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Functional Activity of the M_2 and M_4 Receptor Subtypes in the Spinal Cord Studied with Muscarinic Acetylcholine Receptor Knockout Mice

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List of abbreviations:

muscarinic acetylcholine receptors, mAChRs;

guanosine 5'-O-(3-[^{35}S]thio)triphosphate, [^{35}S]GTPγS

D-Ala^2, N-Me-Phe^4, Gly-ol^5)-enkephalin, DAMGO

guanosine triphosphate, GTP; guanosine diphosphate, GDP

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Abstract

Stimulation of spinal muscarinic acetylcholine receptors (mAChRs) produces potent analgesia. Both M_2 and M_4 mAChRs are coupled to similar G proteins ($G_{i/o}$ family) and play a critical role in the analgesic action of mAChR agonists. To determine the relative contribution of M_2 and M_4 subtypes to activation of $G_{i/o}$ proteins in the spinal cord, we examined the receptor-mediated guanosine 5'-O-(3-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) binding in M_2 and M_4 subtype knockout (KO) mice. Basal [^{35}S]GTPγS binding in the spinal cord was similar in the wild-type controls, M_2 and M_4 single-KO, and M_2/M_4 double-KO mice. The spinal [^{35}S]GTPγS binding stimulated by either muscarine or oxotremorine-M was not significantly different among three groups of wild-type mouse strains. In M_2 single-KO and M_2/M_4 double-KO mice, the agonist-stimulated [^{35}S]GTPγS binding was completely abolished in the spinal cord. Furthermore, the agonist-stimulated [^{35}S]GTPγS binding in the spinal cord of M_4 single-KO mice was significantly reduced (~15%), compared to that in wild-type controls. On the other hand, the spinal [^{35}S]GTPγS binding stimulated by a μ opioid agonist was not significantly different between wild-type and M_2- and M_4-KO mice. This study provides complementary new evidence that M_2 is the most predominant mAChR subtype coupled to the $G_{i/o}$ proteins in the spinal cord. Furthermore, these data suggest that a small but functionally significant population of M_4 receptors exists in the mouse spinal cord. The functional activity of these M_4 receptors appears to require the presence of M_2 receptors.

Introduction

The cholinergic system and muscarinic acetylcholine receptors (mAChRs) in the dorsal horn of the spinal cord are important for regulation of different physiological functions including nociception. In this regard, intrathecal administration of muscarinic receptor agonists or acetylcholinesterase inhibitors produces potent analgesia in both animals and humans (Iwamoto and Marion, 1993; Naguib and Yaksh, 1994; Hood et al., 1997; Naguib and Yaksh, 1997). The analgesic effect produced by muscarinic receptor agonists or acetylcholinesterase inhibitors is blocked by the mAChR antagonist atropine (Naguib and Yaksh, 1994). Furthermore, spinal acetylcholine and mAChRs are involved in the analgesic action produced by morphine and α_2-adrenergic receptor agonists (Pan et al., 1999; Chen and Pan, 2001). It has been shown that neurons and nerve terminals expressing choline acetyltransferase and acetylcholinesterase (enzymes for acetylcholine synthesis and degradation) are located in the spinal dorsal horn (Ribeiro-da-Silva and Cuello, 1990; Wetts and Vaughn, 1994). Autoradiographic studies have demonstrated that the highest density of mAChRs in the spinal cord is distributed in the superficial laminae in both rats and humans (Yamamura et al., 1983; Villiger and Faull, 1985; Maher et al., 2001).

Molecular cloning studies have revealed the existence of five molecularly distinct mAChR subtypes referred to as M_1-M_5 (Wess, 1996; Caulfield and Birdsall, 1998). The M_1-M_5 mAChRs are prototypical members of the superfamily of G protein-coupled receptors. The M_1, M_3, and M_5 receptor subtypes couple preferentially to the $G_{q/11}$ protein, whereas the M_2 and M_4 receptors are preferentially coupled to $G_{i/o}$ proteins (Caulfield, 1993; Felder, 1995). In rodents,

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both the M_2 and M_4 subtypes that are coupled to the pertussis toxin-sensitive $G_{i/o}$ proteins have been implicated in the inhibitory effect of mAChR agonists on nociception and spinal dorsal horn neurons (Ellis et al., 1999; Gomeza et al., 1999a; Duttaroy et al., 2002; Chen and Pan, 2004). Recent studies in mutant mouse lines deficient in M_2 and M_4 mAChRs [knockout (KO) mice] indicate that the spinal analgesic effect produced by mAChR agonists is mediated by M_2 and M_4 subtypes (Gomeza et al., 1999a; Duttaroy et al., 2002). Although behavioral experiments suggest the presence of the M_4 subtype in the spinal cord of mice, this subtype could not be directly demonstrated in the mouse spinal cord using immunocytochemical labeling techniques (Duttaroy et al., 2002). The guanosine 5'-O-(3-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) binding assay primarily determines the functional coupling of G protein-coupled receptors to the G proteins of the $G_{i/o}$ family (Maher et al., 2001; Sim-Selley and Childers, 2002). In this study, we used agonist-stimulated [^{35}S]GTPγS binding and M_2 and M_4 single-KO and M_2/M_4 double-KO mice to determine the relative amount and interaction between functional M_2 and M_4 subtypes in G protein coupling in the spinal cord. This study reveals a close association of M_2 and M_4 subtypes in their coupling to $G_{i/o}$ proteins in the spinal cord.

Methods

Animals

The generation of M_2 and M_4 mAChR single-KO and M_2/M_4 double-KO mice has been described previously (Gomeza et al., 1999a; Gomeza et al., 1999b; Duttaroy et al., 2002). The genetic background of these mice was: M_2 single-KO (129J1/CF-1 hybrids), M_4 single-KO (129SvEv/CF-1 hybrids), and M_2/M_4 double-KO (129/J1/129SvEv/CF1 hybrids). Wild-type mice of the same genetic background as M_2, M_4, and M_2/M_4 KO mice served as controls. To generate mice deficient in both M_2 and M_4 mAChRs, homozygous M_2 receptor KO mice were mated with homozygous M_4 receptor KO mice (Duttaroy et al., 2002). The resulting F1 compound heterozygotes were then intercrossed to generate M_2/M_4 double KO mice (F2 generation). In parallel, the wild-type F2 mice were interbred to obtain wild-type control mice. Thus, both M_2/M_4 double-KO mice and the corresponding wild-type control mice had an equivalent genetic background (129/SvJ1 [25%] x 129SvEv [25%] x CF1 [50%]). Mice were maintained by intermating homozygous KO and the corresponding wild-type strains. All animals used for this study were amplified by Taconic Farms (Germantown, NY) where they were raised and housed under identical conditions. Mouse genotyping was carried out by Southern blotting and polymerase chain reaction analysis of mouse-tail DNA, as described previously (Gomeza et al., 1999a; Gomeza et al., 1999b; Duttaroy et al., 2002).

