

***Functional Activity of the M<sub>2</sub> and M<sub>4</sub> Receptor Subtypes in the Spinal Cord Studied with Muscarinic Acetylcholine Receptor Knockout Mice***

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Running title: Spinal muscarinic receptors and G protein coupling

List of abbreviations:

muscarinic acetylcholine receptors, mAChRs;

guanosine 5'-O-(3-[<sup>35</sup>S]thio)triphosphate, [<sup>35</sup>S]GTP $\gamma$ S

D-Ala<sup>2</sup>, N-Me-Phe<sup>4</sup>, Gly-ol<sup>5</sup>-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]GTP $\gamma$ S) binding in  $M_2$  and  $M_4$  subtype knockout (KO) mice. Basal [ $^{35}$ S]GTP $\gamma$ S 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]GTP $\gamma$ S 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]GTP $\gamma$ S binding was completely abolished in the spinal cord. Furthermore, the agonist-stimulated [ $^{35}$ S]GTP $\gamma$ S 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]GTP $\gamma$ S binding stimulated by a  $\mu$  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  $\alpha_2$ -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,

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]GTP $\gamma$ S) 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]GTP $\gamma$ S 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 (Gomez et al., 1999a; Gomez 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 (Gomez et al., 1999a; Gomez 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  $-80^{\circ}\text{C}$ . 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 [ $^{35}\text{S}$ ]GTP $\gamma$ S binding in spinal membranes**

The [ $^{35}\text{S}$ ]GTP $\gamma$ S binding stimulated by muscarinic agonists was conducted to quantify functional muscarinic receptors in the spinal cord, as described previously (Chen and Pan, 2003). The [ $^{35}\text{S}$ ]GTP $\gamma$ S binding assay is based on the principle that the inactive state of the G protein  $\alpha$  subunit has a relatively high affinity for GDP over GTP, whereas activation of a G protein-coupled receptor by its agonist shifts the  $\alpha$  subunit into a higher affinity for GTP versus GDP. The [ $^{35}\text{S}$ ]GTP $\gamma$ S is a hydrolysis-resistant form of GTP, and the degree to which an agonist stimulates [ $^{35}\text{S}$ ]GTP $\gamma$ S binding can be quantified in tissue membranes. Unlike conventional radioligand binding techniques, the agonist-stimulated [ $^{35}\text{S}$ ]GTP $\gamma$ S 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 [ $^{35}\text{S}$ ]GTP $\gamma$ S 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  $\text{MgCl}_2$  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<sub>2</sub>, 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 [<sup>35</sup>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 [<sup>35</sup>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%. [<sup>35</sup>S]GTPγS (1,250 Ci/mmol) was obtained from PerkinElmer Life Sciences (Boston, MA).

To determine whether the reduced [<sup>35</sup>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 [<sup>35</sup>S]GTPγS binding using a selective opioid agonist, (D-Ala<sup>2</sup>, N-Me-Phe<sup>4</sup>, Gly-ol<sup>5</sup>)-enkephalin (DAMGO), as the control. The DAMGO concentration used was 0.001-10 μM (Chen et al., 2002). [<sup>35</sup>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<sub>2</sub> receptors using immunoprecipitation and radioactive binding assay**

Because we observed that muscarinic agonist-stimulated [<sup>35</sup>S]GTPγS binding was significantly reduced in M<sub>4</sub>-KO mice (see Results), the following immunoprecipitation and radioligand binding experiments were performed to determine if the M<sub>2</sub> receptor levels are reduced in the spinal cord of M<sub>4</sub>-KO mice. An M<sub>2</sub> subtype-specific antiserum was raised against a nonconserved region of the third cytoplasmic loop of the mouse M<sub>2</sub> receptor protein (Gomez et al., 1999a; Gomez 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°C. The pellet was discarded, and the supernatant was centrifuged again at 48,000 g for 20 min at 4°C. Then, the pellet was resuspended in assay buffer (25 mM of phosphate buffer containing 5 mM of MgCl<sub>2</sub> 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 [<sup>3</sup>H]quinuclidinyl benzilate ([<sup>3</sup>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 [<sup>3</sup>H]QNB-labeled receptors with the M<sub>2</sub> subtype-selective antiserum, as described previously (Gomez et al., 1999a; Gomez 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<sub>50</sub> values, [<sup>35</sup>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<sub>50</sub> 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 [<sup>35</sup>S]GTPγS binding in the spinal cord

The basal [<sup>35</sup>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 [<sup>35</sup>S]GTPγS levels were also similar in spinal cord tissues in the muscarine and oxotremorine control groups. Furthermore, the basal [<sup>35</sup>S]GTPγS binding in the spinal cord from M<sub>2</sub> single-KO, M<sub>4</sub> single-KO, and M<sub>2</sub>/M<sub>4</sub> double-KO mice was not significantly different from the corresponding wild-type controls (Figure 1).

