Brief Communications

Involvement of a Subpopulation of Neuronal M_4 Muscarinic Acetylcholine Receptors in the Antipsychotic-like Effects of the M_1/M_4 Preferring Muscarinic Receptor Agonist Xanomeline

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Disturbances in central dopaminergic neurotransmission are believed to be centrally involved in the pathogenesis of schizophrenia. Central dopaminergic and cholinergic systems interact and the cholinergic muscarinic agonist xanomeline has shown antipsychotic effects in clinical studies. Preclinical studies indicate that the M_4 muscarinic cholinergic receptor subtype (mAChR) modulates the activity of the dopaminergic system and that this specific mAChR subtype is involved in mediating the antipsychotic-like effects of xanomeline. A specific neuronal subpopulation that expresses M_4 mAChRs together with D_1 dopamine receptors seems to be especially important in modulating dopamine-dependent behaviors. Using mutant mice that lack the M_4 mAChR only in D_1 dopamine receptor-expressing cells (D1-M4-KO), we investigated the role of this neuronal population in the antipsychotic-like effects of xanomeline in amphetamine-induced hyperactivity and apomorphine-induced climbing. Interestingly, the antipsychotic-like effects of xanomeline in the two models were almost completely abolished in D1-M4-KO mice, suggesting that M_4 mAChRs colocalized with D_1 dopamine receptors are centrally involved in mediating the antipsychotic-like effects of xanomeline. This is consistent with the hypothesis that activation of the M_4 mAChR represents a potential target for the future medical treatment of psychosis.

Introduction

Xanomeline is an M_1/M_4-preferring muscarinic acetylcholine receptor (mAChR) agonist that, despite its lack of affinity for dopamine receptors, has demonstrated antipsychotic-like effects in rodents (Shannon et al., 2000), antipsychotic effects in schizophrenic patients (Shekhar et al., 2008), and robust effects against psychotic-like behavior in Alzheimer's disease patients (Bodick et al., 1997). Xanomeline has been shown to inhibit apomorphine-induced climbing in mice (Shannon et al., 2000) and amphetamine-induced hyperactivity and apomorphine- and scopolamine-induced disruption of prepulse inhibition in rats and mice (Stanhope et al., 2001; Jones et al., 2005; Thomsen et al., 2010), all models possessing predictive validity for alleviation of psychotic symptoms.

The antipsychotic-like effects of xanomeline have been suggested to be primarily mediated via M_4 mAChRs (Bymaster et al., 2002; Woolley et al., 2009; Thomsen et al., 2010). Behavioral and neurochemical analysis of M_4 receptor knock-out mice has shown that the M_4 mAChR plays an important role in the dopamine homeostasis, especially in striatal areas (Gomeza et al., 1999; Felder et al., 2001; Zhang et al., 2002; Tzavara et al., 2004; Fink-Jensen et al., 2011). The M_4 mAChR subtype is the major mAChR expressed in the striatum (Levey, 1993), where the receptor is expressed on cholinergic interneurons, as well as on GABAergic projection neurons, where it is coexpressed with D_1 dopamine receptors (Weiner et al., 1990; Bernard et al., 1992). We have recently created a line of mice in which the M_4 mAChR is selectively abrogated in D_1 dopamine receptor-expressing neurons (D1-M4-KO) and shown that this specific subpopulation of M_4 mAChRs is critically involved in the regulation of dopaminergic neurotransmission (Jeon et al., 2010). Here we describe the effects of xanomeline on amphetamine-induced hyperactivity and apomorphine-induced climbing behavior in D1-M4-KO and wild-type mice to investigate the role of this receptor subpopulation in antipsychotic-like effects.

Materials and Methods

Animals. D1-M4-KO mice were generated as previously described (Jeon et al., 2010). Knock-out mice and their wild-type littermates were bred at the animal facility at the Panum Institute, University of Copenhagen (Copenhagen, Denmark). Mouse genotypes were determined by PCR analysis of mouse ear genomic DNA and genotypes were reconfirmed

Received Jan. 22, 2011; accepted Feb. 15, 2011.

