. The supernatant was removed, adding one-third volume of 7 M NH_4Cl and two volumes of ethanol. After centrifugation, the pellet was washed with 70% ethanol and dried at room temperature. The genomic DNA was then dissolved with TE buffer and stored at 4°C.

Primers used for PCR genotyping were P1 neoR U and P2 neoR D to amplify a 492-bp fragment, and P3 MR-M5-U4 and P4 MR-M5-D4 to amplify a 732-bp fragment of the mouse M5 gene. In all, 200 ng gDNA in a final volume of 50 μl was used. A measure of 10 μl of the products was resolved on 1% agarose gels containing 0.5 μg/ml ethidium bromide, visualized under UV light, and photographed (Polaroid, Cambridge, MA, USA). Thereafter, the cDNAs were transferred onto Hybond™-N membrane by capillary transfer and UV autocrosslinked. Then, PCR Southern blot analysis was completed according to previously described procedures (Wang *et al*, 1997, 1999). Briefly, the filters were prehybridized for 12 h at 42°C, and then hybridized for 24 h at 42°C in a hybridization buffer with a random primed [α-^{32}P]dCTP-labeled cDNA probe for the mouse M5 gene. The probes were cloned by T-vector ligation with the PCR-amplified cDNA, confirmed by sequence analysis, and purified with a G-50 Nick column (Amersham Biosciences, Québec, QC, Canada). Washes were performed in 0.5 × SSC, 0.1% SDS at 65°C. After stripping and prehybridization, the blots were then hybridized sequentially with the neoR gene.

Semiquantitative RT-PCR for Regional Expression of M5 and D2 mRNA

Mice, 8-week old, were decapitated, and their tissues from different brain regions were dissected and snap-frozen on dry ice. The boundaries used for brain region dissections were based on the Franklin and Paxinos (1997) mouse brain atlas. Total RNA was extracted with TRI reagent. Contaminating genomic DNA was removed by DNase I digestion (2 U of DNase I/μg of RNA; Roche), followed by acidic phenol/chloroform extraction. RT-PCR was performed on a Perkin–Elmer DNA Thermal Cycler (GMI, Inc., Albertville, MN, USA). Primer/probe sets were designed using Vector NTI Suite (InforMax, Frederick, Maryland, USA) and the sequences are available in the GenBank database. For the first-strand synthesis, 1 μg of total RNA in a final volume of 20 μl was used (Wang *et al*, 1997). The reaction mixture was incubated at 25°C for 10 min and then followed by another incubation at 42°C, heated to 94°C for 5 min to denature the reverse transcriptase, cooled on ice for 3 min, and stored at −20°C.

For PCR, primer pairs for the detection of the mouse M5 muscarinic (P5 and P6), dopamine D2 receptor (P9 and P10), and α-tubulin mRNA (P7 and P8) were used as described above. To control for PCR products deriving from amplification of residual genomic DNA, an aliquot of RNA from each tissue was subjected to PCR without preceding cDNA synthesis. M5 cDNA was selectively amplified using the gene-specific primers P5 and P6 that generate a 436 bp fragment of the M5 coding region. The PCR products were resolved on 1% agarose gels containing 0.5 μg/ml ethidium bromide, visualized under UV light, and photographed (Polaroid, Cambridge, MA, USA). PCR products were gel-purified (Qiagen, Mississauga, ON, Canada) and sequenced using primers P5 and P6; P7 and P8; P9 and P10. The proper cycles for semiquantification of M5, D2R, and α-tubulin mRNA levels were determined to be 36, 34, and 27, respectively, by varying cycles from 23 to 40.

In order to quantify the levels of mRNA expression, measurements of relative optical density, assessing the mean gray value per pixel of the measured area for the bands of PCR products, were made with Scion Image software (NIH, Bethesda, MD, USA). To calculate the relative optical density, the background density of film was subtracted from the density of the bands. The relative optical density of each band was divided by the optical density of matched α-tubulin bands to determine '% Relative Densitometry'. For further statistical analysis, the data are presented as means ± SD of four independent experiments, performed in triplicate, with each experiment using tissues pooled from three mice. The mRNA expression levels were analyzed separately within each brain region by one-way ANOVA (with the single variable being M5 gene status, 3 levels), followed by *t*-tests.

Motor Tests

Rotarod test. The apparatus consisted of a rod 4.5 cm in diameter, covered with a nylon grid surface, and driven by a variable-speed motor (New England Gear Works, Model G5H, Southington, CT, USA). The top of the rod was 34 cm above the table. Mice were placed individually on the rod for at least 30 s before the motor was engaged, and the rod was rotated at a speed of 5 revolutions per minute (rpm) for a maximum of 90 s, and then rotated at 15 rpm. The time that each animal remained on the rod at each rotation speed was recorded. The maximum trial duration was 240 s, and the intertrial interval was 30 min. Animals were tested in three trials per day for 2 consecutive days (Kihara *et al*, 2001). Rotarod test data were analyzed using Student's *t*-tests.

Pole climbing test. The cylindrical metal pole (1-cm diameter, 60-cm long) was covered with cloth-tape. With the pole inclined 45°, a mouse was first placed at the center with the body axis perpendicular to the pole. The amount of time (seconds) needed for the mouse to turn around and climb the 30 cm down the pole was measured.

Negative geotaxis test. Each mouse was placed at the center of a metal grid, 23 × 23 cm, with the metal wires spaced 1.2 cm apart. At the start of the test, the grid was oriented vertically, with the lowest point approximately 20 cm above the table (ie the mouse was facing vertically downwards about 30 cm above the table). The mouse then was allowed to turn and climb on the vertical grid for up to 30 s. After the 30 s, the mouse was removed from the grid and returned to the middle of the grid, and the grid was turned 90° further, so that the mouse was hanging from the grid. Their ability to hold onto the grid and move freely in the upside down position was tested for another 30 s.

Locomotor Activity Test

Locomotor activity was measured as described by Chintoh *et al* (2003) with some modifications. Locomotor activity was measured in two automated, open-field locomotion boxes ($43 \times 43 \times 30$ cm) (MED Associates Inc., St Albans, VT, USA). Infrared beams across the base of the box, and 15 cm above the floor, recorded horizontal movements made by each mouse (Activity Monitor Version 3.25, MED Associates Inc., St Alban's, VT, USA).

Before starting the locomotion experiments, all mice were placed in individual cages in the same room for at least 3 days. All testing occurred in the dark part of their cycle (1000 to 1800 hours). For each dose, eight wild-type and eight homozygous mutant mice were tested as a set over 6 days, with each mouse receiving three continuous days of 2 h exposure to the open-field apparatus. These mice were matched for age (2–4 mo) and mass (25–35 g). Groups of animals were counterbalanced as fully as possible with respect to genotype.

