Steroids as the novel class of high-affinity allosteric modulators of muscarinic receptors

Eva Dolejší
Institute of Physiology https://orcid.org/0000-0002-5845-182X
Eszter Szánti-Pintér
Institute of Organic Chemistry and Biochemistry
Nikolai Chetverikov
Institute of Physiology https://orcid.org/0000-0003-4462-0319
Dominik Nelic
Institute of Physiology
Alena Randáková
Institute of Physiology
Vladimír Doležal
Institute of Physiology https://orcid.org/0000-0003-3412-256X
Eva Kudová
Institute of Organic Chemistry and Biochemistry
Jan Jakubik (jan.jakubik@fgu.cas.cz)
Institute of Physiology https://orcid.org/0000-0002-1737-1487

Article

Keywords: muscarinic receptors, cholesterol-binding site, membrane cholesterol, allosteric modulators

Posted Date: February 4th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-140412/v1

Version of Record: A version of this preprint was published at Neuropharmacology on November 1st, 2021. See the published version at https://doi.org/10.1016/j.neuropharm.2021.108798.

Steroids as the novel class of high-affinity allosteric modulators of muscarinic receptors

Eva Dolejší[1], Eszter Szánti-Pintér[2], Nikolai Chetverikov[1], Dominik Nelic[1], Alena Randáková[1], Vladimír Doležal[1], Eva Kudová[2*], Jan Jakubík[1*]

[1] Institute of Physiology Czech Academy of Sciences, Prague, 142 20, Czech Republic.

[2], Institute of Organic Chemistry and Biochemistry Czech Academy of Sciences, Prague, 166 10, Czech Republic.

Running title

Allosteric modulation of muscarinic receptors by steroids

Contacts

Eva Dolejší, eva.dolejsi@fgu.cas.cz

Eszter Szánti-Pintér, eszter.szanti-pinter@uochb.cas.cz

Nikolai Chetverikov, nikolai.chetverikov@fgu.cas.cz

Dominik Nelic, Dominik.nelic@fgu.cas.cz

Alena Randáková, alena.randakova@fgu.cas.cz

Vladimír Doležal, vladimir.dolezal@fgu.cas.cz

Eva Kudová, eva.kudova@uochb.cas.cz

Jan Jakubík, jan.jakubik@fgu.cas.cz

[*] To whom correspondence should be addressed: Jan Jakubik (jan.jakubik@fgu.cas.cz) and Eva Kudová (eva.kudova@uochb.cas.cz)

Abstract

The membrane cholesterol was found to bind and modulate the function of several G-protein coupled receptors including muscarinic acetylcholine receptors. We investigated the binding of 20 steroidal compounds including neurosteroids and steroid hormones to muscarinic receptors. Corticosterone, progesterone and some neurosteroids bound to muscarinic receptors with the affinity of 100 nM or greater. We established a structure-activity relationship for steroid-based allosteric modulators of muscarinic receptors. Further, we show that corticosterone and progesterone allosterically modulate the functional response of muscarinic receptors to acetylcholine at physiologically relevant concentrations. It can play a role in stress control or in pregnancy, conditions where levels of these hormones dramatically oscillate. Allosteric modulation of muscarinic receptors via the cholesterol-binding site represents a new pharmacological approach at diseases associated with altered cholinergic signalling.

Introduction

Muscarinic receptors mediate various physiological functions of acetylcholine in the peripheral and central nervous system [1]. Alterations in cholinergic signalling are a cause of a wide range of neurological (e.g. epilepsy, Alzheimer's disease, Parkinson's disease) and psychiatric conditions (e.g. schizophrenia) as well as internal diseases (e.g. asthma, chronic obstructive pulmonary disease, over-reactive bladder) [2,3]. All five subtypes of muscarinic receptors share high structural homology in the orthosteric binding site that makes their pharmacological targeting via orthosteric binding site extremely difficult [4–7]. Therefore, in past decades a lot of effort was dedicated to the research of allosteric modulators that bind to the less conserved sites on muscarinic receptors.

Cholesterol was found co-crystallized with many G-protein coupled receptors (GPCRs). The structural motifs at GPCRs specific for cholesterol binding were identified suggesting the existence of the cholesterol-specific binding sites [8]. Cholesterol changed the affinity of muscarinic ligands and slowed down the activation of muscarinic receptors [9]. The common allosteric binding site of muscarinic receptors has been located between the second and third extracellular loop [10]. In contrast, the cholesterol-binding site is oriented towards membrane and may represent a novel target for allosteric modulation of muscarinic receptors with therapeutic potential [11].

Steroids are physiologically and pharmacologically important compounds. Similarly to their precursor cholesterol, they have a structure of specifically arranged four rings (Fig. 1). Cholesterol is an essential part of the membrane. Steroids *per se* are biosynthesized from cholesterol and exert many different functions, including hormonal signalling of sex hormones or various effects of corticosteroids. Steroid hormones can be divided into several classes – corticosteroids, sex steroids, and bile acids. Corticosteroids play an important role in response to stress, regulation of inflammation, while sex steroids are essential in the function of the reproductive system. Bile acids are secreted from the liver to duodenum, absorbed back from the alimentary tract and enabling various actions mainly through nuclear receptor farnesoid X receptor. A special class of steroids represents neurosteroids. The term of neurosteroids (NS) was introduced [12] for compounds of which the accumulation occurs in the nervous system independently, of the steroidogenic endocrine glands and which can be synthesized *de novo* in the nervous system from cholesterol or steroidal precursors imported from peripheral sources [12,13]. Synthetic analogues of NS are called neuroactive steroids (NAS). The physiological and pathological implications of steroid hormones and NS are related to their genomic vs non-genomic mechanism of action. While gonadal and adrenal steroids

exert mostly long-term genomic effects through nuclear steroid receptors, NS can elicit fast, non-genomic effects *via* stimulation various ligand-gated ion channels, voltage-gated ion channels, or G-protein coupled receptors [13]. Therefore, NS exert different roles in neuronal functions, including cognition, memory processes, and neuroprotection [14]. It is important to note that some sex steroids – progesterone, testosterone, estradiol, and estrone also influence neuronal functions by binding to membrane receptors for neurotransmitters that subsequently afford a variety of effects that are unrelated to reproduction [15].

It was hypothesized that the neuroprotective effect of steroid hormones or NS may include also the modulation of the cholinergic nervous system. However, the mechanisms of action on signalling of muscarinic acetylcholine receptors have not been clarified yet. Steroids have been shown to improve memory by affecting acetylcholine release and cholinergic neurotransmission [16–18]. Moreover, steroid hormones may inhibit binding of muscarinic ligands [19–23]. However, all reports so far describe the effects of steroids on muscarinic receptors only at micromolar or higher concentrations. In this study, we investigated in detail the allosteric modulation of muscarinic receptors by 20 steroidal hormones and neurosteroids at nanomolar concentrations. Further, we measured the functional response of muscarinic receptors to natural neurotransmitter acetylcholine in the presence of steroid hormones corticosterone, progesterone, estradiol and testosterone in nanomolar, i.e. physiologically relevant, concentrations.

Results

Twenty steroidal compounds including hormones (corticosterone, progesterone, estradiol and testosterone), pro-neurosteroids (dihydrotestosterone, deoxycorticosterone and pregnenolone), neurosteroids and their metabolites (Fig. 1) were and pharmacologically analysed in binding (Table 1, 2, S1 and S2, Fig. 2) and functional experiments (Fig. 3 through 6, Table 3). Generic names of tested compounds are used throughout the study to simplify their chemical names.