This work was supported by the Ivan Nielsen Foundation, the Lundbeck Foundation, and the Aase and Ejnar Danielsen Foundation.

The authors declare no competing financial interests.

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DOI:10.1523/JNEUROSCI.0370-11.2011

 0270-6474/11/315905-04$15.00/0

after the completion of experiments. The mice were housed in standard rodent cages (Macrolon type III), enriched with cardboard housing and nesting material. The animals were kept at room temperature (22–24°C) in a 12 h light/dark cycle (lights on at 6:00 A.M.) with *ad libitum* access to food and water. All experiments were performed on experimentally naive 10–16 weeks old male mice in the middle of the light cycle (between 9:00 A.M. and 4:00 P.M.). The mice were allowed to acclimatize to the animal facility for at least 7 d before the start of experiments. Animals were taken to the experimental room at least 2 h before initiating the experiment. All procedures were conducted in accordance with guidelines from the Animal Experimentation Inspectorate, Denmark and the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Drugs. D-amphetamine was obtained from the Copenhagen University Hospital Pharmacy. R-(−)-apomorphine hydrochloride hemihydrate and xanomeline L-tartate hydrate (3-Hexoxy-4-(1-methyl-3,6-dihydro-2H-pyridin-5-yl)-1,2,5-thiadiazole) were purchased from Sigma-Aldrich. All drugs were dissolved in saline and administered by subcutaneous injections (10 ml/kg).

Locomotor activity measurements. Locomotor activity was assessed in an open-field (40 × 40 × 80 cm) setup, placed in a dimly lit room. A camera located on the ceiling above the open field recorded the experiments. The distance the animal moved was analyzed with the video-tracking program Etho Vison (version 3.1; Noldus). Mice were initially placed in the open field for a 1 h habituation period. Hereafter mice were coadministered with xanomeline (1 and 2 mg/kg), vehicle and amphetamine (2 mg/kg), or vehicle, and locomotor activity was monitored for 2 h.

Apomorphine climbing. Climbing behavior was measured using the three-point rating scale of Protais et al. (1976). Immediately after an injection of apomorphine (1 or 2 mg/kg), the mice were placed into a cylindrical individual cage (diameter, 14 cm; height, 15 cm; grid-size, 5 × 5 mm) with the top covered by a gray plastic lid and the floor covered by a scant lining of bedding material. An observer who was blinded to drug treatment and genotype measured climbing behavior at 5, 10, 15, 20, 25, and 30 min after apomorphine administration. Climbing behavior was observed for 30 s at the six time points and the score corresponding to the posture the animal adopted the longest was recorded. The following postures were scored: four paws on the floor, 0 points; forefeet holding the grid, 1 point; four paws holding the grid, 2 points. The scores for the six time points were summed (maximum 12 points) and this sum was used in statistical calculations. Xanomeline was administered 25 min before the injection of apomorphine (Shannon et al., 2000).

Data analysis. Group differences in locomotor and climbing activities were analyzed by two-way ANOVA with genotype and treatment as between-subjects factors, followed by Bonferroni-corrected pairwise comparisons. Within each genotype, treatment groups were analyzed by one-way ANOVA with treatment as the between-subjects factor, followed by Newman–Keuls *post hoc* test. A p value of <0.05 was considered statistically significant.

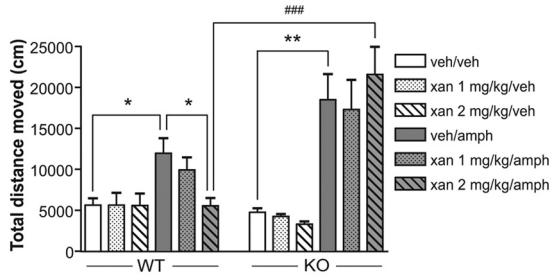

Figure 1. Effects of xanomeline (1 and 2 mg/kg) on amphetamine (2 mg/kg)-induced locomotor activity in D1–M4-KO and wild-type mice. Data are presented as means ± SEM (n = 6–11). WT, Wild-type; KO, D1–M4-KO; veh, vehicle; xan, xanomeline; amph, amphetamine. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{###}p < 0.001$.