### Spinal [<sup>35</sup>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 [<sup>35</sup>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 [<sup>35</sup>S]GTPγS binding in wild-type mice (data not shown). Both 50-100 μM muscarine and 10-25 μM oxotremorine-M produced saturable [<sup>35</sup>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<sub>50</sub> values for muscarine in the M<sub>2</sub><sup>-</sup>, M<sub>4</sub><sup>-</sup>, and M<sub>2</sub>/M<sub>4</sub>-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 [<sup>35</sup>S]GTPγS binding, the EC<sub>50</sub> values in the M<sub>2</sub><sup>-</sup>, M<sub>4</sub><sup>-</sup>, and M<sub>2</sub>/M<sub>4</sub>-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 [<sup>35</sup>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]GTP $\gamma$ S 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]GTP $\gamma$ S 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]GTP $\gamma$ S binding in  $M_4$  single-KO mice was similar in muscarine- and oxotremorine-M-treated groups ( $14.7 \pm 0.5\%$  vs.  $15.1 \pm 0.4\%$ ). Similar to  $M_2/M_4$  double-KO mice, both muscarine and oxotremorine-M failed to cause any increase in [ $^{35}$ S]GTP $\gamma$ S 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 (Gomez et al., 1999a; Gomez 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).

### **DAMGO-stimulated [<sup>35</sup>S]GTP $\gamma$ S binding in the spinal cord of wild-type and mAChR KO mice**

To exclude the possibility that the reduction in mAChR agonist-induced [<sup>35</sup>S]GTP $\gamma$ S binding in the M<sub>4</sub> single-KO mice was due to reduced levels of spinal cord G<sub>i/o</sub> proteins, we carried out a series of control experiments using the  $\mu$  opioid DAMGO as an agonist. DAMGO produced a concentration-dependent increase in the [<sup>35</sup>S]GTP $\gamma$ S binding in the spinal cord of M<sub>2</sub>- and M<sub>4</sub>-KO and wild-type mice (n = 4 in each group, Figure 5). The spinal [<sup>35</sup>S]GTP $\gamma$ S binding stimulated by DAMGO was similar among the three different wild-type strains (data not shown). Furthermore, the DAMGO-stimulated [<sup>35</sup>S]GTP $\gamma$ S binding in the spinal cord of M<sub>2</sub>- or M<sub>4</sub>-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<sub>2</sub> and M<sub>4</sub> subtypes are involved in mediating analgesia produced by muscarinic agonists (Gomez et al., 1999a; Gomez et al., 1999b; Duttaroy et al., 2002). The M<sub>2</sub> and M<sub>4</sub> 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 [<sup>35</sup>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<sub>2</sub> and M<sub>4</sub> subtypes to activation of G<sub>i/o</sub> proteins in the spinal cord using M<sub>2</sub> and M<sub>4</sub> single- and M<sub>2</sub>/M<sub>4</sub> double-KO mice.

Unlike conventional receptor binding technique, [<sup>35</sup>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<sub>i/o</sub> family of G proteins can be readily detected with [<sup>35</sup>S]GTPγS binding techniques (Maher et al., 2001; Sim-Selley and Childers, 2002). Thus, the M<sub>2</sub> and M<sub>4</sub> subtypes likely are the mAChRs measured in the present study since they are both coupled to G<sub>i/o</sub> 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 [<sup>35</sup>S]GTPγS binding in the spinal cord was similar in wild-type and M<sub>2</sub> and M<sub>4</sub> KO mice, suggesting that basal levels of G<sub>i/o</sub> proteins and the affinity of G proteins for GDP and GTP are not altered in M<sub>2</sub> and M<sub>4</sub> KO mice. We observed that both muscarine and oxotremorine-M produced profound increases in the [<sup>35</sup>S]GTPγS binding in the spinal cord of wild-type mice, indicating the presence of functional M<sub>2</sub> and/or M<sub>4</sub> mAChR subtypes in the spinal cord. Interestingly, oxotremorine-M displayed significantly greater potency and efficacy than muscarine in stimulating [<sup>35</sup>S]GTPγS binding in the mouse spinal cord. The level of stimulation of [<sup>35</sup>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 [<sup>35</sup>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 [<sup>35</sup>S]GTPγS binding was completely eliminated in M<sub>2</sub>/M<sub>4</sub> 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 [<sup>35</sup>S]GTPγS binding in the spinal cord of M<sub>2</sub> single-KO mice. Radioligand binding studies suggest that the M<sub>2</sub> subtype represents ~90% of the total spinal cord mAChR population in mice (Duttaroy et al., 2002). The [<sup>35</sup>S]GTPγS binding data therefore provide further evidence that the M<sub>2</sub> subtype is the predominant mAChR

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]GTP $\gamma$ S 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]GTP $\gamma$ S response in the mouse spinal cord. The [ $^{35}$ S]GTP $\gamma$ S 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]GTP $\gamma$ S 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]GTP $\gamma$ S 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<sub>4</sub> mAChRs in the mouse spinal cord.