All experiments were performed using male mice (5 to 6 weeks old). In all experiments, KO mice and age-matched wild-type mice of the proper genetic background were performed in parallel. Mice were first anesthetized with 4-5% halothane, and the whole spinal cord was

rapidly removed by hydraulic extrusion. The spinal cord tissue was immediately frozen in 2-methylbutane (Sigma, St. Louis, MO) in the presence of dry ice and stored at -80°C. The experimental protocols and procedures were approved by the Animal Care and Use Committee of the Pennsylvania State University College of Medicine and conformed to the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Muscarinic agonists-stimulated [^{35}S]GTPγS binding in spinal membranes

The [^{35}S]GTPγS binding stimulated by muscarinic agonists was conducted to quantify functional muscarinic receptors in the spinal cord, as described previously (Chen and Pan, 2003). The [^{35}S]GTPγS binding assay is based on the principle that the inactive state of the G protein α subunit has a relatively high affinity for GDP over GTP, whereas activation of a G protein-coupled receptor by its agonist shifts the α subunit into a higher affinity for GTP versus GDP. The [^{35}S]GTPγS is a hydrolysis-resistant form of GTP, and the degree to which an agonist stimulates [^{35}S]GTPγS binding can be quantified in tissue membranes. Unlike conventional radioligand binding techniques, the agonist-stimulated [^{35}S]GTPγS binding can provide functional information about the receptor (i.e., coupling of G proteins to activated receptors) (Lazareno, 1999; Sim-Selley and Childers, 2002). Two structurally different non-selective mAChR agonists, muscarine and oxotremorine-M, were used to activate mAChRs and stimulate spinal cord [^{35}S]GTPγS binding. To harvest adequate amounts of membrane proteins, the spinal cord from 2-3 mice in the mAChR KO and corresponding wild-type groups was used in each binding experiment. The tissue was homogenized and disrupted by sonication in ice-cold 50 mM Tris buffer containing 3 mM $MgCl_2$ and 1 mM EGTA (pH 7.4). The homogenate was then

centrifuged at 500 g for 10 min at 4°C. The pellet was discarded and the supernatant was centrifuged at 48,000 g for 30 min at 4°C. The pellet was resuspended in fresh assay buffer (50 mM Tris, 3 mM $MgCl_2$, 100 mM NaCl, 0.2 mM EGTA, pH 7.7) and was centrifuged again as described above. The final pellet was resuspended in 2 ml of the assay buffer and sonicated for 5 s. The protein content was measured using the Bio-Rad protein assay kit.

The concentration-dependent effect of agonist-stimulated [^{35}S]GTPγS binding was determined by incubating the tissue homogenate with 0.01-100 μM muscarine or 0.005-25 μM oxotremorine-M, 30 μM GDP, 0.05 nM [^{35}S]GTPγS, 4 mU/ml adenosine deaminase, 20 μg protein, and assay buffer in a final volume of 1 ml (in duplicates). The basal binding was determined in the presence of GDP and absence of muscarinic agonists, and nonspecific binding was assessed in the presence of 10 μM unlabeled GTPγS. The concentrations of muscarine and oxotremorine-M were chosen based on previous experiments (Chen and Pan, 2003) and pilot studies. After incubation at 30°C for 1 hr, the reaction was terminated by filtration through Whatman GF/B filters on a cell harvester with cold 50 mM Tris buffer (pH 7.4). Radioactivity was determined by immersion of filters in scintillation fluid, incubated for 2 hr at room temperature, and counted by a liquid scintillation counter (LS6500, Beckman Coulter, Inc., Fullerton, CA). Nonlinear regression analyses of concentration-effect curves were performed using Prism (GraphPad Software Inc, San Diego, CA). Percent stimulation was calculated as: (net stimulated binding/basal binding) × 100%. [^{35}S]GTPγS (1,250 Ci/mmol) was obtained from PerkinElmer Life Sciences (Boston, MA).

To determine whether the reduced [^{35}S]GTPγS binding stimulated by muscarinic agonists in KO mice was due to altered G protein levels or agonist-induced G protein coupling,

we repeated agonist-stimulated [^{35}S]GTPγS binding using a selective opioid agonist, (D-Ala^2, N-Me-Phe^4, Gly-ol^5)-enkephalin (DAMGO), as the control. The DAMGO concentration used was 0.001-10 μM (Chen et al., 2002). [^{35}S]GTPγS binding assays and data calculation were performed as described above. GDP, muscarine, oxotremorine-M, atropine, DAMGO, and adenosine deaminase were obtained from Sigma (St. Louis, MO). GTPγS was purchased from Boehringer Mannheim (Indianapolis, IN). All other reagent grade chemicals were obtained from Sigma.

Quantification of M_2 receptors using immunoprecipitation and radioactive binding assay

Because we observed that muscarinic agonist-stimulated [^{35}S]GTPγS binding was significantly reduced in M_4-KO mice (see Results), the following immunoprecipitation and radioligand binding experiments were performed to determine if the M_2 receptor levels are reduced in the spinal cord of M_4-KO mice. An M_2 subtype-specific antiserum was raised against a nonconserved region of the third cytoplasmic loop of the mouse M_2 receptor protein (Gomeza et al., 1999a; Gomeza et al., 1999b). The mice were decapitated and the spinal cords were quickly harvested. Two spinal cords were used to obtain adequate membrane proteins for each assay. The spinal tissue was homogenized in ice-cold 0.32 M sucrose in 5 mM Tris-HCl buffer containing 1 mM phenylmethanesulfonyl fluoride. The homogenate was centrifuged at 500 g for 10 min at 4^{o}C. The pellet was discarded, and the supernatant was centrifuged again at 48,000 g for 20 min at 4^{o}C. Then, the pellet was resuspended in assay buffer (25 mM of phosphate buffer containing 5 mM of $MgCl_2$ and 1 mM of phenylmethanesulfonyl fluoride, pH 7.4) and was centrifuged as described above. The final pellet was resuspended in 3 ml of the

same buffer and disrupted by sonication for 5 s. Subsequently, the membrane protein was incubated for 1 hr with 2 nM of the nonselective muscarinic antagonist [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB; 42 Ci/mmol; PerkinElmer Life Sciences) in the final volume of 1 ml. After washing thoroughly, the labeled membranes were solubilized with 1% of digitonin and followed by immunoprecipitation of solubilized [^{3}H]QNB-labeled receptors with the M_2 subtype-selective antiserum, as described previously (Gomeza et al., 1999a; Gomeza et al., 1999b). Radioactivity was quantified using Beckman LS6500 scintillation counter. Data were analyzed using the GraphPad Prism program.