The formal behavioral testing took place over 3 consecutive days: day 1, habituation and sham injection; day 2, saline test; and day 3, amphetamine test. On all 3 days, mice were habituated to the test room in home cages for 15 min before placement into the open-field apparatus. On day 1, each mouse was placed in the apparatus singly for 120 min. Each mouse was taken out of the apparatus after 60 min and given a sham injection (without saline or amphetamine), then returned to the apparatus for 60 min more without collecting data. On day 2, the mice were weighed to determine the dose of saline or amphetamine to be used, and then placed into the open-field apparatus for 60 min. The mice were then given a saline injection (about 0.3 ml i.p., but proportional to the mass of the mouse). Soon after injection, the mice were returned to the apparatus, and the locomotor activity was recorded with a software program (Activity Monitor, Version 3.25, MED Associates Inc., St Alban's, VT, USA) for 60 min as total horizontal distance moved. Day 3 proceeded exactly as day 2, except that each mouse was injected with a single dose of D-amphetamine sulfate, in the same volume of saline as on day 2. Before each mouse was tested, the open-field apparatus was wiped with 70% ethanol and allowed to dry completely.

On subsequent weeks, independent groups of 16 mice were tested in the same way at ascending doses of amphetamine (1 mg/kg i.p. for the first group, then 2, 4, and 8 mg/kg i.p.).

Locomotion data were analyzed using a two-way ANOVA (with genotype and dose of the two variables), followed by individual comparisons between mutant and wild-type mice at each dose using Student's *t*-tests with Bonferroni correction.

Tail-Withdrawal Test

Mice were assessed for nociceptive sensitivity on the 49°C tail-withdrawal test (Kest *et al*, 2001). In this assay of acute, thermal nociception, the mouse was gently restrained by the experimenter and the distal half of the tail was immersed in water maintained at 49.0 ± 0.2°C by an immersion circulator pump (Model JULAO 12B, JULABO Lobortechnik, Germany). Latency to reflexive withdrawal of the tail was measured three times by an experimenter to the nearest 0.1 s, with each determination separated by 20 s, to measure the mean withdrawal latency. This tail-withdrawal test was chosen because of its stability even after repeated exposures at this water temperature (D'Amour *et al*, 1941). A cutoff latency of 15 s was employed to prevent the possibility of tissue damage, and to minimize unnecessary animal distress. All mice were tested in light near the onset of lights-off in their colony room. Tail-withdrawal test data were analyzed using Student's *t*-tests.

Acoustic Startle and Prepulse Inhibition (PPI) Tests

Apparatus. Mice were placed in four animal holders (ENV-263, MED Associates Inc., St Albans, VT, USA). Each holder rested on a solid base inside a sound-attenuated acoustic startle cubicle. A loudspeaker mounted inside each cubicle produced a continuous background noise of 65 dB (white noise) in the holder. Vibrations of the startle chamber during the startle response of the mouse were converted into analog signals by a piezoelectric unit attached to the platform (ENV-250–60). These signals were digitized and stored by a computer. The startle response was recorded for 65 ms by sampling the response every 1 ms starting with the onset of the startle stimulus. The maximum startle amplitude recorded during the 65-ms sampling window was used as the dependent variable. The sensitivity of the stabilimeter was calibrated to ensure consistency between chambers and across sessions.

Procedures. A test session began by placing a mouse in the holder which was then placed in the cubicle with background noise, where the mouse was left undisturbed for 5 min. The hearing and reflex abilities of the mice were tested in a single session, by measuring the acoustic startle reflex following the startling noise (120 dB SPL for 40 ms), and measuring PPI following a 20 ms, 81 dB prepulse presented before the startling noise.

A session consisted of 60 discrete trials, in two blocks of 30 trials, with 30 s between trials. Each trial consisted of the prepulse (or no prepulse), followed by the startling pulse, at interpulse intervals that varied randomly from 20, 50, 100, 200 to 250 ms. A total of 10 trials were tested at each interpulse interval, along with 10 trials of the startling pulse with no prepulse. In total, 10 mice were tested, six (+/+) and four (−/−) for the M5 mutation.

Statistical analyses. Baseline startle amplitudes were compared using Student's *t*-tests. PPI was measured by the reduction of startle magnitude in prepulse trials relative to that in pulse-alone trials. Percent PPI was calculated for each animal according to the following formula: $\%PPI = (1 - (response_{prepulse + startle\ stimulus}/response_{startle\ stimulus\ alone})) \times 100$). Percent PPI was analyzed using two-way between/within repeated measures ANOVA, with genotype as the between-subjects factor (two levels, M5 mutant and wild type) and with interpulse interval as the within-subjects factor (five levels, 20–250 ms). In the absence of sphericity, appropriate adjustments were made to the degrees of freedom using Greenhouse–Geisser's ε. The mean startle amplitudes were calculated first at each interpulse interval, and then each mean was divided by the mean baseline

startle response to calculate the percent startle amplitude. All statistical analyses were carried out using SPSS software.

Latent Inhibition Test

Pretraining. Procedures used to study latent inhibition in mice followed those of Gould and Wehner (1999). Mice were weighed and housed in separate cages with water bottles removed. At 24 h after removal of water bottles, 3 days of pretraining began to adapt the mice to the deprivation and drinking conditions. Mice were weighed and given access to water in a small chamber (25×25 cm) for 15 min. This test chamber was located inside a larger sound-attenuating chamber with a constant background white-noise level of 65 dB SPL delivered via a small speaker to reduce distractions from outside events. Total time of licking the water bottle was measured with a stopwatch on each day to ensure stable baseline responding in all groups. Mice were maintained at 85% of the body mass measured before deprivation, and given 15 min of extra water access in their home cages if their mass ever dipped below 80% of the original body mass.

Pre-exposure. On the fourth day, 12 wild-type and 12 homozygous mutant mice were exposed to 40 tone presentations (81 dB SPL, 60 s intertone interval, with each tone lasting for 5 s) delivered by a large 8-Ω Pacer speaker over a period of 40 min. The remaining mice (10 wild-type and 11 homozygous mutants) were placed in the chamber for 40 min without any tone presentation.

Training. On the fifth day, all mice were exposed to 2 tone-shock pairings. At 5 min after being placed in the chamber, the mice were presented with the tone (CS), and during the last second of the tone a 0.35 mA footshock (US) was presented. At 5 min after the CS–US pairing, the CS–US pairing was presented again. The mice were removed from the test chamber 5 min after this second pairing.

Reinstatement of baseline drinking session. On the sixth day, the mice were placed in the chamber for 15 min, and total time spent licking the water bottle was measured. Before testing, three mice (one wild-type, two homozygous mutants) were removed from the data set because they did not drink on pretraining and baseline reinstatement days, or were unable to maintain 80% body weight or above on these days. A total of 10–12 mice remained in each group.

Test day. On the seventh day, mice were placed in the chambers individually as before. A trained observer blind to the genotype and conditioning history of the mice measured licking times. After 16 total seconds of licking, the time taken for 4 more seconds of licking was measured. At the end of the 4 s of licking, the tone (CS) was presented for 5 s. The time taken for 4 more total seconds of licking during and after the tone was measured. Increased latent inhibition would result in a shorter time taken to complete the 4 s of licking after the tone.