The complete loss of muscarinic agonist-stimulated [<sup>35</sup>S]GTPγS binding in M<sub>2</sub> single-KO mice is an unexpected but important finding. Because the mAChR agonist-stimulated [<sup>35</sup>S]GTPγS binding in the spinal cord was abolished in M<sub>2</sub>/M<sub>4</sub> double-KO mice and reduced by about 15% in M<sub>4</sub> single-KO mice, one would predict a certain degree of residual [<sup>35</sup>S]GTPγS binding in M<sub>2</sub> single-KO mice. Surprisingly, however, the muscarinic agonist-stimulated [<sup>35</sup>S]GTPγS binding was abolished in the spinal cord of M<sub>2</sub> single-KO mice. These data suggest that M<sub>4</sub> receptor activity requires the presence of M<sub>2</sub> mAChRs in the mouse spinal cord. Alternatively, M<sub>4</sub> mAChRs may be able to form functional oligomers with spinal M<sub>2</sub> 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, [<sup>35</sup>S]GTPγS binding assays showed that the mouse spinal cord expresses a small but significant number of functional M<sub>4</sub> mAChRs, in addition to the predominant population of M<sub>2</sub> mAChRs. Importantly, our data suggest that functional activity of spinal M<sub>4</sub> mAChRs may be dependent on the presence of M<sub>2</sub> mAChRs, possibly reflecting the existence of functional M<sub>2</sub>/M<sub>4</sub> mAChR oligomers. Data from this study are important for our understanding of the cellular function and drug action mediated by M<sub>2</sub> and M<sub>4</sub> mAChR subtypes.

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## Footnotes

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## Figure Legends

Figure 1. Basal [<sup>35</sup>S]GTPγS binding in the spinal cord of M<sub>2</sub> and M<sub>4</sub> single-KO and M<sub>2</sub>/M<sub>4</sub> double-KO mice and corresponding wild-type controls. Panels A and B are basal binding [<sup>35</sup>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 [<sup>35</sup>S]GTPγS binding stimulated by muscarine in the spinal cord membranes of M<sub>2</sub> and M<sub>4</sub> single-KO and M<sub>2</sub>/M<sub>4</sub> double-KO mice and the three respective wild-type (WT) strains. Panel B, comparison of maximal stimulated [<sup>35</sup>S]GTPγS binding by 100 μM muscarine in the spinal cord from M<sub>2</sub> and M<sub>4</sub> single-KO and M<sub>2</sub>/M<sub>4</sub> double-KO mice and corresponding wild-type controls. The binding data are expressed as the percent of basal [<sup>35</sup>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 [<sup>35</sup>S]GTPγS binding stimulated by oxotremorine-M in the spinal cord membranes of M<sub>2</sub> and M<sub>4</sub> single-KO and M<sub>2</sub>/M<sub>4</sub> double-KO mice and the three respective wild-type (WT) strains. Panel B, comparison of maximal stimulated [<sup>35</sup>S]GTPγS binding by 25 μM oxotremorine-M in the spinal cord from M<sub>2</sub> and M<sub>4</sub> single-KO and M<sub>2</sub>/M<sub>4</sub> double-KO mice and corresponding wild-type controls. The binding data are expressed as the percent of basal [<sup>35</sup>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<sub>2</sub> mAChRs in the spinal cord of wild-type (WT) and M<sub>2</sub>- and M<sub>4</sub>-KO mice. Spinal cord membranes were incubated with 2 nM of the non-selective muscarinic antagonist, [<sup>3</sup>H]QNB. [<sup>3</sup>H]QNB-labeled muscarinic receptors were solubilized and immunoprecipitated with an M<sub>2</sub> 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 [<sup>35</sup>S]GTPγS binding stimulated by DAMGO in spinal cord membranes of wild-type (WT) and M<sub>2</sub>- and M<sub>4</sub>-KO mice. Data are presented in means ± S.E.M. (n = 4). The data are expressed as the percent increase over basal [<sup>35</sup>S]GTPγS binding.