Data analysis

Data are presented as means ± S.E.M. The saturation binding data were processed and fit using nonlinear regression analysis (Prism; GraphPad Software). For computation of EC_{50} values, [^{35}S]GTPγS binding data were converted to the percentage of stimulation by muscarine and oxotremorine based on the following calculation: [(net stimulated binding/(maximal net stimulated binding)] x 100%. The EC_{50} values of muscarine and oxotremorine-M were determined by nonlinear regression analyses of the concentration-response curves using Prism (GraphPad Software). The binding data between matched pairs were analyzed using the Wilcoxon rank sum test. For comparisons in more than two groups, the Kruskal-Wallis test was performed followed by the Mann-Whitney U *post hoc* test. $P < 0.05$ was considered to be statistically significant.

Results

Basal [^{35}S]GTPγS binding in the spinal cord

The basal [^{35}S]GTPγS binding, in the absence of muscarinic receptor agonists, in the spinal cord from the three wild-type mouse strains was almost identical (Figure 1). Basal [^{35}S]GTPγS levels were also similar in spinal cord tissues in the muscarine and oxotremorine control groups. Furthermore, the basal [^{35}S]GTPγS binding in the spinal cord from M_2 single-KO, M_4 single-KO, and M_2/M_4 double-KO mice was not significantly different from the corresponding wild-type controls (Figure 1).

Spinal [^{35}S]GTPγS binding stimulated by muscarinic agonists in wild-type and KO mice

The two non-selective mAChR agonists, muscarine and oxotremorine-M, produced a significant and concentration-dependent increase in [^{35}S]GTPγS binding in the spinal cord of all three strains of wild-type mice (Figures 2 and 3). In the presence of 10 μM atropine, muscarine and oxotremorine-M failed to cause a detectable increase in the spinal [^{35}S]GTPγS binding in wild-type mice (data not shown). Both 50-100 μM muscarine and 10-25 μM oxotremorine-M produced saturable [^{35}S]GTPγS binding in the spinal cord, the magnitude of which was comparable in three different wild-type strains (Figures 2 and 3). The EC_{50} values for muscarine in the M_2-, M_4-, and M_2/M_4-wild-type groups were 3.54 ± 0.12, 3.61 ± 0.12, and 3.58 ± 0.16 μM ($P < 0.05$), respectively. For oxotremorine-M-stimulated [^{35}S]GTPγS binding, the EC_{50} values in the M_2-, M_4-, and M_2/M_4-wild-type groups were 0.61 ± 0.05, 0.63 ± 0.02, and 0.62 ± 0.02 μM ($P < 0.05$), respectively. Notably, the maximal increase in spinal [^{35}S]GTPγS binding

produced by muscarine was significantly less than that stimulated by oxotremorine-M, and the EC_{50} value of muscarine was about 6-fold greater than that of oxotremorine-M (Figures 2 and 3).

In M_2/M_4 double-KO mice, the [^{35}S]GTPγS binding stimulated by muscarine and oxotremorine-M was completely eliminated (Figures 2 and 3). By comparison, there was a small but significant reduction in stimulated [^{35}S]GTPγS binding in the spinal cord of M_4 single-KO mice, compared to the corresponding wild-type controls (Figures 2 and 3). The level of attenuation of the maximal stimulated [^{35}S]GTPγS binding in M_4 single-KO mice was similar in muscarine- and oxotremorine-M-treated groups (14.7 ± 0.5% vs. 15.1 ± 0.4%). Similar to M_2/M_4 double-KO mice, both muscarine and oxotremorine-M failed to cause any increase in [^{35}S]GTPγS binding in the spinal cord of M_2 single-KO mice (Figures 2 and 3).

Quantification of spinal M_2 receptors in wild-type and mAChR KO mice

To determine M_2 receptor expression levels in the spinal cord of wild-type and mAChR KO mice (n = 4 in each group), mouse spinal cord membranes were labeled with a saturating concentration (2 nM) of the non-selective mAChR antagonist, [^{3}H]QNB, solubilized with 1% digitonin, and then immunoprecipitated using an M_2 receptor-selective antiserum (Gomeza et al., 1999a; Gomeza et al., 1999b). This analysis showed that the three wild-type mouse strains expressed similar levels of spinal M_2 receptors (data not shown). Strikingly, [^{3}H]QNB binding was completely eliminated in spinal cord membranes from M_2-KO mice (Figure 4). However, there was no significant difference in the amount of immunoprecipitated M_2 receptors in the spinal cord between wild-type and M_4-KO mice (Figure 4).

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DAMGO-stimulated [^{35}S]GTPγS binding in the spinal cord of wild-type and mAChR KO mice

To exclude the possibility that the reduction in mAChR agonist-induced [^{35}S]GTPγS binding in the M_4 single-KO mice was due to reduced levels of spinal cord $G_{i/o}$ proteins, we carried out a series of control experiments using the μ opioid DAMGO as an agonist. DAMGO produced a concentration-dependent increase in the [^{35}S]GTPγS binding in the spinal cord of M_2- and M_4-KO and wild-type mice (n = 4 in each group, Figure 5). The spinal [^{35}S]GTPγS binding stimulated by DAMGO was similar among the three different wild-type strains (data not shown). Furthermore, the DAMGO-stimulated [^{35}S]GTPγS binding in the spinal cord of M_2- or M_4-KO mice was not significantly different from that in wild-type mice (Figure 5).

Discussion

The spinal mAChRs are important for regulation of nociception. There are five subtypes of closely homologous mAChRs that are coupled to heterotrimeric G proteins, resulting in a multitude of cellular effects through a variety of signaling pathways (Caulfield, 1993; Wess, 1996). Most available mAChR agonists or antagonists are unable to clearly discriminate among individual mAChR subtypes because of the close homology of the orthosteric binding site. Data from mAChR subtype KO mice have unequivocally demonstrated that both the M_2 and M_4 subtypes are involved in mediating analgesia produced by muscarinic agonists (Gomeza et al., 1999a; Gomeza et al., 1999b; Duttaroy et al., 2002). The M_2 and M_4 subtypes have been demonstrated in the superficial dorsal horn of the spinal cord in rodents and humans (Potter et al., 1996; Hoglund and Baghdoyan, 1997; Duttaroy et al., 2002; Li et al., 2002). Also, spinal [^{35}S]GTPγS binding stimulated by a mAChR agonist is primarily located in the superficial dorsal horn (Maher et al., 2001). In the present study, we determined the relative contribution of the M_2 and M_4 subtypes to activation of $G_{i/o}$ proteins in the spinal cord using M_2 and M_4 single- and M_2/M_4 double-KO mice.