Statistical analyses. All latency scores from the latent inhibition experiments were transformed to the reciprocal of latency to make variances more uniform for parametric analyses. A two-way ANOVA (SPSS version 11) was tested for wild-type and mutant mice, with pre-exposure or non-pre-exposure to the noise being one variable and genotype (2 levels) the second. To investigate performance between genotypes, the Student's *t*-test test with Bonferroni correction was used to compare the pre-exposed mutant and wild-type groups.

Chromosomal Mapping of M5 Muscarinic Receptor Genes in Human and Mouse

The cDNAs encoding human (accession No. AB084282) and mouse M5 muscarinic receptors (accession No. AF264051) were isolated from human neuroblastoma SH-SY5Y cells and mice, respectively. We localized their chromosome sites by blasting human and mouse genome resources in the NCBI data base with the full-length human and mouse cDNA sequences, respectively. Accordingly, we determined their localizations on human and mouse chromosomes. These results were further compared with previous results obtained with cytogenetic methods (Bonner *et al*, 1991).

RESULTS

PCR Genotyping of M5 Mutant Mice

Genotypes of mice used in the latent inhibition experiments were confirmed by fast PCR analysis in four mice from each genotype group. As shown in Figure 1, a1, all wild-type mice had a single band at 0.75 kb (four left lanes), all homozygous mutant mice had a single band at 0.50 kb (right lanes), and all heterozygous mutant mice had both of these bands (middle four lanes). These genotypes were further confirmed by PCR-Southern blots using [α-^{32}P]dCTP-labeled specific M5 cDNA and neoR gene probes (Figure 1, a2).

Regional Expression of M5 and D2 mRNAs in M5 Mutant Mice

We evaluated M5 and D2 mRNA levels using semiquantitative RT-PCR after DNase pretreatment of RNA samples. M5 gene expression levels were highest in wild-type mice with lower expression in heterozygous mutants in all brain areas tested (Figures 1, Part b, and Figure 2a). No M5 messenger RNA was detected in homozygous mutant mice, due to the deleted ic3 loop segment in mutant mice (Figures 1, Parts b and c, and Figure 2a). No band was detected in wild-type mice when the RT reaction was omitted (Figure 1, Parts b and c), indicating that the bands were from M5 mRNAs, and not from DNA contamination.

In wild-type mice, the highest expression of M5 mRNA was found in ventral tegmentum, striatum, hindbrain, and thalamus (Figure 2a). M5 mRNA levels were lower in the hypothalamus, tectum, frontal cortex, middle and posterior cortex, hippocampus, and cerebellum. These results extend previous reports using *in situ* hybridization (Vilaro *et al*, 1990; Weiner *et al*, 1990).

M5 and D2 mRNA were expressed together in many brain regions in wild-type mice. The expression levels of D2 mRNA varied by region, with striatum > thalamus > middle cortex > tectum > hippocampus > ventral tegmentum >