Binding of tested compounds to muscarinic receptors was initially inferred from their ability to change the equilibrium binding of tritiated antagonist N-methylscopolamine ([^{3}H]NMS) to membranes prepared from CHO cells expressing individual subtypes of muscarinic receptors. At 10 μM concentration all tested compounds changed [^{3}H]NMS equilibrium binding with distinct pattern (Table S1). The tested compounds either increased (e.g. progesterone at all subtypes, corticosterone at all subtypes except M_5) or partially decreased (e.g. allopregnanolone at all subtypes) equilibrium [^{3}H]NMS binding indicating allosteric interaction between tested compounds and [^{3}H]NMS. Equilibrium dissociation constant K_A of a tested compound and factor of binding cooperativity α between a tested compound and [^{3}H]NMS was determined by fitting Eq. 2 to binding data (Table 1 and S2). Highest affinity, about 50 nM, was observed for progesterone at all receptors except M_4, for pregnenolone at M_1 and M_4 pregnanolone at M_5 and androstenedione at M_3 receptors (Fig. 2, Table 1). Relatively high binding affinity (about 100 nM) was also found for corticosterone at M_1 and M_3 receptors, 5β-dihydroprogesterone at M_2 and M_4 receptors and androstenedione and testosterone at M_2 receptors. However, for the majority of combinations of receptors and tested compounds affinities could not be determined. The observed changes in [^{3}H]NMS affinity induced by tested compounds were small, in the order of tens of per cent (Table S2). The only exception was a two-fold increase in affinity (two-fold decrease in K_A) of [^{3}H]NMS at M_1 receptor by corticosterone and about a two-fold decrease in affinity at M_4 receptor epipregnanolone and pregnanolone, respectively.

To detect possible high-affinity binding of tested compounds to muscarinic receptors with neutral cooperativity with [^{3}H]NMS, putative effects on receptor binding kinetics of tested compounds at 100 nM concentration were measured. Any change in [^{3}H]NMS dissociation rate is indicative of compound binding at the given concentration. Indeed changes in binding kinetics of [^{3}H]NMS were found in several cases where no effects on [^{3}H]NMS equilibrium binding was observed (e.g. acceleration of [^{3}H]NMS dissociation from M_3 receptors by pregnanolone or deceleration of [^{3}H]NMS dissociation from M_3 receptor by DHEA and androstenedione) (Table 2). The 5α-dihydroprogesterone changed [^{3}H]NMS dissociation from all receptors except M_1. Notably, estradiol slowed [^{3}H]NMS dissociation from the M_5 receptor and accelerated it at rest of the subtypes. The dihydrotestosterone accelerated [^{3}H]NMS dissociation from all receptor subtypes except M_3. However, in many cases tested compounds at 100 nM concentration did not affect [^{3}H]NMS dissociation.

Physiological concentrations of steroid hormones are in the nanomolar range. Thus, effects of steroid hormones corticosterone, progesterone, estradiol and testosterone on the functional response of muscarinic receptors to acetylcholine were tested at 100 nM and 1 μM concentration (Figs. 2 to 5, Table 3). To correctly rank acetylcholine efficacies regardless receptor reserve of the system, concentration-response curves were analysed according to the operational model of agonism (OMA) described previously [24]. Factors of binding and functional cooperativity α and β, respectively, were determined according to the operational model of allosterically modulated agonism (OMAMA) [25]. The maximal response (E_{MAX}) of individual systems and parameters of control response to acetylcholine (pEC_{50}, E'_{MAX}, τ) in the absence of allosteric modulators are summarized in Table S3. Maximal response to acetylcholine E'_{MAX} was about one half of maximal system response E_{MAX}. The factor of operational efficacy τ of acetylcholine ranged from 0.535 at M_4 to 0.756 at M_2 receptors.

Both 100 nM and 1μM corticosterone 3-times increased potency of acetylcholine and decreased E'_{MAX} by 15 % at M_2 receptor (Fig. 3). Effects of corticosterone at M_4 receptors were opposite; it 3-times decreased acetylcholine potency and increased E'_{MAX} by 20 %. No difference in the effects of 100 nM and 1 μM corticosterone indicates saturation of corticosterone binding at these receptors already at 100 nM (Table 3A). Corticosterone also slightly increased acetylcholine E_{MAX} at M_3 receptors. No effects of corticosterone at M_1 and M_5 receptors were observed. Both 100 nM and 1 μM progesterone increased acetylcholine E'_{MAX} by 30 % at M_1 receptors indicating the saturation of progesterone binding at 100 nM (Fig. 4, Table 3B). One μM progesterone increased acetylcholine E'_{MAX} at M_3 receptors (20 %) and decreased it at M_2 (30 %) and M_4 (15 %) receptors. Except for M_4 and M_5 receptors, 1 μM estradiol increased acetylcholine E'_{MAX} (Fig. 5, Table 3C). At M_1 receptors, the effect of estradiol on acetylcholine E'_{MAX} was observed already at 100 nM concentration. Both 100 nM and 1 μM estradiol decreased acetylcholine potency two-fold. Testosterone did not affect the functional response of muscarinic receptors to acetylcholine (Fig. 6 and Table 3D).

Further, functional data were analysed in detail using the operational model of allosterically modulated agonism [25]. Factors of operational cooperativity β and binding cooperativity α were calculated (Table 3). Values greater than one denote positive cooperativity and values smaller than one denote negative cooperativity. Effects of corticosterone at M_2 and M_4 receptor were profound. Corticosterone increased 3.5-times affinity of acetylcholine at M_2 receptor and decreased it 5-times at the M_4 receptor (Table 3A). Progesterone increased efficacy of acetylcholine 2-times at M_1

receptor and by 70 % at M_3 receptor (Table 3B). Progesterone decreased affinity of acetylcholine at the M_2 and M_4 receptor about 2-times. Progesterone also about 2-times decreased affinity of acetylcholine to M_1 and M_3 receptors. Estradiol increased efficacy of acetylcholine at M_1, M_2 and M_3 receptors (Table 3C). The effect was strongest at the M_1 receptor, more than 2-fold increase. Estradiol increased affinity of acetylcholine by 40 % at $_{M5}$ receptor and decreased it at the rest of subtypes. The effect was strongest at the M_4 receptor, more than 2-fold decrease. Testosterone did not affect parameters of functional response to acetylcholine at any receptor subtype (Table 3D). In some cases, the apparent absence of effects of tested hormones on functional response to acetylcholine was due to their opposite effects on operational efficacy β and affinity α. For example, estradiol increased operational efficacy but decreased affinity of acetylcholine at M_2 and M_3 receptors. These data are further evidence for the interaction of tested steroid hormones at submicromolar concentrations with muscarinic receptors.

Based on the results of pharmacological experiments basic structure-activity relationship was established. Progesterone and corticosterone possess common structural features, such as a pregnane skeleton and an enone group (3-oxo-4-ene functionality) in A-ring (Fig. 1). The hydroxylation of progesterone at C-17 (17α-hydroxyprogesterone) or C-21 (11-deoxycorticosterone) leads to steroids with lower affinities to muscarinic receptor subtypes (Table 1). In contrast, corticosterone, bearing hydroxyl moieties at C-11 and C-21 has improved binding affinity. Furthermore, the orientation of C-5 hydrogen has a significant effect on binding, as the 5β-steroids have higher affinities to all subtypes in general than their 5α-analogues. Note, that 5α-reduced steroids are planar molecules, while the 5β-reduced skeleton has a "bent" ring structure. The difference is noticeable in the case of 5β-dihydroprogesterone and 5α-dihydroprogesterone (Table 1), likewise among pregnanolone and allopregnanolone. The final shape of the molecule is additionally affected by the location of the 3-hydroxy reduced substituent. Both epipregnanolone and pregnanolone have similar affinities to the M_4 receptor and induced a two-fold decrease in [^{3}H]NMS affinity at M_4. Taken together, it seems that rather 5α/5β stereochemistry than the orientation of C-3 hydroxy-substituent defines the in vitro effect.