Unlike conventional receptor binding technique, [^{35}S]GTPγS binding assays directly assess the efficiency of receptor-G protein coupling. It has been shown that activation of receptors coupled to the $G_{i/o}$ family of G proteins can be readily detected with [^{35}S]GTPγS binding techniques (Maher et al., 2001; Sim-Selley and Childers, 2002). Thus, the M_2 and M_4 subtypes likely are the mAChRs measured in the present study since they are both coupled to $G_{i/o}$ proteins. The recently developed mAChR subtype KO mice are particularly useful

experimental tools because inactivation of one specific subtype does not seem to affect the expression levels of the remaining mAChR subtypes (Wess, 2004). We found that basal [^{35}S]GTPγS binding in the spinal cord was similar in wild-type and M_2 and M_4 KO mice, suggesting that basal levels of $G_{i/o}$ proteins and the affinity of G proteins for GDP and GTP are not altered in M_2 and M_4 KO mice. We observed that both muscarine and oxotremorine-M produced profound increases in the [^{35}S]GTPγS binding in the spinal cord of wild-type mice, indicating the presence of functional M_2 and/or M_4 mAChR subtypes in the spinal cord. Interestingly, oxotremorine-M displayed significantly greater potency and efficacy than muscarine in stimulating [^{35}S]GTPγS binding in the mouse spinal cord. The level of stimulation of [^{35}S]GTPγS binding is directly proportional to the magnitude of biological effects displayed by these two mAChR agonists in other pharmacological assays (Kurihara et al., 1993; Duttaroy et al., 2002; Chen and Pan, 2004). Thus, these data reinforce the concept that agonist-stimulated [^{35}S]GTPγS binding is a valuable functional assay that can be used to estimate the efficacy and potency of the biological action of G protein-coupled receptor agonists.

In the spinal cord, muscarinic agonist-stimulated [^{35}S]GTPγS binding was completely eliminated in M_2/M_4 double-KO mice. This observation is consistent with our recent finding that the inhibitory effect of muscarinic agonists on spinal dorsal horn neurons is abolished in rats pretreated with intrathecal pertussis toxin (Chen and Pan, 2004). Furthermore, both muscarine and oxotremorine-M failed to increase [^{35}S]GTPγS binding in the spinal cord of M_2 single-KO mice. Radioligand binding studies suggest that the M_2 subtype represents ~90% of the total spinal cord mAChR population in mice (Duttaroy et al., 2002). The [^{35}S]GTPγS binding data therefore provide further evidence that the M_2 subtype is the predominant mAChR

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coupled to $G_{i/o}$ proteins in the spinal cord.

The presence of spinal cord M_4 mAChRs has been predicted based on competition radioligand binding using rat spinal cord homogenates (Hoglund and Baghdoyan, 1997). Also, because intrathecal injection of the selective M_4 receptor antagonist, muscarinic toxin-3, reduces the antinociceptive effect of mAChR agonists in mice (Ellis et al., 1999), functional M_4 mAChRs are likely to exist in the mouse spinal cord dorsal horn. Additionally, the analgesic action of intrathecal muscarinic agonists is largely attenuated (by ~60-90%), but not abolished, in M_2 single-KO mice (Duttaroy et al., 2002). Because the analgesic effect of intrathecal muscarinic agonists is completely eliminated in M_2/M_4 double-KO mice (Duttaroy et al., 2002), spinal M_4 mAChRs are considered responsible for the residual muscarinic analgesic effect in M_2 single-KO mice. However, spinal M_4 mAChRs could not be detected by using immunocytochemical techniques in mice (Duttaroy et al., 2002). One possibility is that the number of M_4 mAChRs in the mouse spinal cord is too low to be detected by immunocytochemical techniques. In the present study, by comparing agonist-stimulated [^{35}S]GTPγS binding in M_4 single-KO and M_2/M_4 double-KO and wild-type mice, we found that the M_4 subtype mediated a small but significant [^{35}S]GTPγS response in the mouse spinal cord. The [^{35}S]GTPγS assay therefore appears to be more sensitive than the immunocytochemistry method in detecting spinal M_4 mAChRs. The reduction in muscarinic agonist-stimulated spinal [^{35}S]GTPγS binding in M_4-KO mice is not due to a decrease in M_2 mAChR levels because the amount of spinal M_2 receptors measured by a [^{3}H]QNB binding/immunoprecipitation assay was not significantly different between the wild-type and M_4-KO mice. Also, we found that DAMGO-stimulated [^{35}S]GTPγS binding was similar in wild-type and M_2- and M_4-KO mice,

suggesting that the G protein levels and receptor-G protein coupling are not generally altered in mAChR KO mice. Therefore, this study provides complementary new information for the presence of a small population of functionally significant M_4 mAChRs in the mouse spinal cord.

The complete loss of muscarinic agonist-stimulated [^{35}S]GTPγS binding in M_2 single-KO mice is an unexpected but important finding. Because the mAChR agonist-stimulated [^{35}S]GTPγS binding in the spinal cord was abolished in M_2/M_4 double-KO mice and reduced by about 15% in M_4 single-KO mice, one would predict a certain degree of residual [^{35}S]GTPγS binding in M_2 single-KO mice. Surprisingly, however, the muscarinic agonist-stimulated [^{35}S]GTPγS binding was abolished in the spinal cord of M_2 single-KO mice. These data suggest that M_4 receptor activity requires the presence of M_2 mAChRs in the mouse spinal cord. Alternatively, M_4 mAChRs may be able to form functional oligomers with spinal M_2 mAChRs. Consistent with this notion, it has been demonstrated that most G protein-coupled receptors can exist structurally or functionally as dimers or as part of larger oligomeric complexes (Angers et al., 2002; George et al., 2002).

In summary, [^{35}S]GTPγS binding assays showed that the mouse spinal cord expresses a small but significant number of functional M_4 mAChRs, in addition to the predominant population of M_2 mAChRs. Importantly, our data suggest that functional activity of spinal M_4 mAChRs may be dependent on the presence of M_2 mAChRs, possibly reflecting the existence of functional M_2/M_4 mAChR oligomers. Data from this study are important for our understanding of the cellular function and drug action mediated by M_2 and M_4 mAChR subtypes.