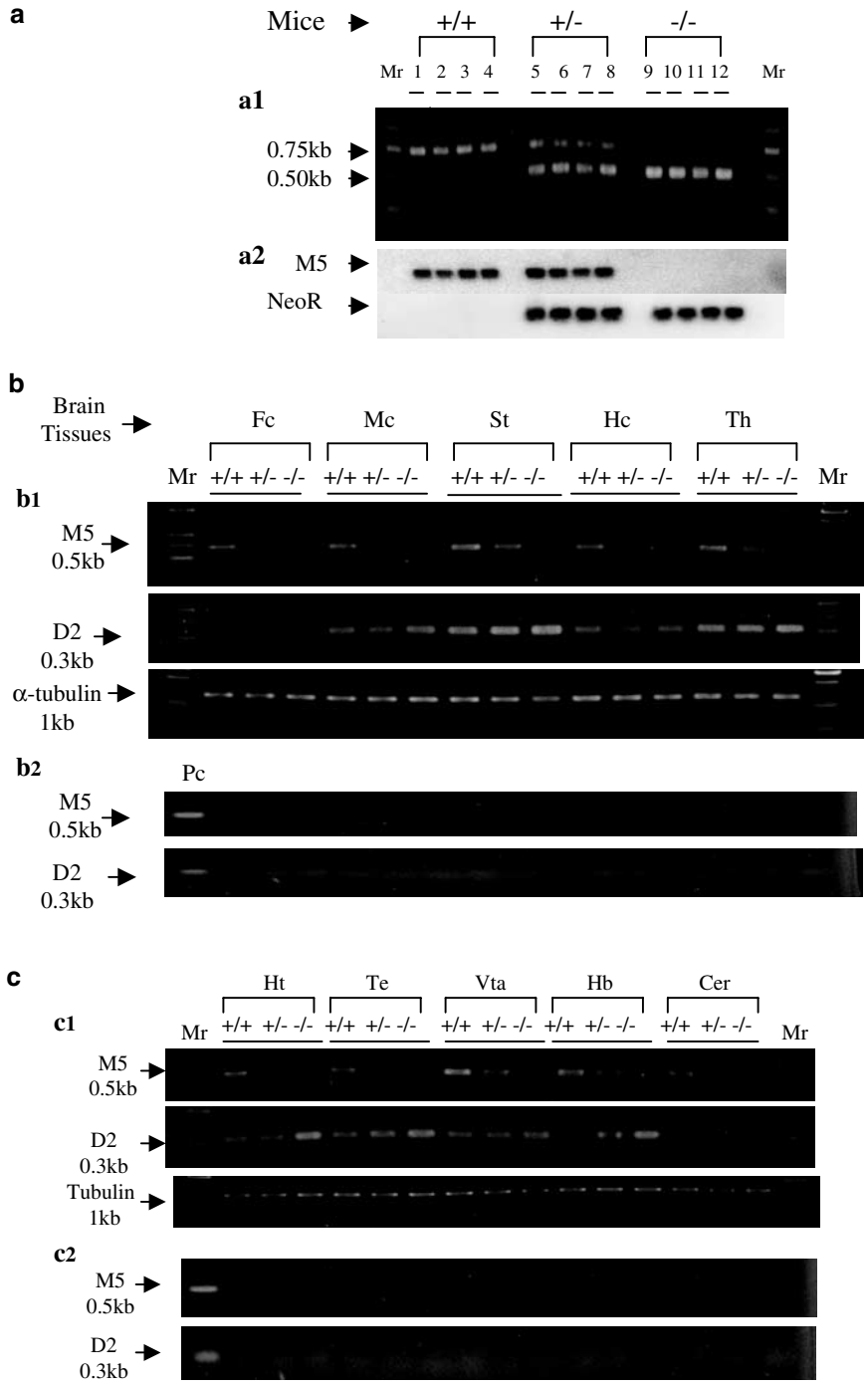

Figure 1 (a) PCR genotyping of M5 mutant mice. a1. PCR results obtained from tail DNA of 12 mice. Primer pairs P1 + P2 yield a 0.50 kb product (M5 mutant allele). Primers P3 + P4 yield a 0.75 kb DNA fragment (wild-type allele). a2. PCR-Southern blot hybridization with [α-^{32}P]dCTP-labeled specific cDNA probes for the mouse M5 gene and neomycin-resistance gene, respectively. Mr = marker; NeoR = neomycin-resistance gene. Both methods confirm the genotypes of wild-type (+/+), heterozygous (+/−), and homozygous (−/−) mice. (b) RT-PCR analysis of M5 and D2 receptor mRNA expression in brain regions of M5 mutant and wild-type mice. b1. Expression of M5 and D2 receptors in frontal cortex (Fc), middle and posterior cortex (Mc), striatum (St), hippocampus (Hc), and thalamus (Th) from a typical experiment. M5 expression was reduced in all heterozygous mutant tissues, and lost in homozygous mutants. D2 expression was increased in mutants. α-Tubulin is a control gene expressed uniformly in all regions. The number of cycles needed for proper expression of M5, D2R, and α-tubulin were 36, 34, and 27, respectively. b2. When RT was omitted, no mRNA was detected, indicating that results of Panel A are not from genomic DNA contamination or a technical artifact. The leftmost lane is a positive control (Pc). (c) RT-PCR analysis of M5 and D2 receptor mRNA expression in hypothalamus (Ht), tectum (Te), ventral tegmentum (Vta), hindbrain (Hb), and cerebellum (Cer) of M5 mutant and wild-type mice (as described above in Part b).

hypothalamus > frontal cortex, hindbrain, and cerebellum (Figure 2b). In M5 homozygous mutant mice, D2 mRNA was upregulated in all regions except frontal cortex, cerebellum, and hippocampus, as compared with those of wild-type mice. Upregulation of D2 mRNA was strongest in the hindbrain, hypothalamus, striatum, and tectum. For

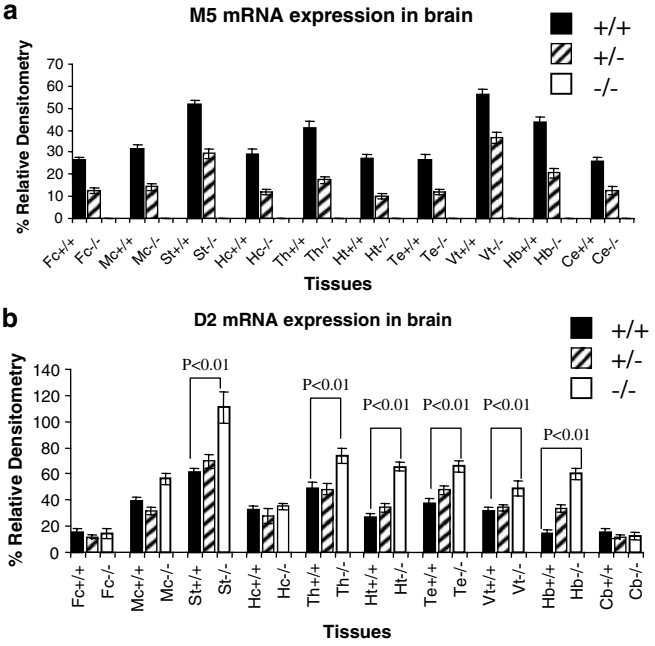

Figure 2 RT-PCR relative densitometry results for M5 and D2 mRNA in M5 mutant mice. (a) Expression of M5 mRNA in brain regions defined in Figure 1. The data are presented as ratios of calibrated optical densities of M5 and α-tubulin bands, such as those shown in Figure 1 part b. Each column represents the mean ± standard deviation from four independent PCR experiments. M5 expression was always significantly reduced in heterozygous mice and was not detected in homozygous mutant mice. Other abbreviations: hypothalamus (Ht), tectum (Te), ventral tegmentum (Vt), hindbrain (Hb), and cerebellum (Ce). (b) RT-PCR relative densitometry results for D2 mRNA in M5 mutant mice in brain regions defined in Figure 1, parts b and c. The data are presented as ratios of calibrated optical densities of D2 and α-tubulin using the same methods and abbreviations as above in Part a. D2 expression was increased in homozygous M5 mutant mice in many tissues.

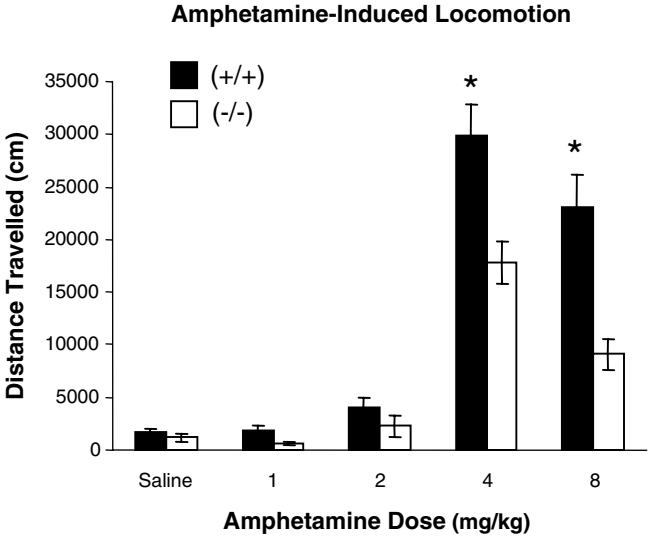

Figure 3 Amphetamine-induced locomotion was reduced in M5 mutant (−/−) mice as compared with wild-type (+/+) mice. Total distance traveled in cm during the 60 min test period is shown at each dose. Error bars are standard errors of the mean. * indicates $p<0.05$ in Bonferroni corrected *post hoc t*-tests.

example, striatal D2 receptor mRNA increased by over 80% in homozygous mutant mice *vs* wild-type mice. In heterozygous mice, D2 mRNA expression levels were between wild-type and homozygous mutants in many regions where the highest expression of D2 receptors were found (striatum, tectum, hindbrain) in wild-type mice, but were as low or lower than the wild types in brain regions where lower expression of D2 receptors was found (cerebellum, frontal cortex, hippocampus, motor cortex) in wild-type mice.

Motor Functions and Amphetamine-Induced Locomotion in M5 Mutant Mice

We first examined motor skills of wild-type and homozygous M5 mutant mice by using rotarod, pole climbing, and negative geotaxis tests. On the rotarod test, all mice were able to stay on the rod at 0 and 5 rpm for 30 and 90 s of testing, respectively. When the speed increased to 15 rpm, all mice fell off within 130 s, but no significant differences between M5 mutants and wild-type mice were found ($t(8)=0.924$, $p>0.05$). The mean duration for wild-type mice was 51.3 ± 4.3 s, and for M5 mutants the mean was 45.2 ± 4.1 s.