The nature of C-17 substituent on the steroid skeleton can modulate not only the affinities but also selectivity to individual muscarinic receptor subtypes. For example, progesterone possessing C-17 acetyl moiety has a high affinity to almost all subtypes, while androstenedione bearing C-17 keto group shows higher selectivity to M_2 and M_3 receptors. The reduction of the 17-keto group to 17-hydroxyl affords testosterone with remarkable affinity only on the M_2 subtype. Finally, the aromatization of A-ring at testosterone that gives estradiol structure abolishes the binding to the M_2 receptor.

Discussion

The major finding of this study is the demonstration of specific binding of neurosteroids and steroid hormones to muscarinic acetylcholine receptors at physiologically relevant concentrations. Further, we have shown that steroid hormones at physiological concentrations allosterically modulate the functional response of muscarinic receptors to acetylcholine.

Several lines of evidence led to the postulation of the cholesterol-binding site at G-protein coupled receptors (GPCRs). Cholesterol was found co-crystallized with many GPCRs, e.g β2-adrenergic or adenosine A_{2A} receptor [26,27]. The structural motifs at GPCRs specific for cholesterol binding were identified suggesting the existence of the cholesterol-specific binding sites [8]. Although cholesterol

has not been found co-crystallized in any crystallographic structure of the muscarinic receptor, cholesterol changes the affinity of muscarinic ligands and slows down the activation of muscarinic receptors [9]. The common allosteric binding site of muscarinic receptors is located between the second and third extracellular loop and can accommodate structurally various ligands [10]. The cholesterol-binding site(s), on the other hand, is oriented towards membrane and may bind membrane soluble compounds that share structural similarity with cholesterol, like steroid hormones or neurosteroids (NS). This site may represent a novel target for allosteric modulation of muscarinic receptors [11].

Non-genomic effects of neurosteroids are fast responses to them that do not depend on gene transcription or protein synthesis. Several GPCRs were implicated in non-genomic signalling of NS [28]. Only a small number of studies reported the action of NS at muscarinic receptors. Pregnenolone and progesterone inhibited the [^{3}H](-)QNB binding to M_1 and M_3 receptors expressed in Xenopus oocytes while dehydroepiandrosterone (DHEA) was found to be inactive [21]. Presumably, pregnenolone and progesterone exert their effect as negative allosteric modulators. Next, the analgesic neurosteroid alfaxalone (3α-hydroxy-5β-pregnane-11,20-dione) inhibited acetylcholine-induced currents in oocytes expressing M_1 and M_3 receptors with IC_{50} values of 1.8 μM and 5.3 μM, respectively [29]. Finally, the M_2 receptor, the most abundant muscarinic receptor subtype in the heart [30] was studied as a potential target of steroid hormones. Progesterone inhibited the binding of [^{3}H]N-methylscopolamine (NMS) to M_2 receptor in cardiac tissues slices IC_{50} value of 50 μM while estradiol and testosterone were inactive in this assay [22]. Metabolites of progesterone such as 17α-hydroxyprogesterone and 17α,21-dihydroxyprogesterone were found to be even more potent (5 μM and 1 μM, respectively). Interestingly, the authors demonstrated [23] that the inhibition of NMS binding to cardiac M_2 receptors is not mediated via the [^{3}H]NMS binding site and that the modulatory effect is possibly non-genomic.

All reports so far described NS effects with micromolar potency. Our results demonstrate that several steroids (e.g. corticosterone, pregnenolone, progesterone, 5β-dihydroprogesterone, androstenedione, and testosterone) allosterically modulate muscarinic receptors with nanomolar affinity (Table 1). Steroids acting at nanomolar concentrations at muscarinic receptors represent the novel pharmacophore for development of new highly potent and efficacious steroids. Further, binding of steroid hormones corticosterone and progesterone to the muscarinic receptors with about 100 nM affinity (Table 1 and 2) indicates that steroid hormones may bind to muscarinic receptors under physiological conditions. Thus, the effects of four hormones (corticosterone, progesterone, estradiol and testosterone) on functional response to acetylcholine were measured (Fig. 3 through 6).

The basal plasma concentration of corticosterone in human is about 100 nM and rises to 500 nM during stress. Interestingly, corticosterone increases the potency of acetylcholine at M_2 and decreases the potency of acetylcholine at M_4 (Fig. 3, Table 3A). At the periphery, M_2 receptors are mainly post-synaptic, mediating the response to acetylcholine released from parasympathetic neurons. In contrast, M_4 receptors are mainly parasympathetic pre-synaptic receptors inhibiting the release of acetylcholine [1]. Therefore, the action of corticosterone at these two receptors synergistically facilitates parasympathetic action and may counter-balance increased activity of sympathetic neurons under stress.

The plasma concentration of progesterone oscillates between 0.3 and 50 nM during the menstruation cycle and rises to 770 nM at the end of pregnancy. The effects of progesterone were

the greatest at M_1 receptors. Effects of 100 nM and 1 μM progesterone were the same indicating saturation within the range of physiological concentrations (Fig. 4, Table 3B). Progesterone at 100 nM concentration increased efficacy of acetylcholine at the M_1 receptor and decreased efficacy of acetylcholine at M_2 receptors. Similarly to progesterone, the plasma concentration of estradiol oscillates between 0.07 and 2.3 nM during the menstruation cycle and rises to 22 nM at the end of pregnancy. However, the estradiol binding to muscarinic receptors was observed at concentrations much higher (about 1 μM) than physiological ones (Table 1 and S1). In functional experiments, estradiol affected only the M_1 receptor (Fig. 5, Table 3C). At 100 nM concentration, it increased E_{MAX} only by 24 %. Thus, at the physiological concentrations, the effects of estradiol on signalling via M_1 receptors are negligible. Although physiological determinants of epilepsy are not fully understood, the role of cholinergic signalling in neuronal firing activity has been implicated [31]. E.g. overstimulation of cholinergic signalling is the basis of pilocarpine model of status epilepticus [32]. The incidence of status epilepticus correlates with menstrual oscillations in the level of progesterone and estradiol [33,34]. The hormonal influence on epilepsy is supported by findings that progesterone increases a seizure threshold while estradiol decreases it. Therefore, it is likely that allosteric modulation of cholinergic transmission by progesterone may contribute to its anti-epileptic effects. In contrast, muscarinic receptors do not mediate the pro-epileptic effect estradiol. Initiation of seizures in the lithium-pilocarpine model is mediated by hippocampal M_1 receptors [35]. Thus, observed positive modulation of M_1 receptors may seem pro-convulsive. However, seizures are evoked by prolonged overstimulation of cholinergic system that leads to a several-fold increase in acetylcholine levels [36]. In contrast, acute short-term selective stimulation of M_1 or M_4 receptors normalizes hippocampal activity [37]. Thus, moderate positive modulation of hippocampal M_1 receptors restricted in time and space to ongoing cholinergic signalling may contribute to the anti-convulsive effect of progesterone.