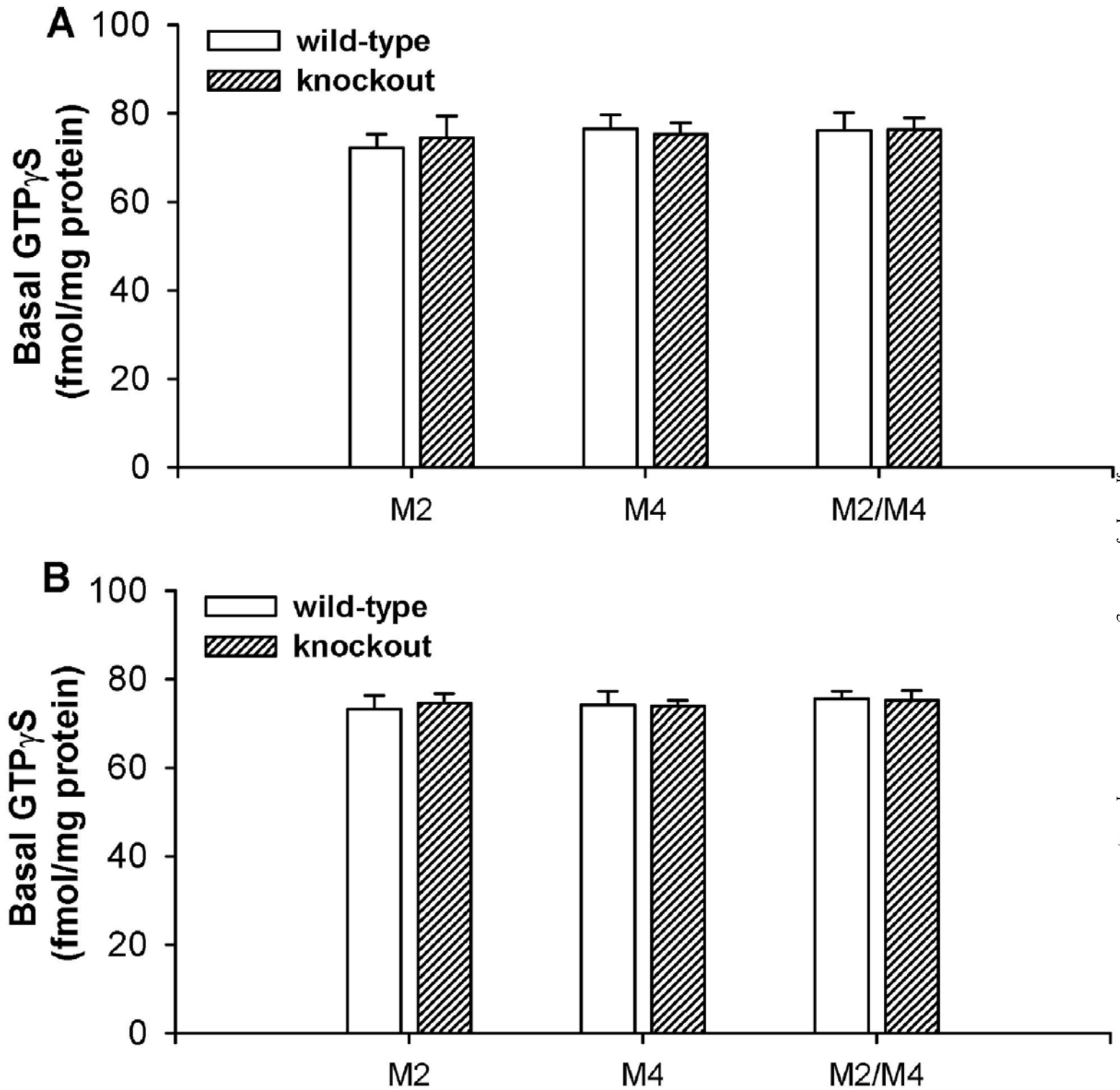


Fig. 1