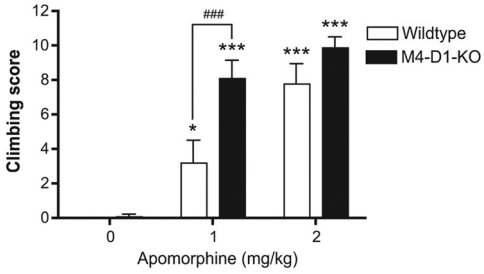

Figure 2. Apomorphine-induced climbing in D1–M4-KO and wild-type mice. Data are given as means ± SEM (n = 8–12). $^{*}p < 0.05$, $^{***}p < 0.001$ versus corresponding control group, and $^{###}p < 0.001$ versus same treatment group in wild-type controls.

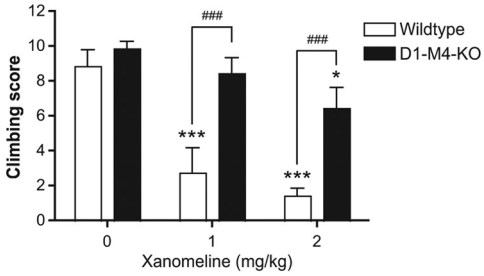

Figure 3. Effects of xanomeline on apomorphine-induced (2 mg/kg) climbing in D1–M4-KO and wild-type controls. Data are given as means ± SEM (n = 7–9). $^{*}p < 0.05$, $^{***}p < 0.001$ versus corresponding control group, and $^{###}p < 0.001$ versus same treatment group in wild-type controls.

Results

Xanomeline does not attenuate amphetamine-induced locomotor activity in D1-M4-KO mice

Overall two-way ANOVA showed a significant effect of genotype ($F_{(1,81)} = 11.33$; $p = 0.0012$) and treatment ($F_{(5,81)} = 11.42$; $p < 0.0001$), as well as a significant interaction ($F_{(5,81)} = 5.14$; $p = 0.0004$). One-way ANOVAs within genotype showed significant effects of treatment in both wild-type and D1-M4-KO mice ($F_{(5,47)} = 4.00$; $p = 0.005$ and $F_{(5,44)} = 9.32$; $p < 0.0001$, respectively). *Post hoc* tests showed that administration of amphetamine (2 mg/kg) induced a significant increase in distance moved when compared with vehicle in both wild-type ($p < 0.05$) and D1-M4-KO ($p < 0.01$) mice (Fig. 1). In wild-type mice, xanomeline dose dependently attenuated the amphetamine-induced hyperactivity, reaching significance at the 2 mg/kg dose ($p < 0.05$). In D1-M4-KO mice, xanomeline had no effect on the induced hyperactivity. Xanomeline (1 or 2 mg/kg) had no effect on baseline locomotor activity in either genotype. Bonferroni-corrected *post hoc* tests revealed a significant difference of the effect of xanomeline (2 mg/kg) between the two genotypes ($p < 0.001$).

Reversal of apomorphine climbing by xanomeline is reduced in D1-M4-KO mice

In the first experiment, climbing behavior of D1-M4-KO mice and wild-type littermates was tested following treatment with vehicle or apomorphine (1 or 2 mg/kg) (Fig. 2). Overall two-way ANOVA showed a significant effect of genotype ($F_{(1,54)} = 12.26$; $p < 0.0009$), treatment ($F_{(2,54)} = 61.96$; $p < 0.0001$), and interaction ($F_{(2,54)} = 3.90$; $p < 0.0026$). One-way ANOVA showed a significant effect of treatment with apomorphine on the climbing score in both wild-type ($F_{(2,28)} = 15.66$; $p < 0.0001$) and D1-M4-KO ($F_{(2,27)} = 60.17$; $p < 0.0001$) mice. In wild-type mice, *post hoc* analysis showed a significant and dose-dependent effect of apomorphine (1 and 2 mg/kg; $p < 0.05$ and $p < 0.001$, respectively) on climbing behavior. Also in D1-M4-KO mice, apomorphine induced climbing behavior at both doses ($p < 0.001$). *Post*