An elevated level of progesterone during pregnancy may lead via muscarinic receptors to increase in heart rate, decrease in gastrointestinal mobility and nausea. In the heart, M_2 receptors mediate acetylcholine-induced bradycardia [1]. Negative modulation by progesterone may decrease the bradycardic effects of acetylcholine in the heart. Gastrointestinal motility is driven by stimulation of M_2 and M_3 receptors in the smooth muscle [38]. Negative modulation of these receptors by progesterone may lead to a decrease in gastrointestinal motility. Stimulation of M_1 receptors in the vestibular nuclei leads to nausea [39]. Positive modulation of these receptors by progesterone may contribute to pregnancy nausea.

No effects of sex hormones were observed at physiological concentrations. Estradiol even at 100 nM concentration, that is 50 times higher than normal, increased efficacy of acetylcholine only at M_1 receptor (Fig. 5, Table 3C). The plasma level of testosterone ranges from 8 to 30 nM in males. The affinity of testosterone for the M_2 receptor was 70 nM (Table 1). However, even at one μM concentration, testosterone did not affect the functional response of muscarinic receptors to acetylcholine (Fig. 6, Table 3D). The possible explanation may be that while testosterone binds to muscarinic receptors at these concentrations (Table 1 and 2), it has opposing effects on efficacy and affinity of acetylcholine that nullify its effect on functional response (Table 3D).

Structure-activity relationship of 20 related steroidal derivates pointed to certain structural features influencing binding affinity to muscarinic receptors and its selectivity to individual muscarinic subtypes. Anti-inflammatory corticosteroids and their synthetic analogues certainly deserve further experiments and structure-activity relationship studies as corticosterone was identified as one of the

most potent compounds. Second, 11-deoxycorticosterone and 5α-dihydrodeoxycorticosterone, on the other hand, display intriguing selectivity towards M_3 and M_4 receptors, respectively. Pregnenolone, progesterone and their 5α and 5β-reduced derivatives (5α/5β-dihydroprogesterone) offer a great variety of possible synthetic modifications (e.g. modifications at C-7) that presumably should afford biologically active compounds as these steroids, out of all tested compounds, display the most profound effect on the affinity of all M_1-M_5 receptors. Contrarily, it seems that the 3-hydroxy reduced derivatives of 5α- and 5β-dihydroprogesterone lack desired effect on muscarinic receptors, excluding pregnanolone. Finally, compounds bearing androstane skeleton, in general, demonstrate poor ability to modulate muscarinic receptors. However, testosterone is an obvious exception as it exhibits strong selectivity towards M_2 receptor with affinity in nanomolar concentrations. As such, synthetic modifications of testosterone that would maintain selectivity and affinity towards muscarinic receptors while having reduced androgenic activity or even antiandrogenic effect might be of interest for further development. These structural findings may allow the design of novel steroid allosteric modulators with high-affinity for muscarinic receptors and possible therapeutic use.

In conclusion, at physiological concentrations, corticosterone and progesterone allosterically modulate the functional response of muscarinic receptors to acetylcholine. Although their effects are subtle, the effects may be cumulative during long-lasting physiological processes (e.g. pregnancy or distress) or chronic pathological processes (e.g. epilepsy) in which muscarinic receptors are involved. Steroids acting at nanomolar concentrations at muscarinic receptors represent the novel pharmacophore for the synthesis of high-affinity and efficacious steroids. Targeting the cholesterol-binding site or interfering with the action of steroid hormones at muscarinic receptors represents a new approach in the pharmacotherapy of pathologies associated with altered muscarinic signalling.

Materials and methods

Materials and Synthesis

Steroid compounds were dissolved in water-free DMSO to 20 mM concentration. All radiolabeled compounds (N-[^{3}H]methyl scopolamine, myo-[2-^{3}H(N)]inositol) were purchased from American Radiolabeled Chemicals, Inc. (Saint Louis, MO). Common chemicals were purchased from (Sigma, Prague, CZ) in the highest available purity.

Pregnenolone (3β-hydroxypregn-5-en-20-one), progesterone (pregn-4-ene-3,20-dione), estradiol (estra-1,3,5(10)-triene-3,17β-diol), dehydroepiandrosterone (DHEA, 3β-hydroxyandrost-5-en-17-one), and testosterone (17β-hydroxyandrost-4-en-3-one) were purchased from Steraloids (Newport, RI, USA). 17α-Hydroxyprogesterone (17α-hydroxypregn-4-ene-3,20-dione) and androstenedione (4-androstene-3,17-dione) were purchased from Sigma Aldrich (Prague, Czech Republic). 11-Deoxycorticosterone (21-hydroxypregn-4-ene-3,20-dione), corticosterone (11β,21-dihydroxypregn-4-ene-3,20-dione), 5α-dihydrodeoxycorticosterone (21-hydroxy-5α-pregnane-3,20-dione), and 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one) were purchased from Toronto Research Chemicals Inc. (North York, Canada). 5α-Dihydroprogesterone (5α-pregnane-3,20-dione) was prepared by hydrogenation of pregnenolone, followed by oxidation of 3β-hydroxy group [40]. 5β-Dihydroprogesterone (5β-pregnane-3,20-dione) was prepared by hydrogenation of progesterone under basic conditions [40]. Pregnanolone (3α-hydroxy-5β-pregnan-20-one) by selective reduction of 5β-dihydroprogesterone with sodium borohydride at low temperature under slightly basic conditions [41]. Epipregnanolone (3β-hydroxy-5β-pregnan-20-one) was prepared from pregnanolone

by a two-step inversion of 3-hydroxy configuration using Mitsunobu reaction via *p*-nitrobenzoate [42]. Isopregnanolone (3β-hydroxy-5α-pregnan-20-one) was prepared by hydrogenation of pregnenolone under neutral conditions [43]. Allopregnanolone (3α-hydroxy-5α-pregnan-20-one) was prepared from isopregnanolone by a two-step inversion of 3-hydroxy configuration using Mitsunobu reaction via 3α-formate followed by hydrolysis [44]. Androstenediol (androst-5-ene-3β,17β-diol) was prepared by the reduction of dehydroepiandrosterone by sodium borohydride [45]. 5α-Androstenedione (5α-androstane-3,17-dione) was prepared by hydrogenation of dehydroepiandrosterone, followed by oxidation of 3β-hydroxy group [46]. 5β-Androstenedione (5β-androstane-3,17-dione) was prepared by hydrogenation of testosterone under basic conditions, followed by oxidation of 17β-hydroxy group [47].