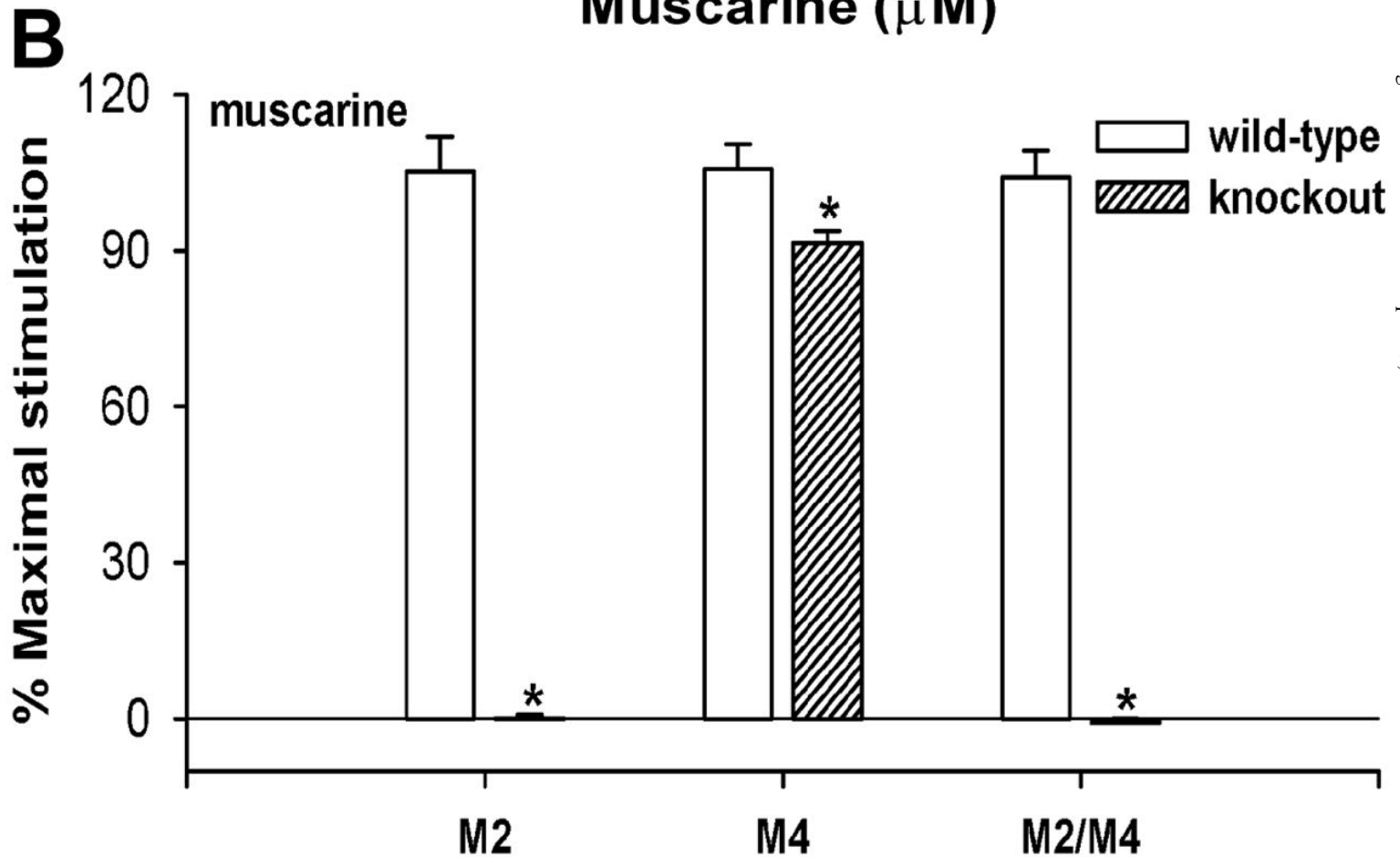
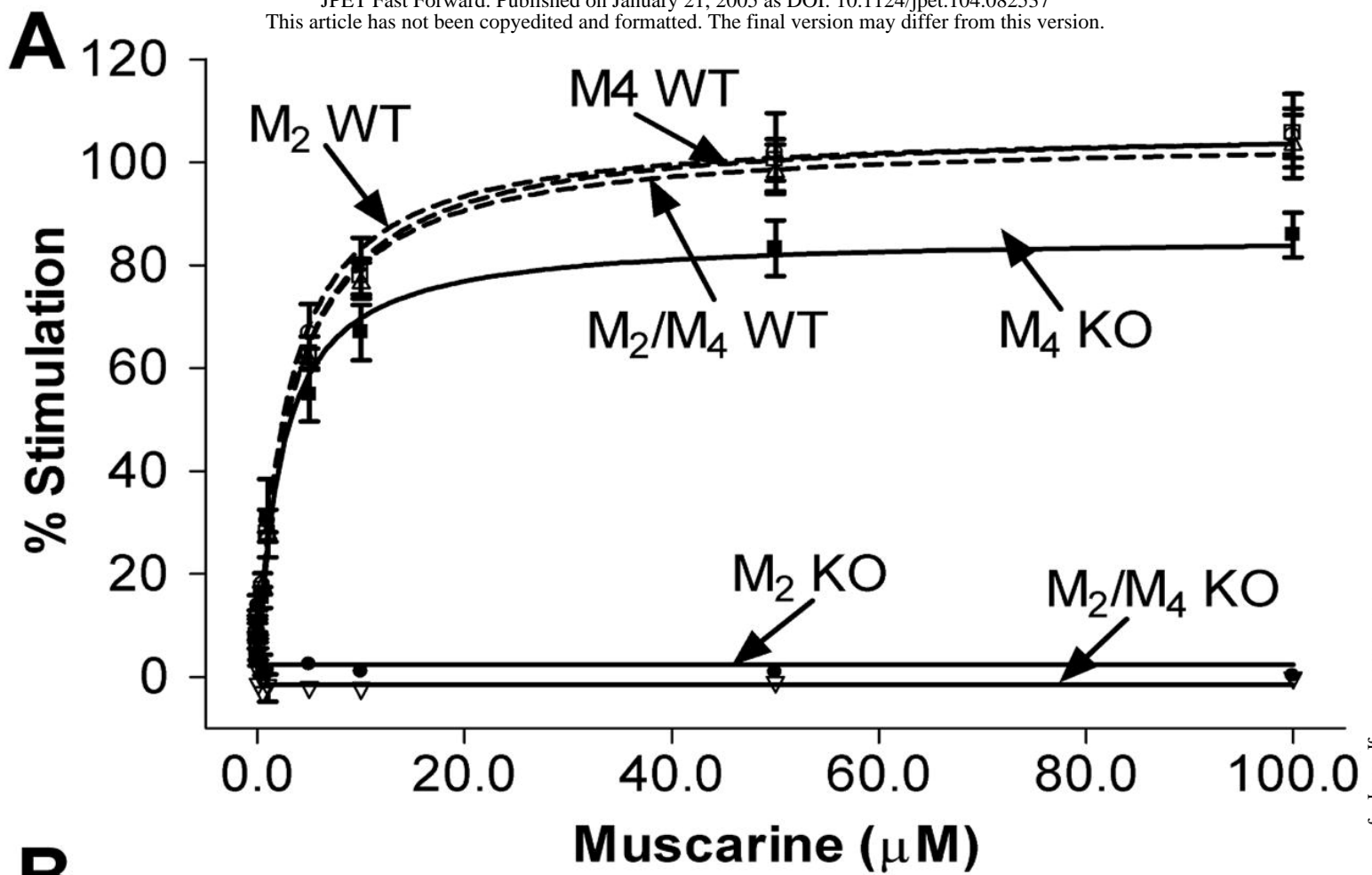