hoc tests comparing the effect between genotypes found a significantly higher climbing score in D1-M4-KO mice following treatment with 1 mg/kg apomorphine ($p < 0.001$). Apomorphine at 2 mg/kg resulted in comparable climbing scores in both genotypes. In the next experiment, the attenuating effect of xanomeline on apomorphine (2 mg/kg)-induced climbing was investigated. Overall two-way ANOVA showed a significant effect of genotype ($F_{(1,42)} = 23.42$; $p < 0.0001$) and treatment ($F_{(2,42)} = 15.26$; $p < 0.0001$), and a near-significant interaction ($F_{(2,42)} = 3.19$; $p = 0.051$). In wild-type animals, one-way ANOVA revealed a significant effect of xanomeline treatment on apomorphine-induced climbing ($F_{(2,21)} = 13.36$; $p = 0.0002$) (Fig. 3). Compared with vehicle treatment, xanomeline (both 1 and 2 mg/kg) significantly attenuated climbing scores ($p < 0.001$). In D1-M4-KO mice, one-way ANOVA showed a significant effect of treatment ($F_{(2,25)} = 3.49$; $p < 0.05$), but in contrast to wild-type animals, xanomeline was only able to significantly reduce climbing at the highest dose in the D1-M4-KO mice ($p < 0.05$). Bonferroni-corrected *post hoc* analysis showed a significant difference between genotypes at both xanomeline doses ($p < 0.001$ and $p < 0.01$, respectively).

Discussion

The results obtained in the present study show that the antipsychotic-like effects of xanomeline, investigated by amphetamine-induced hyperactivity and apomorphine-induced climbing in mice, were almost completely abolished in D1-M4-KO mice. The reported overall effects of xanomeline are in accordance with previous studies (Shannon et al., 2000; Woolley et al., 2009). We previously found that 2 mg/kg amphetamine induced a significantly larger increase in locomotor activity in D1-M4-KO mice relative to wild-type (Jeon et al., 2010). In the present study, D1-M4-KO mice similarly showed a trend toward an increased hyperlocomotor response to amphetamine compared with wild-type controls, although this did not reach significance. Methodological differences are likely responsible for this discrepancy. Interestingly, xanomeline had no effect on the amphetamine-induced hyperactivity in D1-M4-KO mice (Fig. 1), similar to findings obtained with whole-body M_4 knock-out mice (Woolley et al., 2009).

D1-M4-KO mice displayed increased climbing behavior in response to apomorphine compared with wild-type mice (Fig. 2). This finding is in agreement with previous studies in these mice, where D1-M4-KO mice displayed a dopamine hyperresponsive phenotype with increased locomotor response to dopamine agonists, amphetamine-induced behavioral sensitization, and decreased response to antipsychotic-induced catalepsy (Jeon et al., 2010).

Shannon et al. (2000) found that xanomeline attenuated apomorphine climbing in mice at doses ≥3 mg/kg. In the present study, a 1 mg/kg dose was effective in wild-type mice. This is probably due to strain differences in responsiveness. More interestingly, the attenuating effect of xanomeline on apomorphine-induced climbing was significantly reduced in D1-M4-KO mice (Fig. 3). However, in contrast to the amphetamine-induced locomotor hyperactivity study, xanomeline was able to inhibit apomorphine-induced climbing at the highest dose in D1-M4-KO mice. Whether higher doses of xanomeline could inhibit amphetamine-induced hyperactivity in D1-M4-KO mice is unknown. However, higher xanomeline doses would probably affect baseline locomotor activity (Woolley et al., 2009).

The present findings extend our earlier data (Jeon et al., 2010) showing that M_4 mAChRs present on D_1 dopamine receptor-expressing neurons play an important role in the regulation of dopaminergic neurotransmission. D_1 dopamine receptors activate, whereas M_4 mAChRs inhibit, adenylyl cyclase and thereby regulate cAMP production through $G_{\alpha s}$ and $G_{\alpha i/0}$ proteins, respectively (Onali and Olianas, 2002). The present findings therefore suggest that one mechanism by which xanomeline exerts its antipsychotic effect could be via M_4 receptors, acting as functional D_1 dopamine antagonists on the cAMP-dependent signaling pathways.