The commercially available steroids had purity >98.0% and were used without further purification. Steroids that were prepared by synthetic protocols described above were characterized by general organic methods (e.g. 1H, 13C NMR, HR-MS) and their purity (>98.0%) was analysed using LC-MS system. Samples were measured using LC-MS equipped with UV-VIS detector. Equipment: TSP quaternary pump P4000, TSP autosampler AS3000, UV/VIS detector UV6000LP and ion-trap mass spectrometer Advantage. Solvent A was 98%water/ 2%acetonitrile and B was 95%acetonitrile/ 3% isopropanol/ 2%water with 5mM ammonium formate both. Gradient setup: 0-25-30-30.1-45min 50-99-99-50-50% solvent B and flow rate 150 ul/min. For separation has been used C18 column: 250 mm length, 2 mm width, Nucleosil 120-5 C15. UV6000LP is diode array detector witch 512 diodes. UV/VIS setup: wavelength range 190-700nm, bandwidth 1 nm, step 1 nm and scan rate 1 Hz. Ions have been detected in positive mode witch *m/z* range from 280 to 2000 Da

Cell Culture and Membrane Preparation

Chinese hamster ovary cells stably transfected with the genes of human variants of muscarinic receptors were purchased from Missouri S&T cDNA Resource Center (Rolla, MO, USA). Cell cultures and crude membranes were prepared as described previously [48]. Cells were grown to confluence in 75 cm^2 flasks in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 2 million cells were sub-cultured to 100 mm Petri dishes. The medium was supplemented with 5 mM butyrate for the last 24 hours of cultivation to increase receptor expression. Cells were detached by mild trypsinization on day 5 after subculture. Detached cells were washed twice in 50 ml of phosphate-buffered saline and 3 min centrifugation at 250 x g. Washed cells were suspended in 20 ml of ice-cold incubation medium (100 mM NaCl, 20 mM Na-HEPES, 10 mM $MgCl_2$, pH = 7.4) supplemented with 10 mM EDTA and homogenized on ice by two 30 sec strokes using Polytron homogenizer (Ultra-Turrax; Janke& Kunkel GmbH & Co. KG, IKA-Labortechnik, Staufen, Germany) with a 30-sec pause between strokes. Cell homogenates were centrifuged for 30 min at 30,000 x g. Supernatants were discarded, pellets suspended in the fresh incubation medium, incubated on ice for 30 minutes and centrifuged again. Resulting membrane pellets were kept at -80 ºC until assayed within 10 weeks at a maximum.

[^{3}H]NMS Binding Assay

All radioligand binding experiments were optimized and carried out as described previously [48]. Briefly, membranes were incubated in 96-well plates at 30 ºC in the incubation medium described above. Incubation volume was 400 µl or 800 µl for competition and saturation experiments with [^{3}H]NMS, respectively. Approximately 30 µg of membrane proteins per sample were used. N-methylscopolamine binding was measured directly in saturation experiments using six concentrations (30 pM to 1000 pM) of [^{3}H]NMS during incubation for 1 hour (M_2), 3 hours (M_1,

M_3 and M_4) or 5 hours (M_5). For calculations of the equilibrium dissociation constant (K_D), concentrations of free [^{3}H]NMS were calculated by subtraction of bound radioactivity from total radioactivity in the sample and fitting Eq. 1 (Data analysis section). Binding of tested ligands was determined in competition experiments with 100 pM [^{3}H]NMS. The equilibrium dissociation constant K_A and factor of binding cooperativity α were calculated according to Eq. 2. Samples were first preincubated 1 hour with [^{3}H]NMS. Then tested compound was added and incubation continued for an additional 15 hours. Non-specific binding was determined in the presence of 10 μM unlabelled atropine. Incubations were terminated by filtration through Whatman GF/C glass fibre filters (Whatman) using a Brandel cell harvester (Brandel, Gaithersburg, MD, USA). In kinetic experiments membranes were pre-incubated 15-hours with tested compounds at a final concentration of 100nM and the with 1 nM [^{3}H]NMS for additional 1 hour (M_2), 3 hours (M_1, M_3, M_4) or 5 hours (M_5). Then dissociation was started by addition of atropine to the final concentration of 10 μM. Dissociation lasted up to 90 min (M_1, M_4), 15 min (M_2), 150 min (M_3) or 240 min (M_5). Dissociation was terminated by filtration. Filters were dried in a microwave oven and then solid scintillator Meltilex A was melted on filters (105 °C, 70 s) using a hot plate. The filters were cooled and counted in WallacMicrobeta scintillation counter.

Functional Response to Acetylcholine

Effects of tested compounds on activation of receptors by acetylcholine were quantified by measurement of changes in the accumulation of inositol phosphates in cells (M_1, M_3 and M_5) or in [^{35}S]GTPγS binding to cell membranes (M_2 and M_4).

Measurement of inositol phosphate level

For determination of the level of metabolically-labelled inositol phosphates (IP_X), simple separation of precipitated membrane-bound phosphatidylinositol bisphosphate and soluble IP_X described earlier was used [49]. Cells were seeded in 96-well plates, 12 thousand cells per well in 100 μl of DMEM. On the next day, DMEM was removed and 50 μl of DMEM supplemented with 30 nM [^{3}H]myo-inositol for 12 hours. Then cell were washed by Krebs-HEPES buffer (KHB; 138 mM NaCl; 4 mMKCl; 1.3 mM $CaCl_2$; 1 mM $MgCl_2$; 1.2 mM NaH_2PO_4; 10 mM glucose; 20 mM Na-HEPES; pH = 7.4) supplemented with 10 mMLiCl. Cells were pre-incubated at 37ºC with tested compound at the desired concentration and acetylcholine for 30 min. Then KHB was removed and the accumulation of inositol phosphates was stopped by addition of 50 μl of 20 % trichloracetic acid (TCA). Plates were put to 4ºC for 1 hour, then 40 μl of TCA extract were transferred to another 96-well plate, mixed with 200 μl of Rotiszint scintillation cocktail and counted in WallacMicrobeta. Rest of TCA extract was discarded, individual wells were washed with 50 μl of 20 % TCA, 50 μl of 1 M NaOH was added to each well and plates were shaken at room temperature for 15 min. Then 40 μl of NaOH lysate were transferred to another 96-well plate, mixed with 200 μl of Rotiszint scintillation cocktail and counted in WallacMicrobeta. Level of inositol phosphates was calculated as a fraction of soluble (TCA extract) to total (TCA extract plus NaOH lysate) radioactivity.

[^{35}S]GTPγS Binding Assay

Measurement of [^{35}S]GTPγS binding was carried out on membranes in 96-well plates at 30 ºC in the incubation medium described above supplemented with freshly prepared dithiothreitol at a final concentration of 1 mM, essentially as described by [49]. Membranes at concentrations 10 μg of protein per well were used. The final volume was 200 μl, the final concentration of [^{35}S]GTPγS was 500 pM and the final concentration of GDP was 50 μM. Non-specific binding was determined in the presence of 1 μM unlabelled GTPγS. When the effects of tested compounds on ACh-stimulated

[^{35}S]GTPγS binding were measured, tested compounds were added to membranes 30 min before ACh and [^{35}S]GTPγS. Incubation with [^{35}S]GTPγS was carried out for 20 min and free ligand was removed by filtration through Whatman GF/C glass fibre filters (Whatman) using a Brandel cell harvester (Brandel, Gaithersburg, MD, USA). Filtration and washing with ice-cold water lasted for 9 s. Filters were dried in a microwave oven and then solid scintillator Meltilex A was melted on filters (105 °C, 70 s) using a hot plate. The filters were cooled and counted in WallacMicrobeta scintillation counter.

Data Analysis

Data from biological evaluation experiments were processed in Libre Office and then analysed and plotted using program Grace (http://plasma-gate.weizmann.ac.il/Grace/). Statistical analysis was performed using the statistical package R (http://www.r-project.org). For non-linear regression analysis following equations were used:

Saturation of [^{3}H]NMS Binding

Binding of [^{3}H]NMS at various concentrations was measured. After subtraction of non-specific binding and calculation of free radioligand concentration, Eq. 1 was fitted to the data.

$$y = \frac{B_{MAX} * x}{x + K_D} \qquad \text{Eq. 1}$$

Where y is specific binding at free concentration x, B_{MAX} is maximum binding capacity, and K_D is the equilibrium dissociation constant of [^{3}H]NMS.