Fig. 2

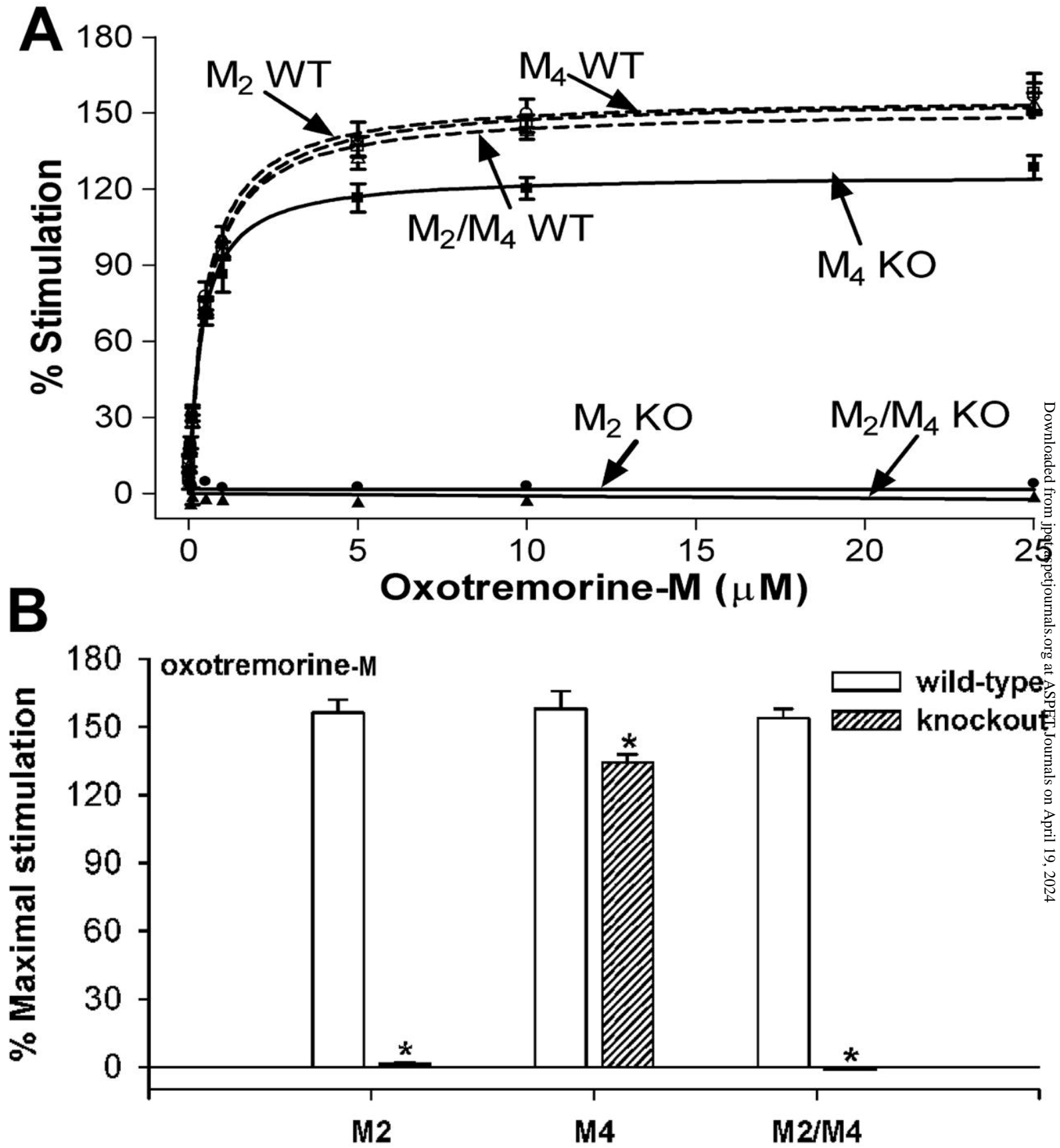