All mice of both genotypes were able to turn and climb to the top of the grid to show negative geotaxis for 30 s, then were able to mobilize on the metal grid for 30 s upside down. On the pole climbing task (45° angle), all mice showed excellent turning and balance, with wild-type mice reaching the base of the pole in a mean of 17.7 ± 4.5 s and mutant mice reaching the base in a mean of 13.0 ± 4.0 s. Consequently, no significant differences were found between the M5 mutant and wild-type mice in any of the tests ($t(8)=0.680$, $p>0.05$). This indicated that the two genotypes of mice were similar in locomotor skills.

Open-field locomotor activity after drug administration was measured in independent groups of mice at four doses of amphetamine. In Figure 3 (left bars), the means of all pre-amphetamine saline tests across all groups are shown for mutant and wild-type groups. Spontaneous locomotion following saline injections did not differ significantly between M5 homozygous mutants and wild-type mice ($t(62)=-1.468$, $p>0.05$). These were not included in the ANOVA, since saline tests were always carried out on day 2 before amphetamine tests on day 3 at each dose. Two-way ANOVA found a significant main effect of amphetamine dose ($F=91.6$; $p<0.001$) and a significant main effect of genotype ($F=24.0$; $p<0.001$), with no significant interaction between dose and genotype ($F=1.99$; $p>0.05$). *Post hoc t*-test comparisons (using Bonferroni corrections) at each dose of amphetamine found a significant difference between mutant and wild-type mice at 4 mg/kg ($t(14)=-3.121$, $p<0.05$) and 8 mg/kg ($t(14)=-3.10$, $p<0.05$), but not at 1 mg/kg ($t(14)=-2.423$, $p>0.05$) and 2 mg/kg ($t(14)=-1.158$, $p>0.05$). Taken together, these results suggest that M5 gene disruption in mice decreased amphetamine-induced locomotion, but not basal locomotion, suggesting that M5 receptors are required for the normal response of amphetamine-induced locomotion.

Nociceptive Sensitivity

The results of pain testing are shown in Figure 4. Both wild-type and M5 mutant mice showed tail–withdrawal responses in about 7 s with small error bars after immersion of the tail into the $49.0 \pm 0.2^{\circ}$C water bath. This indicates that both groups had normal pain sensitivity with no reliable differences due to genotype ($t(8) = -0.378$, $p > 0.05$).

Startle and Prepulse Inhibition

Both wild-type ($n = 6$) and mutant mice ($n = 4$) showed strong startle responses to 120 dB acoustic stimuli (Figure 5). Although wild type mice were slightly more responsive, no reliable differences were found between the two groups ($t(8) = -1.045$, $p > 0.3$). Both mutant and wild-type mice showed much lower responses when prepulses (81 dB) preceded the startling pulses by 20–250 ms ($F(1.95, 15.6) = 16.111$, $p < 0.01$, with Greenhouse–Geisser correction to degrees of freedom). Startle responses following the prepulse were slightly lower in the M5 mutant mice at all interstimulus intervals as compared with wild-type mice. However, when startle responses following prepulses were measured as a percentage of the baseline startle responses (without prepulses), no statistically significant overall differences between wild-type and M5 mutant mice were seen ($F(1, 8) = 2.444$, $p > 0.1$).

Latent Inhibition in M5 Mutant Mice

Drinking time was measured on days 2 and 3 (pretraining) and day 6 (reinstatement of drinking) for all groups (Figure 6a). Under these conditions of repeated deprivation and repeated tests of drinking in mice matched for mass, no significant differences were observed between genotypes ($p > 0.05$). Similarly, on day 7 of testing with the observer blind to the genotype, no significant differences were found between the groups in the latency to drink for 4 s before the tone was presented in either pre-exposed or non-pre-exposed conditions (Figure 6b).

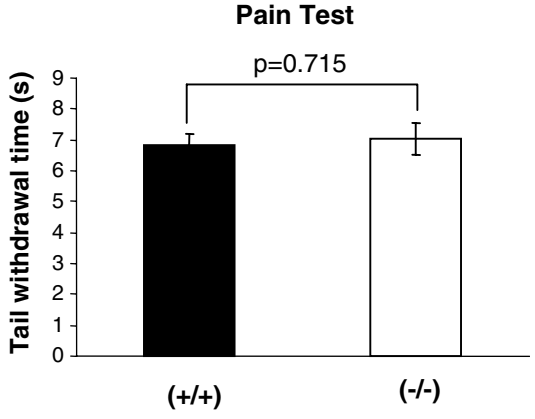

Figure 4 Nociceptive sensitivity on the tail-withdrawal test was not significantly different between wild-type (+/+) and M5 mutant (−/−) mice. Latency to reflexive withdrawal of the tail from 49°C water was measured three times. All bars represent mean values, with error bars indicating standard errors of the mean.

After the tone on the test day (day 7), the time taken to drink for 4 s was significantly greater than the time taken to drink for 4 s before the tone in all groups, as shown by the lower scores in Figure 7 for all 'PE after' and 'NPE after' groups. For statistical analysis, these are all shown as reciprocal of the time taken to drink for 4 s. This indicates that suppression of drinking time by the tone that had been paired with shock was strong in all groups, independent of genotypes. Mutant mice that were pre-exposed (PE after, open bars), however, were significantly different from non-pre-exposed mice (NPE after, open bars), returning to drinking in a shorter time after the tone ($t = 2.619$, $p < 0.05$).

After the tone, two-way ANOVA showed a significant difference for both pre-exposure *vs* non-pre-exposure ($F(1, 39) = 4.523$; $p < 0.05$) and for genotype ($F(1, 39) = 4.791$; $p < 0.05$). There was no significant interaction between the two variables ($F(1, 39) = 2.74$; $p = 0.106$). In the non-pre-exposed groups (NPE after), there were no significant differences between mutant and wild-type groups (Figure 7, right). The pre-exposed mutant mice took less time to drink after the tone compared to the pre-exposed wild types, further indicating that M5 mutant mice learned the latent inhibition task better than wild-type mice. If sex is added as a third factor, there were no significant differences between male and female mice ($F(1, 35) = 3.791$, $p > 0.05$).