Since xanomeline is not entirely M_1/M_4-specific (Jakubík et al., 2006; Noetzel et al., 2009), classical cholinomimetic side effects, e.g., gastrointestinal side effects, were observed in humans (Bodick et al., 1997; Shekhar et al., 2008). Previous efforts to develop highly selective orthosteric agonists of the individual muscarinic subtypes have not been successful. However, selective positive allosteric modulators of the M_4 mAChR have been developed recently (Brady et al., 2008; Chan et al., 2008), and in preclinical studies, positive allosteric modulation of M_4 mAChR has been shown to attenuate apomorphine-induced deficits in prepulse inhibition (Chan et al., 2008) and amphetamine-induced hyperactivity in rats (Brady et al., 2008), supporting the potential role of the M_4 mAChR as a future target for treatment of psychosis.

In conclusion, the antipsychotic-like effects of xanomeline investigated by amphetamine-induced hyperactivity and apomorphine-induced climbing were markedly attenuated in D1-M4-KO mice. This suggests that the specific subpopulation of M_4 mAChRs localized on D_1-containing neurons plays an important role in regulation of dopamine homeostasis and in the antipsychotic effects of xanomeline. Consequently, this study suggests a possible mechanism of action for the antipsychotic effects of xanomeline. The current data further support the hypothesis that activation of the M_4 mAChR represents a potential target for the medical treatment of psychosis.

References

Bernard V, Normand E, Bloch B (1992) Phenotypical characterization of the rat striatal neurons expressing muscarinic receptor genes. J Neurosci 12:3591–3600.

Bodick NC, Offen WW, Levey AI, Cutler NR, Gauthier SG, Satlin A, Shannon HE, Tollefson GD, Rasmussen K, Bymaster FP, Hurley DJ, Potter WZ, Paul SM (1997) Effects of xanomeline, a selective muscarinic receptor agonist, on cognitive function and behavioral symptoms in Alzheimer disease. Arch Neurol 54:465–473.

Brady AE, Jones CK, Bridges TM, Kennedy JP, Thompson AD, Heiman JU, Breininger ML, Gentry PR, Yin H, Jadhav SB, Shirey JK, Conn PJ, Lindsley CW (2008) Centrally active allosteric potentiators of the M_4 muscarinic acetylcholine receptor reverse amphetamine-induced hyperlocomotor activity in rats. J Pharmacol Exp Ther 327:941–953.

Bymaster FP, Felder C, Ahmed S, McKinzie D (2002) Muscarinic receptors as a target for drugs treating schizophrenia. Curr Drug Targets CNS Neurol Disord 1:163–181.

Chan WY, McKinzie DL, Bose S, Mitchell SN, Witkin JM, Thompson RC, Christopoulos A, Lazareno S, Birdsall NJ, Bymaster FP, Felder CC (2008) Allosteric modulation of the muscarinic M_4 receptor as an approach to treating schizophrenia. Proc Natl Acad Sci U S A 105:10978–10983.

Felder CC, Porter AC, Skillman TL, Zhang L, Bymaster FP, Nathanson NM, Hamilton SE, Gomeza J, Wess J, McKinzie DL (2001) Elucidating the role of muscarinic receptors in psychosis. Life Sci 68:2605–2613.

Fink-Jensen A, Schmidt LS, Dencker D, Schülein C, Wess J, Wörtwein G, Woldbye DP (2011) Antipsychotic-induced catalepsy is attenuated in mice lacking the M_4 muscarinic acetylcholine receptor. Eur J Pharmacol 656:39–44.

Gomeza J, Zhang L, Kostenis E, Felder C, Bymaster F, Brodkin J, Shannon H, Xia B, Deng C, Wess J (1999) Enhancement of D_1 dopamine receptor-mediated locomotor stimulation in M_4 muscarinic acetylcholine receptor knockout mice. Proc Natl Acad Sci U S A 96:10483–10488.

Jakubík J, El-Fakahany EE, Dolezal V (2006) Differences in kinetics of xanomeline binding and selectivity of activation of G proteins at M_1 and M_2 muscarinic acetylcholine receptors. Mol Pharmacol 70:656–666.