Binding Parameters of Tested Compounds

Tested compounds are allosteric modulators of [^{3}H]NMS binding. [^{3}H]NMS binding was determined in the presence of tested compounds at various concentrations ^{50}. After subtraction of non-specific binding and normalization to binding in the absence of the tested compound, Eq. 4 was fitted to the data.

$$y = \frac{[D] + K_D}{[D] + K_D \frac{K_A + x}{K_A + \alpha * x}} \qquad \text{Eq. 2}$$

Where y is specific radioligand binding at concentration x of the tested compound expressed as a per cent of binding in its absence, K_A is the equilibrium dissociation constant of the tested compound and α is the factor of binding cooperativity. Value of α greater than 1 denotes positive cooperativity. K_D is the equilibrium dissociation constant and [D] is the concentration of [^{3}H]NMS.

[^{3}H]NMS Dissociation

After subtraction of non-specific binding and normalization to binding at the beginning of dissociation, Eq. 4 was fitted to the data.

$$y = 100 * e^{-k_{off} * x} \qquad \text{Eq. 3}$$

Where y is [^{3}H]NMS binding at time x, and koff is the observed dissociation rate constant.

Concentration Response to Acetylcholine

The intracellular level of inositol phosphates or [^{35}S]GTPγS binding to membranes at various concentrations of acetylcholine was measured. After subtraction of background values or non-specific binding and normalization to the level in the absence of acetylcholine Eq. 3 was fitted to the data.

$$y = 1 + \frac{(E'_{MAX}-1)*x^{nH}}{x^{nH}+EC_{50}}$$ Eq. 4

Where y is the normalized response at acetylcholine concentration x, E'_{MAX} is the observed maximal response, EC_{50} is the concentration of acetylcholine causing half-maximal effect, and nH is Hill coefficient.

From EC_{50} and E'_{MAX} values of series of agonists, the maximal system response E_{MAX} was determined and then operational efficacy acetylcholine τ was calculated according to Eq. 5 [24].

$$\tau = \frac{E'_{MAX}}{E_{MAX}-E'_{MAX}}$$ Eq. 5

The factor of operational cooperativity β and factor of binding cooperativity α was calculated from E'_{MAX} and EC'_{50} values induced by tested allosteric modulator at saturating concentration according to Eq. 6 and Eq. 7, respectively [25].

$$\beta = \frac{E'_{MAX}}{\tau E_{MAX}-\tau E'_{MAX}}$$ Eq. 6

$$\alpha = \frac{EC_{50}(\tau+1)}{EC'_{50}(\beta\tau+1)}$$ Eq. 7

Where E_{MAX} is a maximal system response, E'_{MAX} is the maximal response to acetylcholine in the presence of tested allosteric modulator at saturating concentration, τ is operational efficacy of acetylcholine, EC_{50} and EC'_{50} are concentrations of acetylcholine causing a half-maximal effect in the absence and presence of allosteric modulator at saturating concentration, respectively.

Acknowledgements

This research was funded by the Czech Academy of Sciences institutional support [RVO:67985823] (ED, NC, DN, AR, VD and JJ) and [RVO:61388963], Programs of Strategy AV21 (ES-P and EK) and the Grant Agency of the Czech Republic grant [19-05318S].

Contributions

JJ and EK conceived experimental design, ES-P synthesized, purified and verified chemical compounds, ED, NC, DN and AR conducted experiments, ED, NC, DN, AR and JJ analysed experimental data and performed the statistical analysis, ES-P and EK performed the analysis of the structure-activity relationship. All authors contributed to writing the manuscript, read and approved the final version of the manuscript.

Competing interests

Authors declare no competing interest.

Data and materials availability

Data supporting the findings of this manuscript are available from the corresponding authors upon reasonable request.

References

1. Eglen, R. M. Overview of muscarinic receptor subtypes. in *Handb Exp Pharmacol* (eds. Fryer, A. D., Arthur Christopoulos & Nathanson, N. M.) 3–28 (Springer, 2012).
2. Bock, A., Schrage, R. & Mohr, K. Allosteric modulators targeting CNS muscarinic receptors.

Neuropharmacology **136**, 427–437 (2018).

3. Gosens, R., Zaagsma, J., Meurs, H. & Halayko, A. J. Muscarinic receptor signaling in the pathophysiology of asthma and COPD. *Respir. Res.* **7**, 73 (2006).

4. Haga, K. *et al.* Structure of the human M2 muscarinic acetylcholine receptor bound to an antagonist. *Nature* **482**, 547–551 (2012).

5. Kruse, A. C. *et al.* Structure and dynamics of the M3 muscarinic acetylcholine receptor. *Nature* **482**, 552–556 (2012).

6. Thal, D. M. *et al.* Crystal structures of the M1 and M4 muscarinic acetylcholine receptors. *Nature* **531**, 335–340 (2016).

7. Vuckovic, Z. *et al.* Crystal structure of the M5 muscarinic acetylcholine receptor. *Proc. Natl. Acad. Sci. U. S. A.* **116**, 26001–26007 (2019).

8. Gimpl, G. Interaction of G protein coupled receptors and cholesterol. *Chem. Phys. Lipids* **199**, 61–73 (2016).

9. Randáková, A. *et al.* Role of membrane cholesterol in differential sensitivity of muscarinic receptor subtypes to persistently bound xanomeline. *Neuropharmacology* **133**, 129–144 (2018).

10. Jakubík, J. & El-Fakahany, E. E. Allosteric Modulation of Muscarinic Acetylcholine Receptors. *Pharmaceuticals (Basel).* **3**, 2838–2860 (2010).

11. Jakubik, J. & El-Fakahany, E. E. Current Advances in Allosteric Modulation of Muscarinic Receptors. *Biomolecules* **10**, 325 (2020).

12. Baulieu, E. E. Neurosteroids: a novel function of the brain. *Psychoneuroendocrinology* **23**, 963–87 (1998).

13. Rupprecht, R. & Holsboer, F. Neuroactive steroids: mechanisms of action and neuropsychopharmacological perspectives. *Trends Neurosci.* **22**, 410–6 (1999).

14. Ratner, M. H., Kumaresan, V. & Farb, D. H. Neurosteroid Actions in Memory and Neurologic/Neuropsychiatric Disorders. *Front. Endocrinol. (Lausanne).* **10**, 169 (2019).

15. Baulieu, E. E. & Schumacher, M. Progesterone as a neuroactive neurosteroid, with special reference to the effect of progesterone on myelination. *Hum. Reprod.* **15 Suppl 1**, 1–13 (2000).

16. Darnaudéry, M., Koehl, M., Piazza, P. V., Le Moal, M. & Mayo, W. Pregnenolone sulfate increases hippocampal acetylcholine release and spatial recognition. *Brain Res.* **852**, 173–9 (2000).

17. Pallarés, M., Darnaudéry, M., Day, J., Le Moal, M. & Mayo, W. The neurosteroid pregnenolone sulfate infused into the nucleus basalis increases both acetylcholine release in the frontal cortex or amygdala and spatial memory. *Neuroscience* **87**, 551–8 (1998).

18. Daniel, J. M., Hulst, J. L. & Lee, C. D. Role of hippocampal M2 muscarinic receptors in the estrogen-induced enhancement of working memory. *Neuroscience* **132**, 57–64 (2005).

19. Klangkalya, B. & Chan, A. Inhibition of hypothalamic and pituitary muscarinic receptor binding by progesterone. *Neuroendocrinology* **47**, 294–302 (1988).

20. Klangkalya, B. & Chan, A. Structure-activity relationships of steroid hormones on muscarinic receptor binding. *J. Steroid Biochem.* **29**, 111–8 (1988).