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Footnotes

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Figure Legends

Figure 1. Basal [^{35}S]GTPγS binding in the spinal cord of M_2 and M_4 single-KO and M_2/M_4 double-KO mice and corresponding wild-type controls. Panels A and B are basal binding [^{35}S]GTPγS data in the muscarine and oxotremorine-M group, respectively. Data are presented in means ± S.E.M. (n = 4 experiments in each group).

Figure 2. Panel A, concentration-effect curves of [^{35}S]GTPγS binding stimulated by muscarine in the spinal cord membranes of M_2 and M_4 single-KO and M_2/M_4 double-KO mice and the three respective wild-type (WT) strains. Panel B, comparison of maximal stimulated [^{35}S]GTPγS binding by 100 μM muscarine in the spinal cord from M_2 and M_4 single-KO and M_2/M_4 double-KO mice and corresponding wild-type controls. The binding data are expressed as the percent of basal [^{35}S]GTPγS binding. Data are presented in means ± S.E.M. (n = 4). * $P < 0.05$ compared to the value in the respective wild-type controls.

Figure 3. Panel A, concentration-effect curves of [^{35}S]GTPγS binding stimulated by oxotremorine-M in the spinal cord membranes of M_2 and M_4 single-KO and M_2/M_4 double-KO mice and the three respective wild-type (WT) strains. Panel B, comparison of maximal stimulated [^{35}S]GTPγS binding by 25 μM oxotremorine-M in the spinal cord from M_2 and M_4 single-KO and M_2/M_4 double-KO mice and corresponding wild-type controls. The binding data are expressed as the percent of basal [^{35}S]GTPγS binding. Data are presented in means ± S.E.M. (n = 4). * $P < 0.05$ compared to the value in the respective wild-type controls.

Figure 4. Quantification of M_2 mAChRs in the spinal cord of wild-type (WT) and M_2- and M_4-KO mice. Spinal cord membranes were incubated with 2 nM of the non-selective muscarinic antagonist,[^{3}H]QNB. [^{3}H]QNB-labeled muscarinic receptors were solubilized and immunoprecipitated with an M_2 receptor-selective antiserum. Data are presented in means ± S.E.M. (n = 4). * $P < 0.05$ compared to the wild-type control.

Figure 5. Concentration-effect curves of [^{35}S]GTPγS binding stimulated by DAMGO in spinal cord membranes of wild-type (WT) and M_2- and M_4-KO mice. Data are presented in means ± S.E.M. (n = 4). The data are expressed as the percent increase over basal [^{35}S]GTPγS binding.

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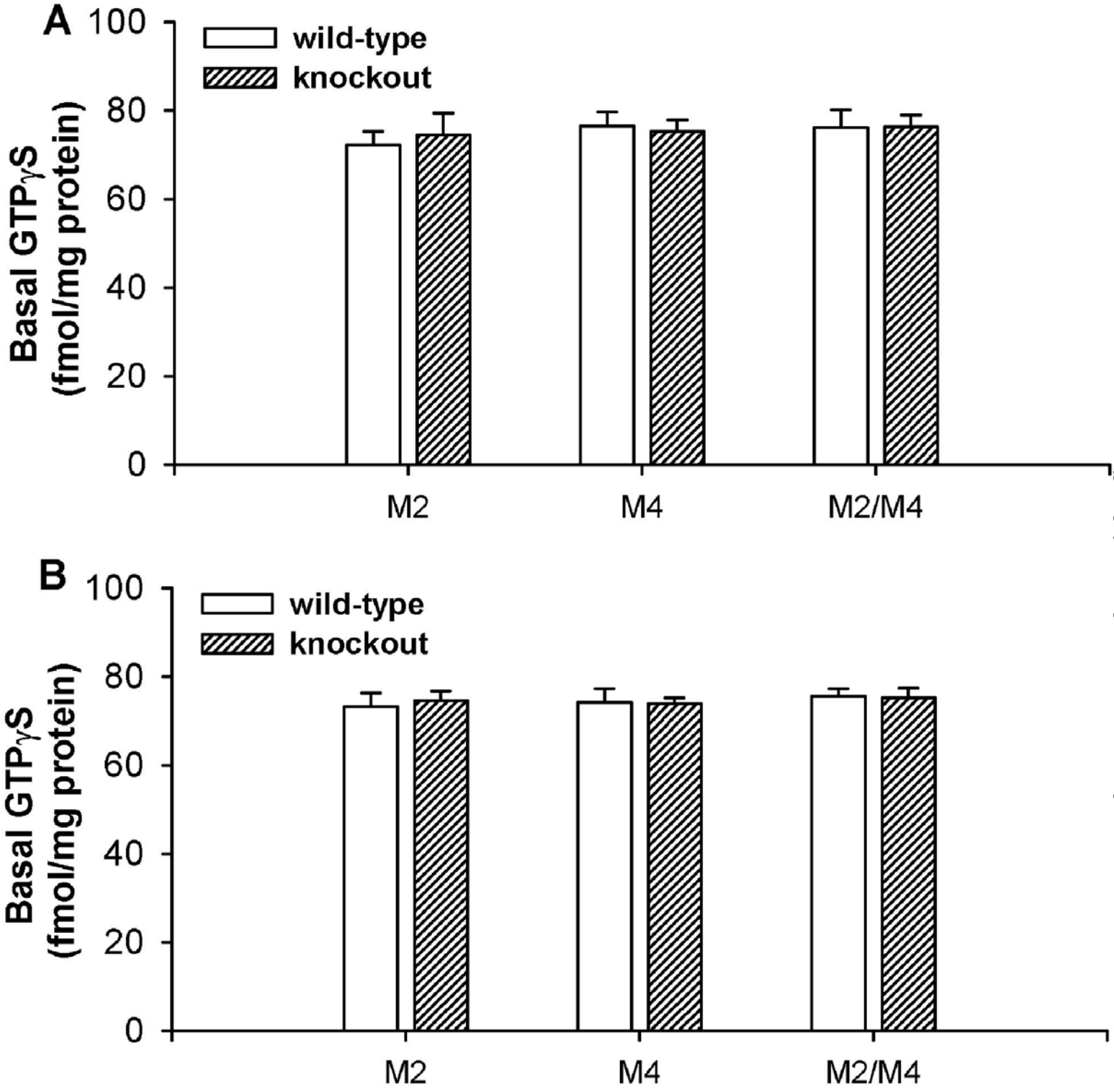