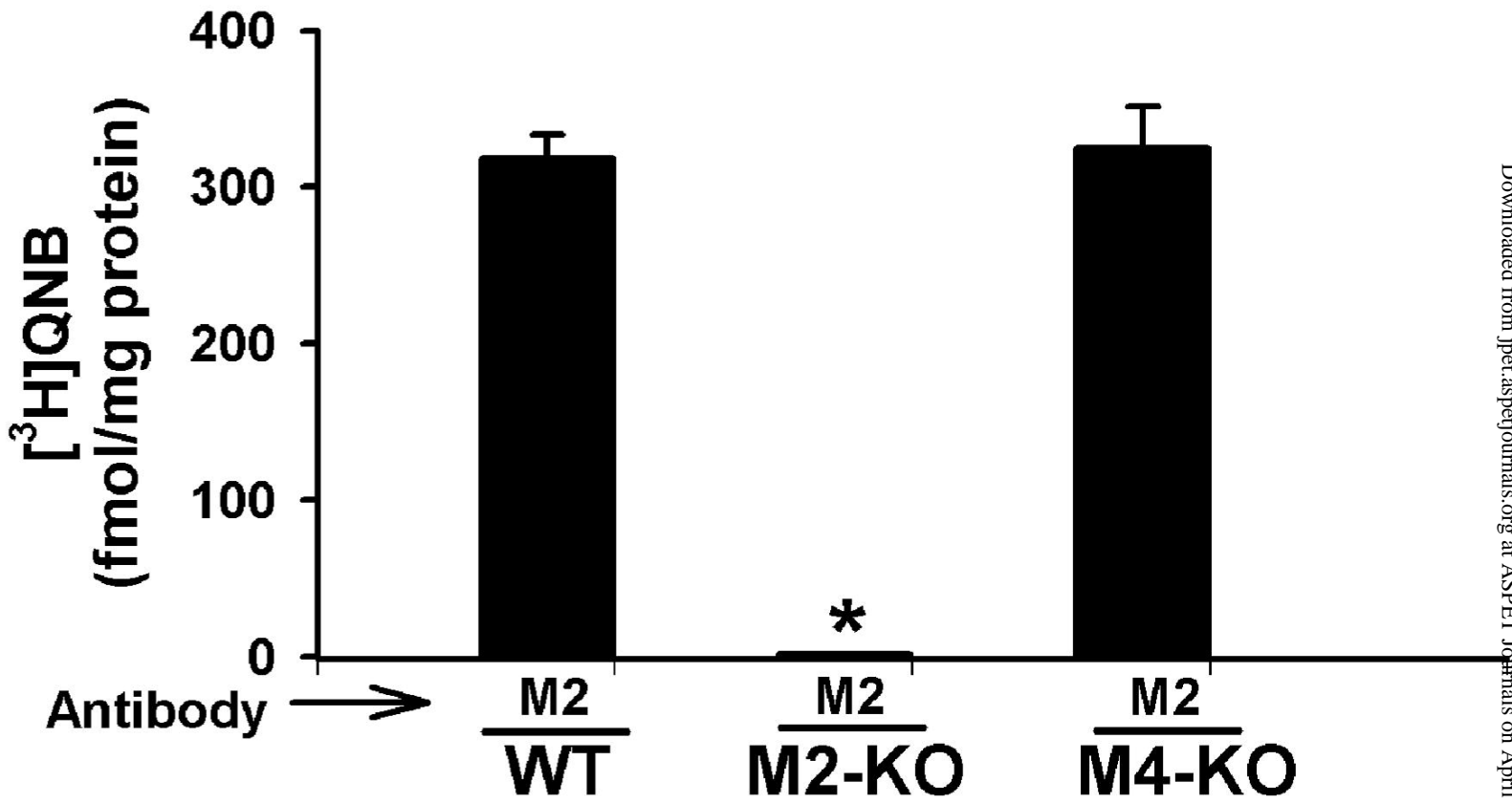
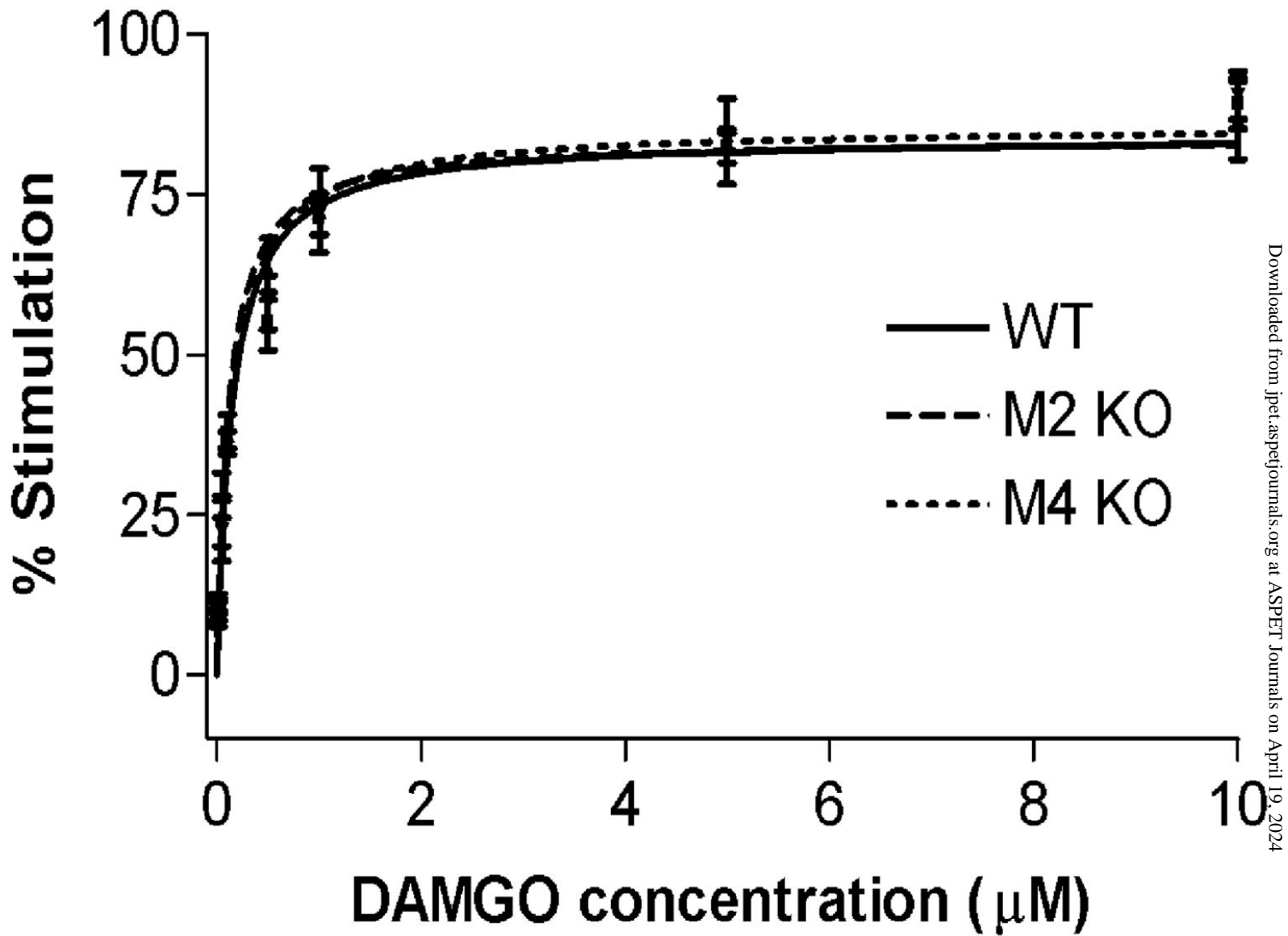


Fig. 3



**Fig. 4**



**Fig. 5**