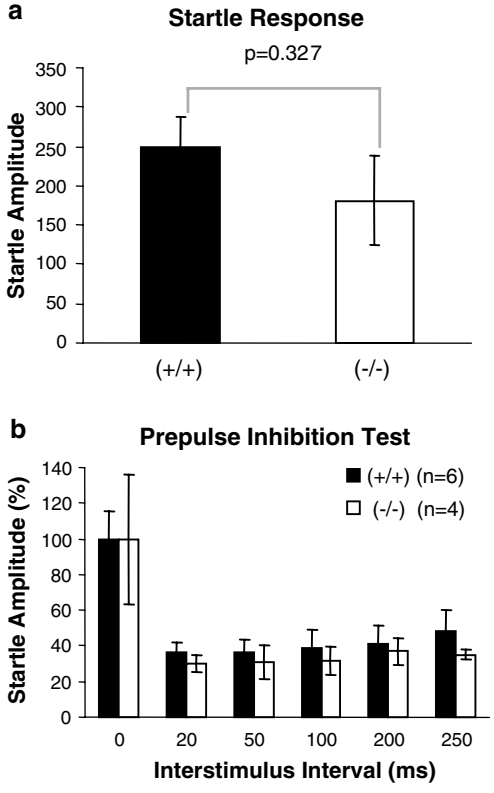

Figure 5 (a) Startle responses to 120 dB noises were not significantly different between wild-type (+/+) and M5 mutant (−/−) mice. The mice were tested with 65 db background white noises. (b) Prepulse inhibition (81 dB prepulses presented before the 120 dB noises) was measured at five interstimulus intervals. When measured as a percentage of baseline startle responses, startle amplitudes were reduced by prepulses in all conditions, but overall no statistically significant differences were observed between wild-type (+/+) and M5 mutant (−/−) mice.

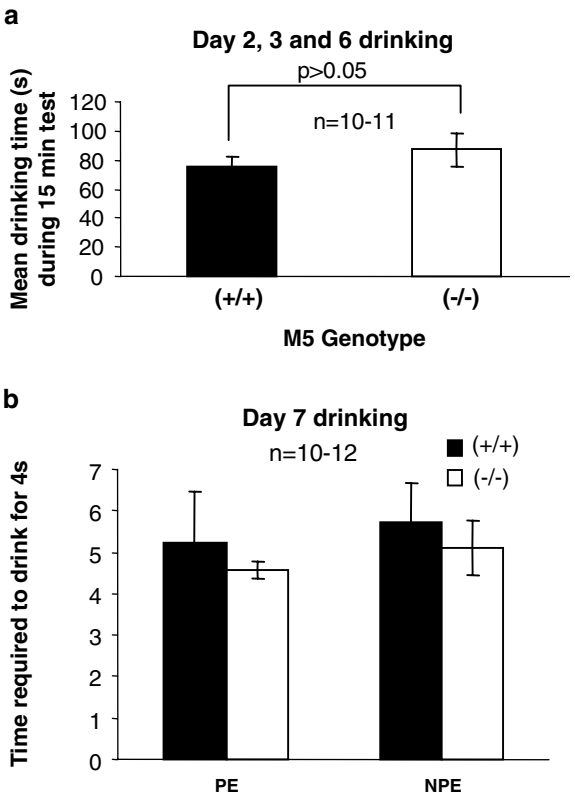

Figure 6 (a) Mean drinking time (±SEM) on days 2, 3, and 6 in M5 wild-type (+/+) and homozygous mutant (−/−) mice. All mice showed vigorous drinking, without significant differences between genotypes. (b) Latency to drink for 4 s on test day 7 before tone presentation. All groups drank vigorously during this period with no reliable differences, whether pre-exposed (PE) to the tone or not pre-exposed (NPE).

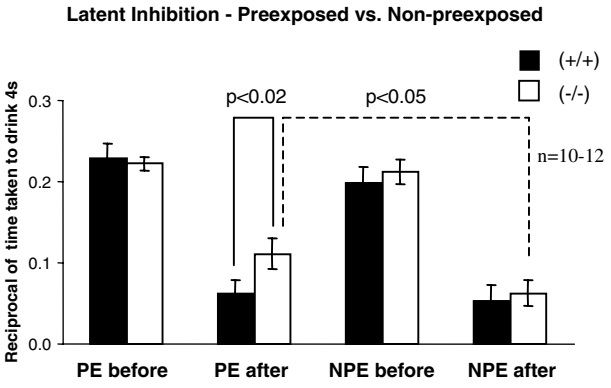

Figure 7 Latent inhibition on test day 7 before and after tone presentation. Data are shown as the reciprocal of the time taken to drink for 4 s. All mice showed strong suppression of drinking following the tone (PE after and NPE after *vs* PE before and NPE before). M5 mutant mice pre-exposed (PE) to tones (PE after, open bar) returned to drinking sooner than wild-type mice (PE after, dark bar), indicating significantly less suppression in pre-exposed mutant mice than wild-type mice (ie improved latent inhibition) ($p<0.02$). M5 mutant mice that were pre-exposed also showed significantly less suppression than non-pre-exposed mutant mice (NPE after, open bar) ($p=0.05$). Wild-type mice that were not pre-exposed to tones (NPE after, right bars) showed no differences from non-pre-exposed mutants.

As shown in Figure 7, the homozygous mutants mice took significantly less time than the wild-type mice to drink after tone presentation (PE after), as indicated by greater reciprocal latency scores ($t(20)=2.619$, $p<0.02$). This clearly indicates latent inhibition improvement in the M5 mutant mice.

Chromosomal Localization of Mouse and Human M5 Muscarinic Receptor Genes

We first compared the cDNA sequences isolated from neuroblastoma cells (see Materials and Methods) to the cDNA sequences for mouse and human M5 muscarinic acetylcholine receptor genes that have been reported previously (Bonner *et al*, 1988; Liao *et al*, 1989). Thereafter, we localized the M5 chromosome sites by blasting human and mouse genome resources in the NCBI database with the full-length human and mouse cDNA sequences, respectively. The unique locations of the M5 muscarinic receptor (CHRM5) were on human chromosome 15 at 31.7 Mb (15q13) (Figure 8, left) (confirmed by Lander *et al*, 2001), and on mouse chromosome 2 at 114.0 Mb from the *p* terminus in region 2E4 (Figure 8, right).

Figure 8 (left) also shows the region of the human chromosome 15q12–14. Several genes located near CHRM5 in the schizophrenia linkage region 15q12–14 are also found in the corresponding mouse region around CHRM5 (2E4) (Figure 8, right).

DISCUSSION

In the present study, amphetamine-induced locomotion was reduced in M5 mutant mice, and latent inhibition was improved. The present results are consistent with previous findings that M5 gene deletion reduces nucleus accumbens and striatal dopamine release (Forster *et al*, 2001; Zhang *et al*, 2002).

Furthermore, at the molecular level, deletion of the M5 receptor gene in the ic3 loop resulted in corresponding deletion of M5 mRNA expression measured by RT-PCR. Homozygous M5 mutant mice showed increased mRNA for D2 receptors, especially in the striatum, hindbrain, hypothalamus, and tectum. These results imply that removal of M5 excitatory input to dopamine neurons leads to compensatory increases in D2 receptor expression. Together, these data further suggest that M5 muscarinic receptors are important for normal dopaminergic function.

Gene-targeted mice are especially useful for studying long-term genetic diseases, because alteration in one gene is maintained over the lifetime of the mouse, as in the disease. Testing of dopamine functions in mice may help evaluation of possible genes for schizophrenia, because of the importance of nucleus accumbens dopamine receptors for latent inhibition and amphetamine-induced locomotion (Joseph *et al*, 2000; Moser *et al*, 2000; Russig *et al*, 2002; Gray *et al*, 1997; Weiner, 2003; Joyce and Koob, 1981).