21. Horishita, T. *et al.* The effects of the neurosteroids: pregnenolone, progesterone and dehydroepiandrosterone on muscarinic receptor-induced responses in Xenopus oocytes

expressing M1 and M3 receptors. *Naunyn. Schmiedebergs. Arch. Pharmacol.* **371**, 221–8 (2005).

22. Wilkinson, M., Giles, A. & Wilkinson, D. A. M2 muscarinic ([3H]N-methyl scopolamine) binding in micropunches of rat ventricular myocardium: characterization and modification by progesterone. *Can. J. Physiol. Pharmacol.* **70**, 943–8 (1992).

23. Wilkinson, M., Siauw, M. & Horackova, M. Modulation of cardiac M2 muscarinic receptor binding by progesterone-related steroids. *J. Mol. Cell. Cardiol.* **27**, 1831–9 (1995).

24. Jakubík, J. *et al.* Applications and limitations of fitting of the operational model to determine relative efficacies of agonists. *Sci. Rep.* **9**, 4637 (2019).

25. Jakubík, J., Randáková, A., Chetverikov, N., El-Fakahany, E. E. & Doležal, V. The operational model of allosteric modulation of pharmacological agonism. *Sci. Rep.* **10**, 14421 (2020).

26. Hanson, M. A. *et al.* A specific cholesterol binding site is established by the 2.8 A structure of the human beta2-adrenergic receptor. *Structure* **16**, 897–905 (2008).

27. Liu, W. *et al.* Structural basis for allosteric regulation of GPCRs by sodium ions. *Science (80-.).* **337**, 232–236 (2012).

28. Simoncini, T. & Genazzani, A. R. Non-genomic actions of sex steroid hormones. *Eur. J. Endocrinol.* **148**, 281–92 (2003).

29. Shiraishi, M. *et al.* The inhibitory effects of alphaxalone on M1 and M3 muscarinic receptors expressed in Xenopus oocytes. *Anesth. Analg.* **97**, 449–55, table of contents (2003).

30. Saternos, H. C. *et al.* Distribution and function of the muscarinic receptor subtypes in the cardiovascular system. *Physiol. Genomics* **50**, 1–9 (2018).

31. Pitkänen, A. & Lukasiuk, K. Mechanisms of epileptogenesis and potential treatment targets. *Lancet. Neurol.* **10**, 173–86 (2011).

32. Ahmed Juvale, I. I. & Che Has, A. T. The evolution of the pilocarpine animal model of status epilepticus. *Heliyon* **6**, e04557 (2020).

33. Pennell, P. B. Hormonal aspects of epilepsy. *Neurol. Clin.* **27**, 941–965 (2009).

34. Velísková, J., De Jesus, G., Kaur, R. & Velísek, L. Females, their estrogens, and seizures. *Epilepsia* **51 Suppl 3**, 141–4 (2010).

35. Hamilton, S. E. *et al.* Disruption of the m1 receptor gene ablates muscarinic receptor-dependent M current regulation and seizure activity in mice. *Proc. Natl. Acad. Sci. U. S. A.* **94**, 13311–6 (1997).

36. Hillert, M. H. *et al.* Dynamics of hippocampal acetylcholine release during lithium-pilocarpine-induced status epilepticus in rats. *J. Neurochem.* **131**, 42–52 (2014).

37. Thorn, C. A., Popiolek, M., Stark, E. & Edgerton, J. R. Effects of M1 and M4 activation on excitatory synaptic transmission in CA1. *Hippocampus* **27**, 794–810 (2017).

38. Ehlert, F. J. Contractile role of M2 and M3 muscarinic receptors in gastrointestinal, airway and urinary bladder smooth muscle. *Life Sci.* **74**, 355–66 (2003).

39. Zajonc, T. P. & Roland, P. S. Vertigo and motion sickness. Part I: vestibular anatomy and physiology. *Ear. Nose. Throat J.* **84**, 581–4 (2005).

40. Martinez Botella, G., Salituro, F. G., Robichaud, A. J. & Harrison, B. L. Compositions and methods for treating CNS disorders. (2016).

41. Kapras, V. *et al.* Synthesis of deuterium labeled NMDA receptor inhibitor - 20-Oxo-5β-[9,12,12-(2)H(3)]pregnan-3α-yl-L-glutamyl 1-ester. *Steroids* **77**, 282–7 (2012).

42. Varasi, M., Walker, K. A. M. & Maddox, M. L. A Revised Mechanism for the Mitsunobu Reaction. *J. Org. Chem.* **52**, 4235–4238 (1987).

43. Hamilton, N. M. *et al.* Novel steroid inhibitors of glucose 6-phosphate dehydrogenase. *J. Med. Chem.* **55**, 4431–45 (2012).

44. Kapras, V., Šťastná, E., Chodounská, H., Pouzar, V. & Krištofíková, Z. Preparation of steroid sulfamates and their interaction with gaba A receptor. *Collect. Czechoslov. Chem. Commun.* **74**, 643–650 (2009).

45. Roleira, F. M. F. *et al.* C-6α- vs C-7α-Substituted Steroidal Aromatase Inhibitors: Which Is Better? Synthesis, Biochemical Evaluation, Docking Studies, and Structure-Activity Relationships. *J. Med. Chem.* **62**, 3636–3657 (2019).

46. Robichaud, A. J. *et al.* C17, c20, and c21 substituted neuroactive steroids and their methods of use. (2018).

47. Katona, B. W. *et al.* Neurosteroid analogues. 12. Potent enhancement of GABA-mediated chloride currents at GABAA receptors by ent-androgens. *Eur. J. Med. Chem.* **43**, 107–13 (2008).

48. Boulos, J. M. F., Jakubik, J., Boulos, J. M. F., Randakova, A. & Momirov, J. Synthesis of novel and functionally selective non- - competitive muscarinic antagonists as chemical probes. *Chem Biol Drug Des* **91**, 93–104 (2018).

49. Randáková, A., Rudajev, V., Doležal, V., Boulos, J. & Jakubík, J. Novel long-acting antagonists of muscarinic ACh receptors. *Br. J. Pharmacol.* **175**, 1731–1743 (2018).

50. Jakubík, J., Randáková, A., El-Fakahany, E. E. & Doležal, V. Analysis of equilibrium binding of an orthosteric tracer and two allosteric modulators. *PLoS One* **14**, e0214255 (2019).

Figure legends

Fig. 1 – Structures and biosynthesis of tested compounds
Structures of tested steroid compounds and their biosynthesis from cholesterol. Dashed arrows, multi-step reaction.

Fig. 2 – Allosteric modulation of [^{3}H]NMS binding by corticosterone and progesterone
Allosteric modulation of 100 pM [^{3}H]NMS binding to individual subtypes of muscarinic receptors (indicated in legend) by corticosterone (left) and progesterone (right). Data are means ± SD from 3 independent experiments performed in quadruplicates.

Fig. 3 – Modulation of functional response to acetylcholine by corticosterone
The functional response of muscarinic receptors to acetylcholine was measured as an increase in the intracellular level of inositol phosphates (M_1, M_3 and M_5) or increase in [^{35}S]GTPγS binding to membranes (M_2 and M_4). Controls and concentrations of corticosterone are indicated in the legend. Functional response is expressed as fold-over basal level. Data are means ± SD from 3 independent experiments performed in quadruplicates.