Fig. 1

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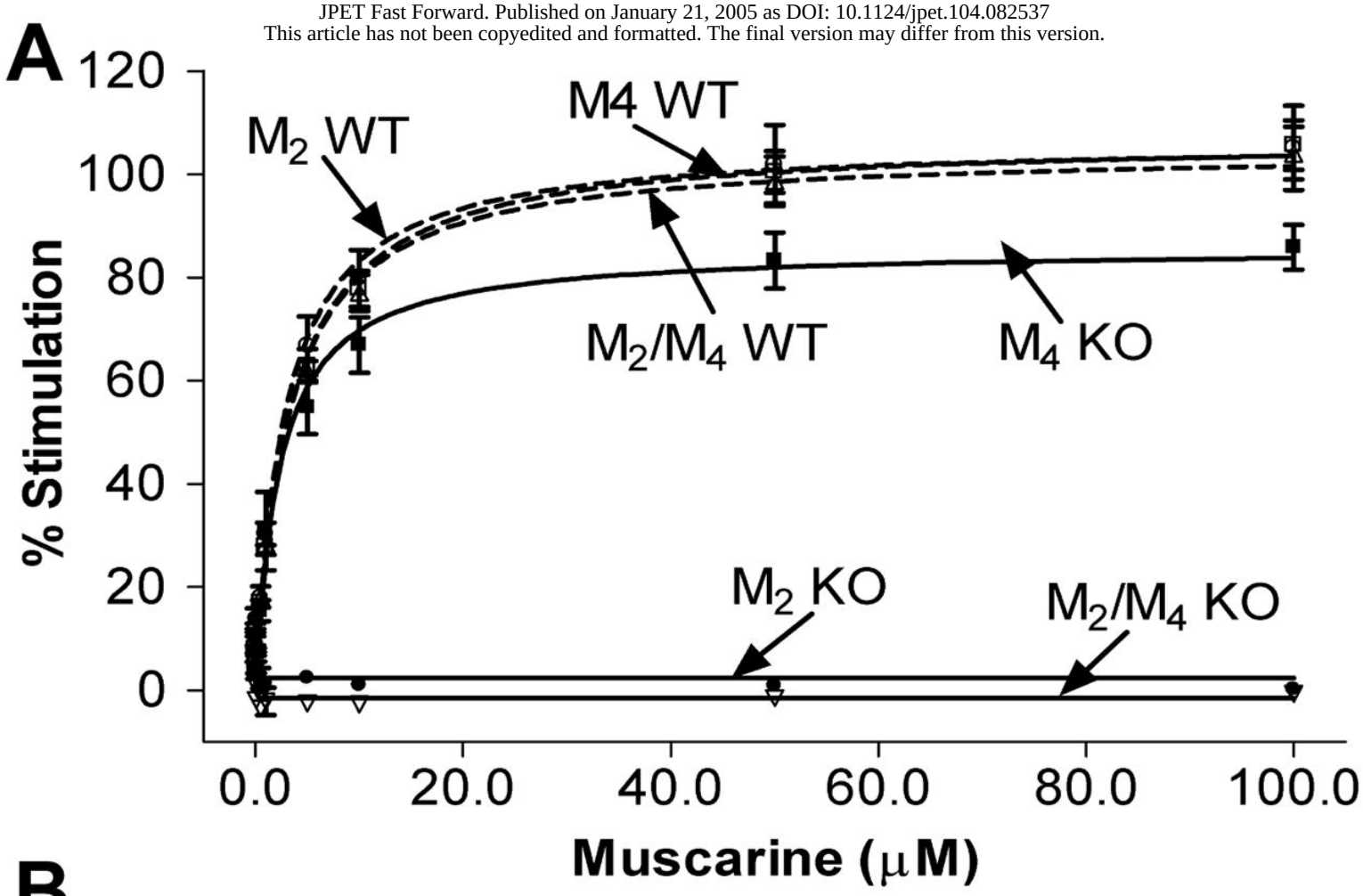