Amphetamine-Induced Locomotion in M5 Mutant Mice

The present study found increased open-field locomotor activity following amphetamine, consistent with many

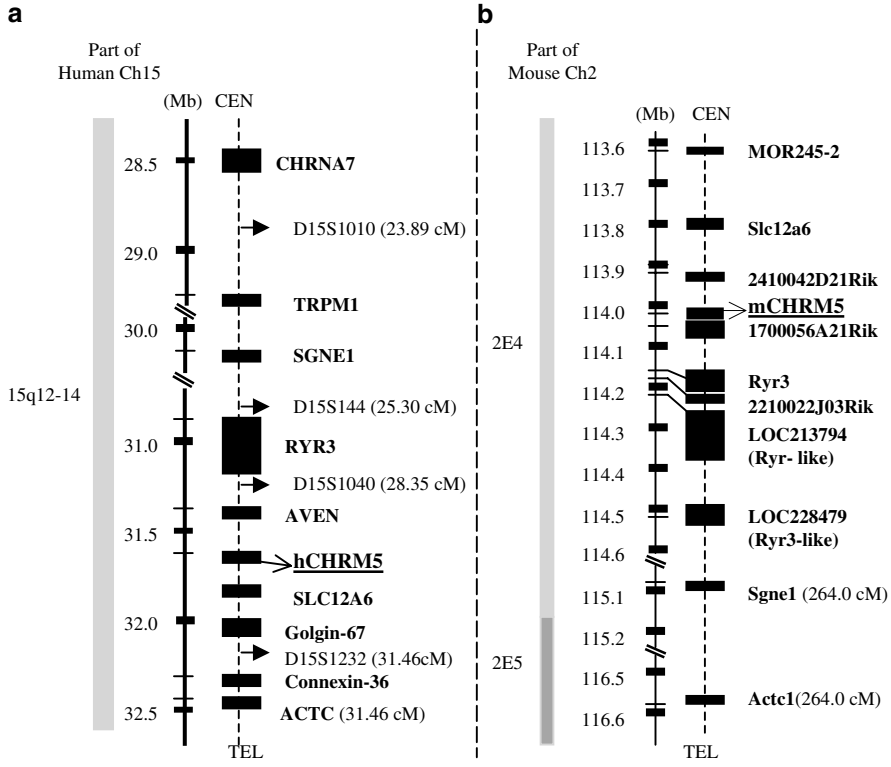

Figure 8 Chromosomal mapping of human and mouse M5 muscarinic receptor genes. (a) Map of human 15q12–14 region from 28.5 to 32.5 megabases (Mb) showing human cholinergic receptor M5 (hCHRM5) at 31.7 Mb, along with other genes from the NCBI database. Genetic markers linked with schizophrenia are shown as D15S numbers with genetic locations in centiMorgans (cM) in parentheses. Directions of centromere (CEN) and q telomere (TEL) are shown above and below, respectively. (b) Map of mouse Ch2E4-5 region from 113.6 to 116.6 Mb showing mouse cholinergic receptor M5 (mCHRM5) along with many nearby genes obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/mapview/maps.cgi?ORG = mouse&CHR = 2) using GenomeScan, Contig (NW_000178.1) and gene analysis. The loci were obtained from two NCBI websites (http://www.ncbi.nlm.nih.gov/LocusLink/) (http://www.ncbi.nlm.nih.gov/mapview/maps.cgi?ORG = hum&CHR = 15&BEG = &END = &MAP0 = ideogr%2Cmarsh%2Cscan%2Ccntg%2Cloc&LINKS). Many homologous genes were identified in both the human schizophrenia 15q12–14 region and the mouse 2E4-5 region around the mCHRM5 gene.

previous studies. In mutant mice, however, locomotor activity was lower than in wild-type mice at all doses (although not significantly different at 1 or 2 mg/kg). Previous work in rats showed that amphetamine-induced locomotion is blocked by 6-hydroxydopamine lesions of dopamine terminals in the nucleus accumbens (Joyce and Koob, 1981). These results suggest that dopamine release induced by amphetamine in the nucleus accumbens is reduced in M5 mutant mice. This result is consistent with studies showing reduced dopamine release in nucleus accumbens of M5 homozygous mutant mice in response to the muscarinic agonist oxotremorine (Zhang *et al*, 2002).

These M5 mutant mice showed increased locomotor responses to scopolamine (Chintoh *et al*, 2003). In that study, as here, no difference was found between groups in spontaneous locomotion. Furthermore, both M5 mutant and wild-type mice showed excellent motor function on tests of rotarod, pole climbing, and negative geotaxis, with no differences in performance. Consistent with this result, scopolamine-induced locomotion was unaffected by 6-hydroxydopamine lesions of the nucleus accumbens (Joyce and Koob, 1981).

Together, these results support the idea that nucleus accumbens dopamine terminals are critical for the locomotor effects of amphetamine in M5 mutant mice, but not of scopolamine. However, because M5 receptors are disrupted throughout the mice, the present studies in M5 mutant mice do not exclude contributions of brain regions other than the nucleus accumbens in these behavioral changes.

Latent Inhibition

A previous report of latent inhibition in knockout mice found no change in mice with a deletion of the beta-2 nicotinic receptor subunit (Caldarone *et al*, 2000). To our knowledge, the present study is the first to show gene-dependent changes in latent inhibition in mice. In this study, truncation of the M5 receptor gene resulted in improved latent inhibition in M5 mutant mice as compared to wild-type mice.

These M5 mutant mice previously showed a profound loss of prolonged dopamine release in the nucleus accumbens core, lasting from 8–50 min following stimulation of mesopontine cholinergic nuclei (Forster *et al*, 2001). Also, excitotoxic lesions of mesopontine cholinergic neurons dampened dopamine activity within the nucleus accumbens 21 days after the lesion (Forster *et al*, 2002). Therefore, M5 receptors are required for prolonged mesolimbic dopamine release in the nucleus accumbens

driven from mesopontine sites. Pharmacological studies suggest that M5 receptors tonically regulate mesolimbic and striatal dopamine functions (Blaha *et al*, 1996; Miller and Blaha, 2002; Zhang *et al*, 2002; Basile *et al*, 2002; Forster and Blaha, 2003). Since decreased dopaminergic activity in the nucleus accumbens enhances latent inhibition in rats (Joseph *et al*, 2000; Moser *et al*, 2000; Russig *et al*, 2002; Gray *et al*, 1997), the improvement in latent inhibition in M5 mutant mice appears to result from decreased mesolimbic dopamine activation by M5 receptors.