Jeon J, Dencker D, Wörtwein G, Woldbye DP, Cui Y, Davis AA, Levey AI, Schütz G, Sager TN, Mørk A, Li C, Deng CX, Fink-Jensen A, Wess J (2010) A subpopulation of neuronal M_4 muscarinic acetylcholine receptors plays a critical role in modulating dopamine-dependent behaviors. J Neurosci 30:2396–2405.

Jones CK, Eberle EL, Shaw DB, McKinzie DL, Shannon HE (2005) Pharmacologic interactions between the muscarinic cholinergic and dopaminergic systems in the modulation of prepulse inhibition in rats. J Pharmacol Exp Ther 312:1055–1063.

Levey AI (1993) Immunological localization of M_1–M_5 muscarinic acetylcholine receptors in peripheral tissues and brain. Life Sci 52:441–448.

Noetzel MJ, Grant MK, El-Fakahany EE (2009) Immediate and delayed consequences of xanomeline wash-resistant binding at the M_3 muscarinic receptor. Neurochem Res 34:1138–1149.

Onali P, Olianas MC (2002) Muscarinic M_4 receptor inhibition of dopamine D_1-like receptor signalling in rat nucleus accumbens. Eur J Pharmacol 448:105–111.

Protais P, Costentin J, Schwartz JC (1976) Climbing behavior induced by apomorphine in mice: a simple test for the study of dopamine receptors in striatum. Psychopharmacology (Berl) 50:1–6.

Shannon HE, Rasmussen K, Bymaster FP, Hart JC, Peters SC, Swedberg MD, Jeppesen L, Sheardown MJ, Sauerberg P, Fink-Jensen A (2000) Xanomeline, an M_1/M_4 preferring muscarinic cholinergic receptor agonist, produces antipsychotic-like activity in rats and mice. Schizophr Res 42:249–259.

Shekhar A, Potter WZ, Lightfoot J, Lienemann J, Dubé S, Mallinckrodt C, Bymaster FP, McKinzie DL, Felder CC (2008) Selective muscarinic receptor agonist xanomeline as a novel treatment approach for schizophrenia. Am J Psychiatry 165:1033–1039.

Stanhope KJ, Mirza NR, Bickerdike MJ, Bright JL, Harrington NR, Hesselink MB, Kennett GA, Lightowler S, Sheardown MJ, Syed R, Upton RL, Wadsworth G, Weiss SM, Wyatt A (2001) The muscarinic receptor agonist xanomeline has an antipsychotic-like profile in the rat. J Pharmacol Exp Ther 299:782–792.

Thomsen M, Wess J, Fulton BS, Fink-Jensen A, Caine SB (2010) Modulation of prepulse inhibition through both M_1 and M_4 muscarinic receptors in mice. Psychopharmacology (Berl) 208:401–416.

Tzavara ET, Bymaster FP, Davis RJ, Wade MR, Perry KW, Wess J, McKinzie DL, Felder C, Nomikos GG (2004) M_4 muscarinic receptors regulate the dynamics of cholinergic and dopaminergic neurotransmission: relevance to the pathophysiology and treatment of related CNS pathologies. FASEB J 18:1410–1412.

Weiner DM, Levey AI, Brann MR (1990) Expression of muscarinic acetylcholine and dopamine receptor mRNAs in rat basal ganglia. Proc Natl Acad Sci U S A 87:7050–7054.

Woolley ML, Carter HJ, Gartlon JE, Watson JM, Dawson LA (2009) Attenuation of amphetamine-induced activity by the non-selective muscarinic receptor agonist, xanomeline, is absent in muscarinic M_4 receptor knockout mice and attenuated in muscarinic M_1 receptor knockout mice. Eur J Pharmacol 603:147–149.

Zhang W, Basile AS, Gomeza J, Volpicelli LA, Levey AI, Wess J (2002) Characterization of central inhibitory muscarinic autoreceptors by the use of muscarinic acetylcholine receptor knock-out mice. J Neurosci 22:1709–1717.