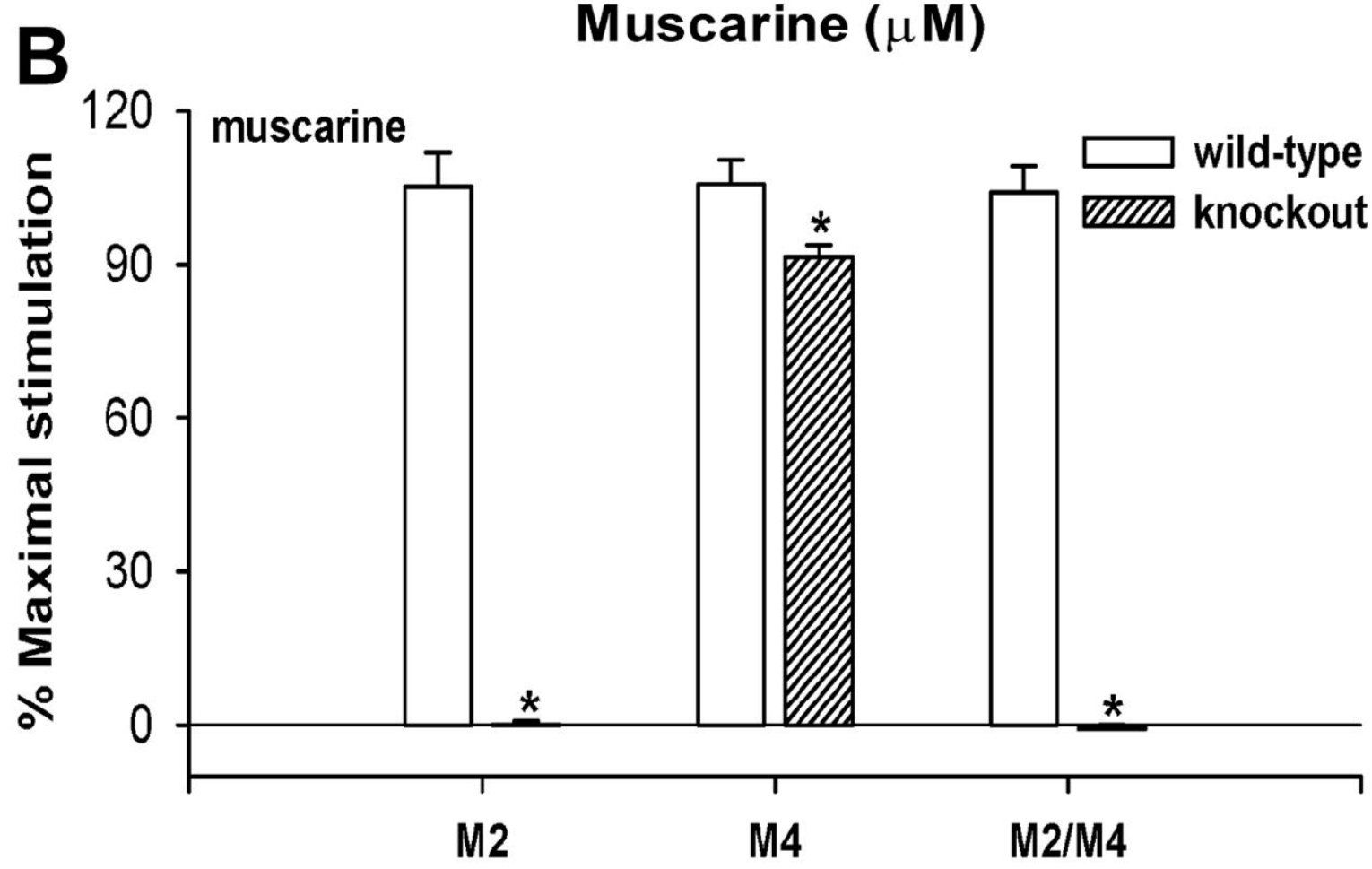

Fig. 2

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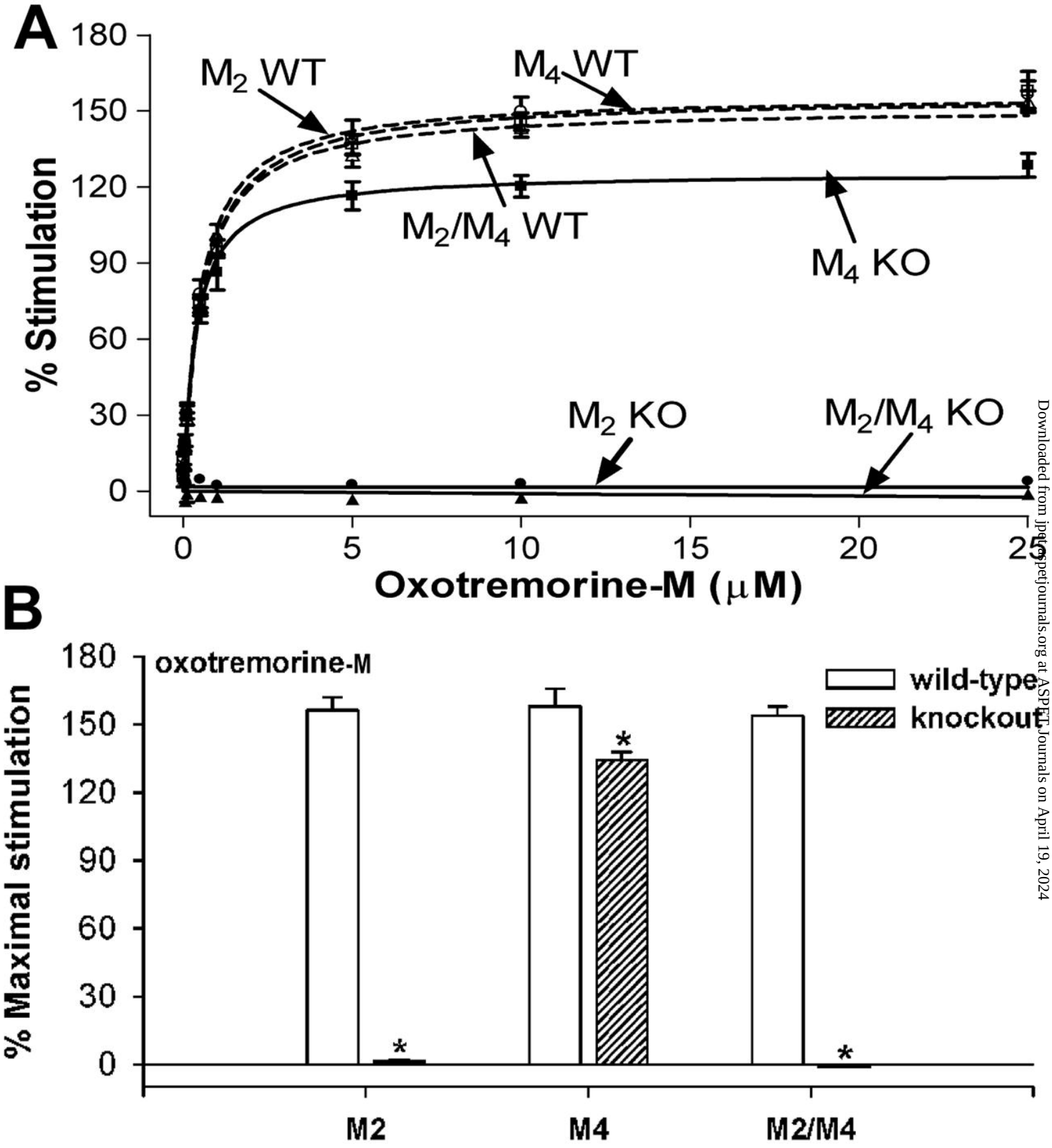