Previously, 8-week-old M5 mutant mice drank more water than age-matched wild-type mice after 14 h water deprivation (Takeuchi *et al*, 2002). In that experiment, the mutant mice averaged 5–10 g lighter than controls at the time of weaning, possibly due to larger litter sizes and reduced milk access, and 2–3 g lighter at the time of testing. Therefore, testing in their first deprivation experience postweaning might have led to compensatory drinking in M5 mutant mice. In the present experiment, deprivation was tested repeatedly over several days in 9–17-week-old mass-matched mice after 23 h deprivation, after which all groups drank intensely in all prestimulus conditions, with no significant differences in drinking. These results further support the conclusion that postnatal milk deprivation led to reduced drinking in underweight M5 mutant mice (Takeuchi *et al*, 2002. p. 122). In the present tests, all groups were matched for weight, were similar in litter sizes, were better adapted to the deprivation conditions at the time of training and testing, and were strongly motivated to drink.

Furthermore, all groups showed reduced drinking when footshocks were delivered, or when tones that were paired with footshock were delivered. This indicates that Pavlovian conditioning (CS–US association) was strong in all conditions. Latent inhibition was poor in wild-type mice (as indicated by nonsignificant differences between pre-exposed and non-pre-exposed groups), consistent with previous data showing poor latent inhibition in 129SvJ strain mice (Gould and Wehner, 1999). Latent inhibition was stronger in M5 mutant mice, suggesting that low dopamine function in M5 mutant mice facilitates memory of the pre-exposure to the CS (Gray *et al*, 1997; Weiner and Feldon, 1997).

The differences between the groups in latent inhibition might be attributed to differences in responsivity to tones or to painful stimuli. However, the M5 mutant mice showed no reliable difference in sensitivity to hot water on the tail-withdrawal test, and no reliable difference in startle response to 120 dB tones. These results helped to rule out the possibility of such artefacts.

The slightly increased sensitivity to prepulse tones (81 dB) was not reliable, but may hint at slightly better sensory gating in M5 mutant mice, consistent with reduced dopamine function (Geyer *et al*, 2001). The stronger difference found here in latent inhibition than in prepulse inhibition in M5 mutant mice indicates a stronger role for M5 receptors in latent inhibition than in sensory gating. M5 receptors are found postsynaptic to mesopontine cholinergic neurons on VTA and substantia nigra dopamine neurons, and on dopamine terminals. The results are consistent with a stronger involvement of M5 receptors in dopamine reward learning mechanisms (Gray *et al*, 1997; Weiner and Feldon, 1997) than in mesopontine induction of sensory gating (Fendt *et al*, 2001; Miyazaki *et al*, 1996).

M5 and D2 Expression Changes

The anatomical association between D2 and M5 mRNA, first noted by Weiner *et al* (1990), is important because D2 receptors are the primary target of typical neuroleptic drugs (Seeman, 1977). The present study shows that M5 and D2 receptors are coexpressed in many brain regions. The upregulation of D2 receptors in M5 mutant mice indicates that loss of M5 receptors results in compensatory increases in D2 receptors, especially in forebrain targets of dopamine neurons. According to this hypothesis, increased D2 expression results from the loss of excitatory input to midbrain dopamine neurons from mesopontine cholinergic neurons (Yeomans, 1995; Forster *et al*, 2002; Miller and Blaha, 2002).

Improvements in latent inhibition in rats occur when D2 receptors are blocked rather than activated (Joseph *et al*, 2000; Moser *et al*, 2000; Russig *et al*, 2002). This suggests that the increased D2 expression in M5 mutant mice found here is not the direct cause of improved latent inhibition in M5 mutant mice, but does not rule out the possibility that the increased D2 mRNA expression may be involved in the improved latent inhibition, since D2 receptors appear to be weakly linked to schizophrenia by meta-analysis (Glatt *et al*, 2003). More work will be required to specify whether presynaptic or postsynaptic D2 receptors are increased, or whether other genes and receptors are altered in M5 mutant mice.

Chromosomal Localization of Mouse and Human M5 Muscarinic Receptor Genes and Their Possible Linkage to Schizophrenia

The localizations of the M5 gene on human and mouse genomes here are consistent with the most recent synteny data associating mouse 2E4 with human 15q12–15 (Waterston *et al*, 2002, their Figure 4), but are inconsistent with the previously reported 15q26 localization using cytogenetic methods in which the data were not shown (Bonner *et al*, 1991). Several genes located near CHRM5 in the schizophrenia linkage region 15q12–14 are also found in the corresponding mouse region around CHRM5 (2E4) (Figure 8), supporting the mouse/human synteny.

Chromosome 15q12–15 has been linked with schizophrenia (Freedman *et al*, 1997, 2001; Gejman *et al*, 2001; Liu *et al*, 2001; Tsuang *et al*, 2001) and familial catatonic schizophrenia (periodic catatonia) (Stober *et al*, 2002; Meyer *et al*, 2002) in several, but not all, studies (Waterwort *et al*, 2002). The exact genetic markers on 15q12–15 most strongly associated with schizophrenia have varied in different studies, suggesting that more than one gene may be involved. All of these markers are near the human M5 muscarinic gene locus (hCHRM5). The closest genetic marker to the M5 gene previously studied is D15S1040, which was strongly linked with schizophrenia in the largest study (Tsuang *et al*, 2001).

The leading candidate genes for schizophrenia in this 15q region have been two copies of the $\alpha 7$ nicotinic receptor gene (CHRNA7) (Freedman *et al*, 1997) located at 28.5 Mb

from the p terminus, according to the NCBI database (June 2003). The M5 gene site is located at 31.7 Mb, that is, 3.2 Mb closer to the center of 15q12–15 region than the α7 nicotinic gene sites. The M5 gene is interesting because of the sustained activation of dopamine neurons by the M5 receptor (Forster *et al*, 2001). The present finding that D2 expression increases in M5 mutant mice may be relevant to schizophrenia, because increased D2 receptors have been found in the striatum of schizophrenics in several studies (Wong, 2002), and an association was recently found between the D2 and schizophrenia (Glatt *et al*, 2003). Direct linkage analysis of polymorphisms of the M5 gene with schizophrenia (De Luca *et al*, in press), especially in populations with 15q linkage, would be of interest (Yeomans, 1995).

In conclusion, our results indicate that M5 muscarinic receptors play a role in amphetamine-induced locomotion and latent inhibition, and that deletion of M5 receptors increases the gene expression of dopamine D2 receptors. These results are consistent with the critical role of M5 receptors in prolonged activation of mesolimbic and striatal dopamine neurons following mesopontine stimulation (Forster *et al*, 2001; Forster and Blaha, 2003). Consequently, M5 mutant mice may serve as a model of reduced dopamine function, and M5 blockers may be useful as neuroleptic drugs.

The localization of the M5 human gene in the 15q12–15 region linked to schizophrenia in several studies provides circumstantial evidence for M5 gene linkage to human schizophrenia (Yeomans, 1995). M5 mutant mice may prove useful for studying the interactions between muscarinic and dopaminergic receptor functions in neuropsychiatric disorders.

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