Fig. 3

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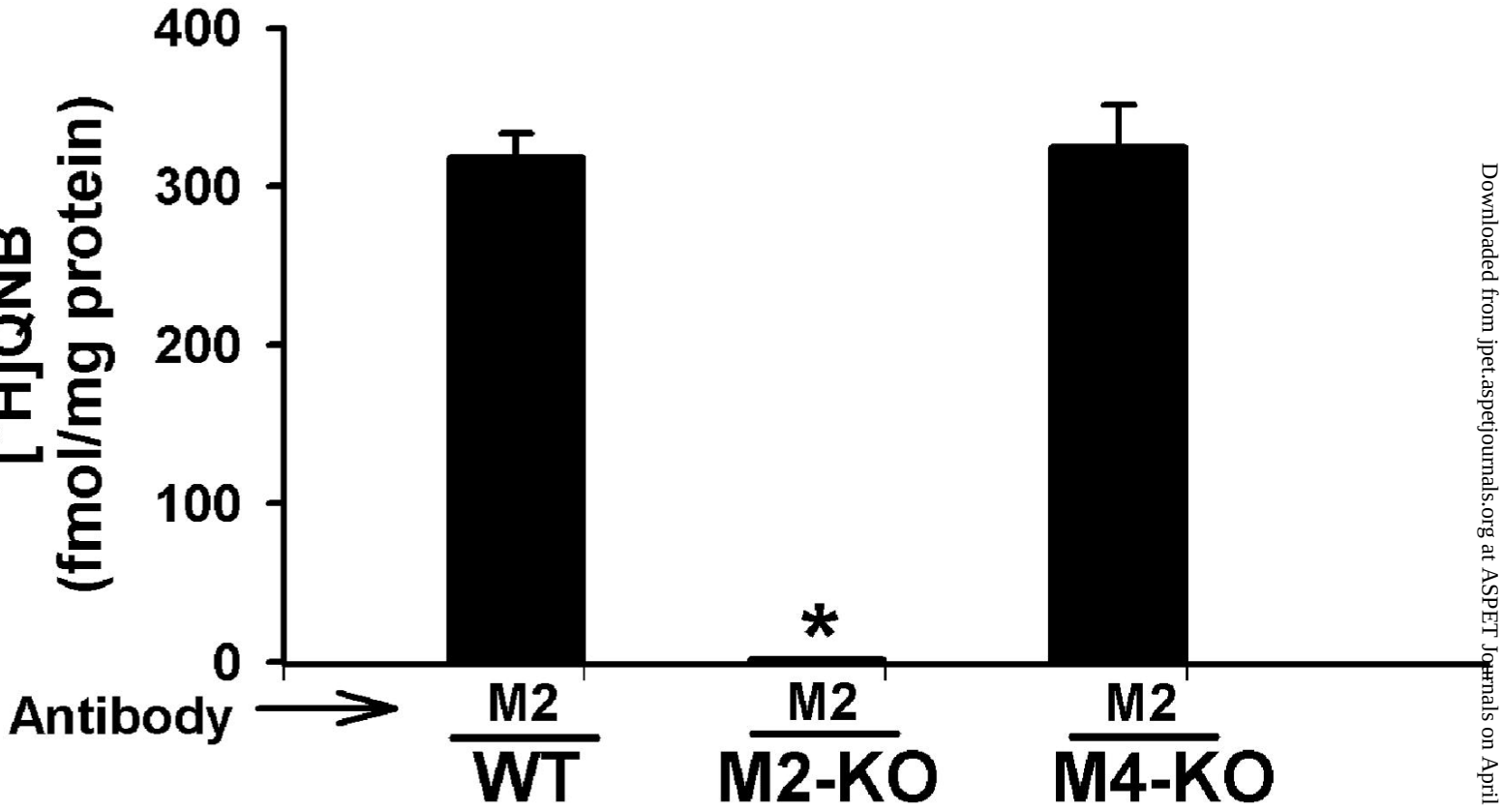

Fig. 4

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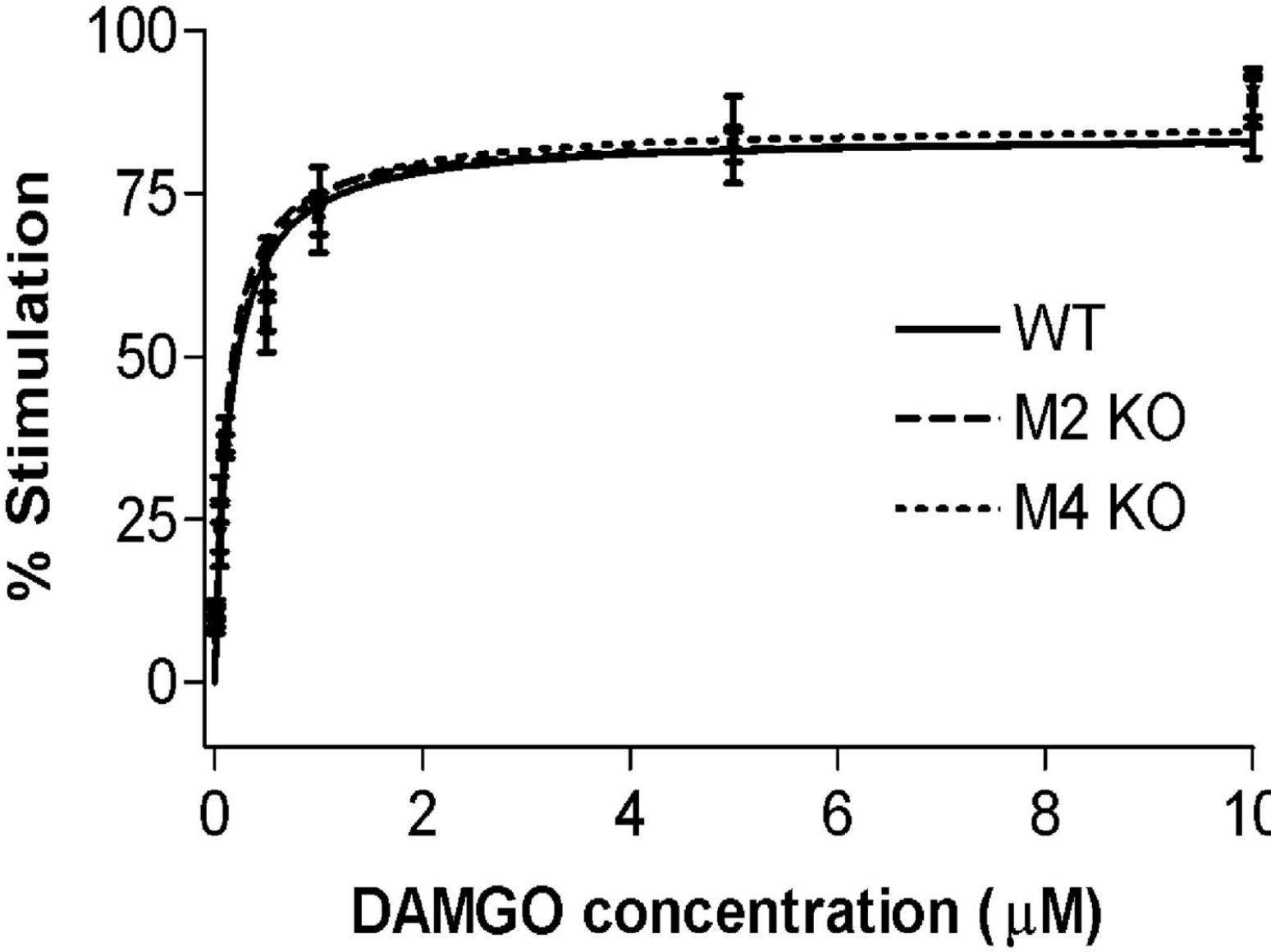

Fig. 5