Fig. 4 – Modulation of functional response to acetylcholine by progesterone
The functional response of muscarinic receptors to acetylcholine was measured as an increase in the intracellular level of inositol phosphates (M_1, M_3 and M_5) or increase in [^{35}S]GTPγS binding to

membranes (M_2 and M_4). Controls and concentrations of corticosterone are indicated in the legend. Functional response is expressed as fold-over basal level. Data are means ± SD from 3 independent experiments performed in quadruplicates.

Fig. 5 – Modulation of functional response to acetylcholine by estradiol
The functional response of muscarinic receptors to acetylcholine was measured as an increase in the intracellular level of inositol phosphates (M_1, M_3 and M_5) or increase in [^{35}S]GTPγS binding to membranes (M_2 and M_4). Controls and concentrations of corticosterone are indicated in the legend. Functional response is expressed as fold-over basal level. Data are means ± SD from 3 independent experiments performed in quadruplicates.

Fig. 6 – Modulation of functional response to acetylcholine by testosterone
The functional response of muscarinic receptors to acetylcholine was measured as an increase in the intracellular level of inositol phosphates (M_1, M_3 and M_5) or increase in [^{35}S]GTPγS binding to membranes (M_2 and M_4). Controls and concentrations of corticosterone are indicated in the legend. Functional response is expressed as fold-over basal level. Data are means ± SD from 3 independent experiments performed in quadruplicates.

Figures:

Fig. 1

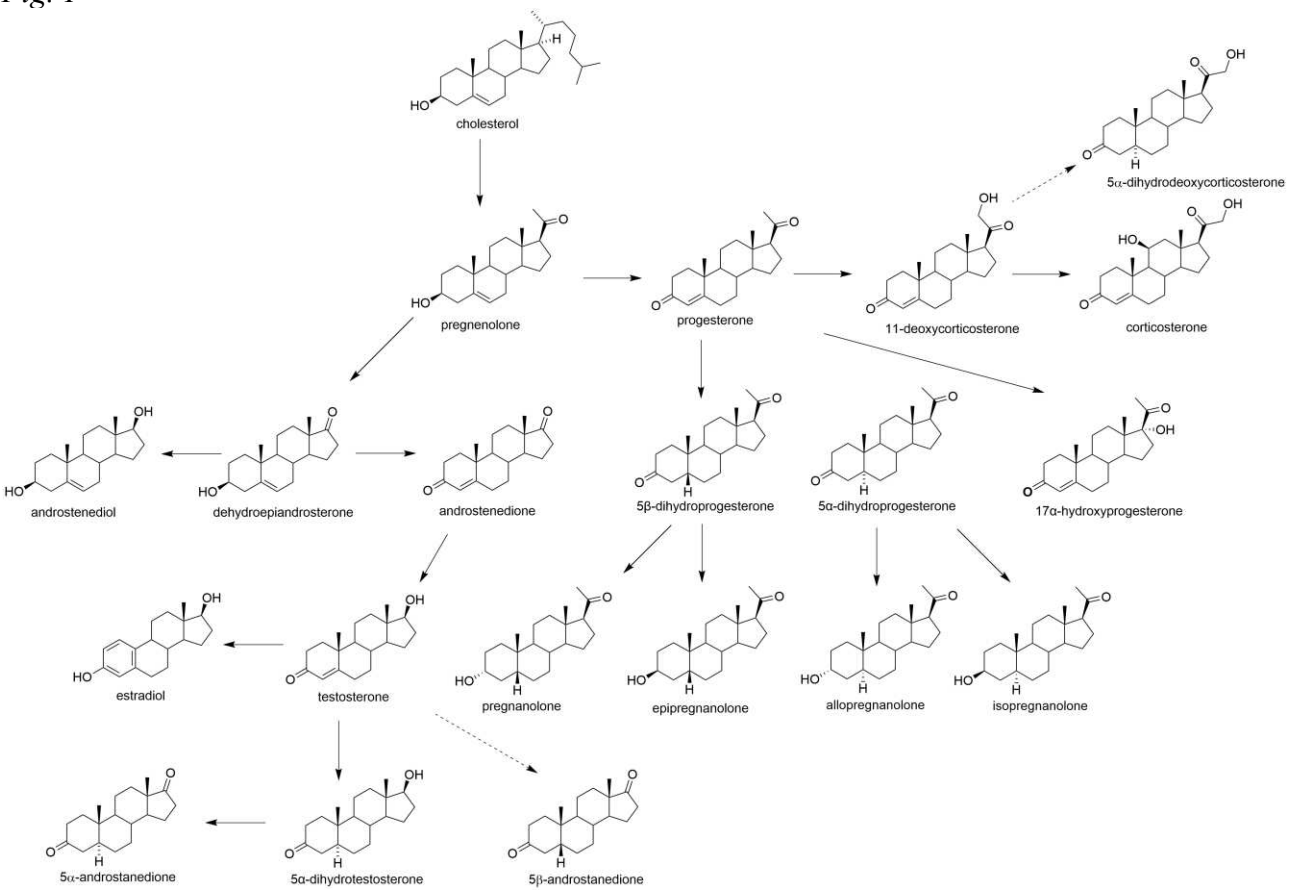
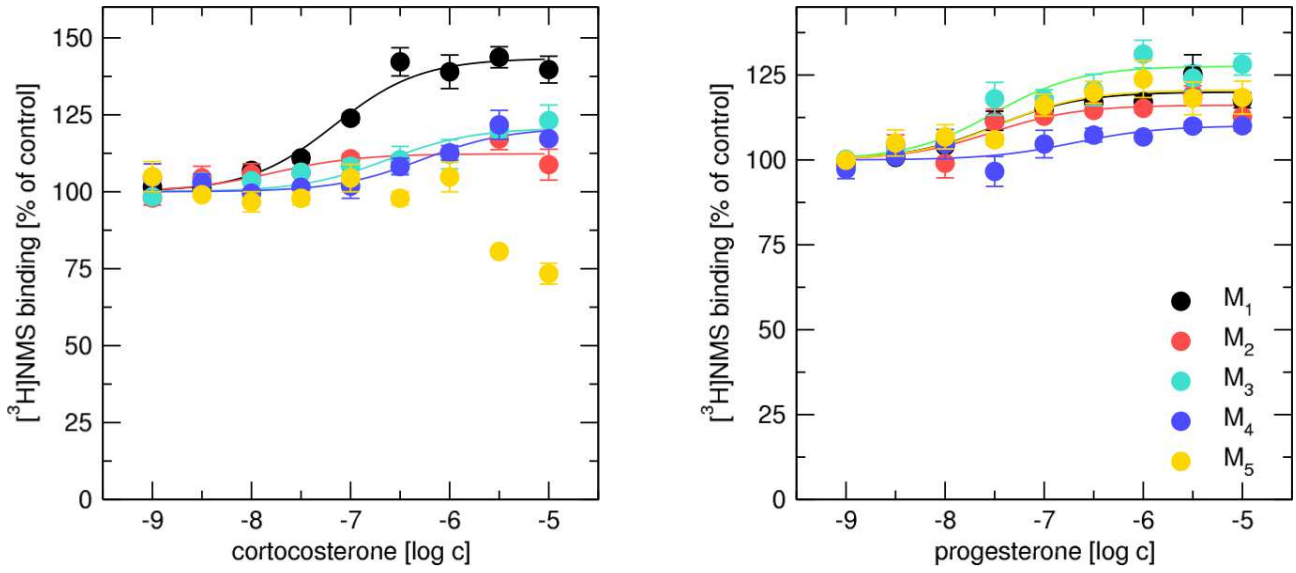
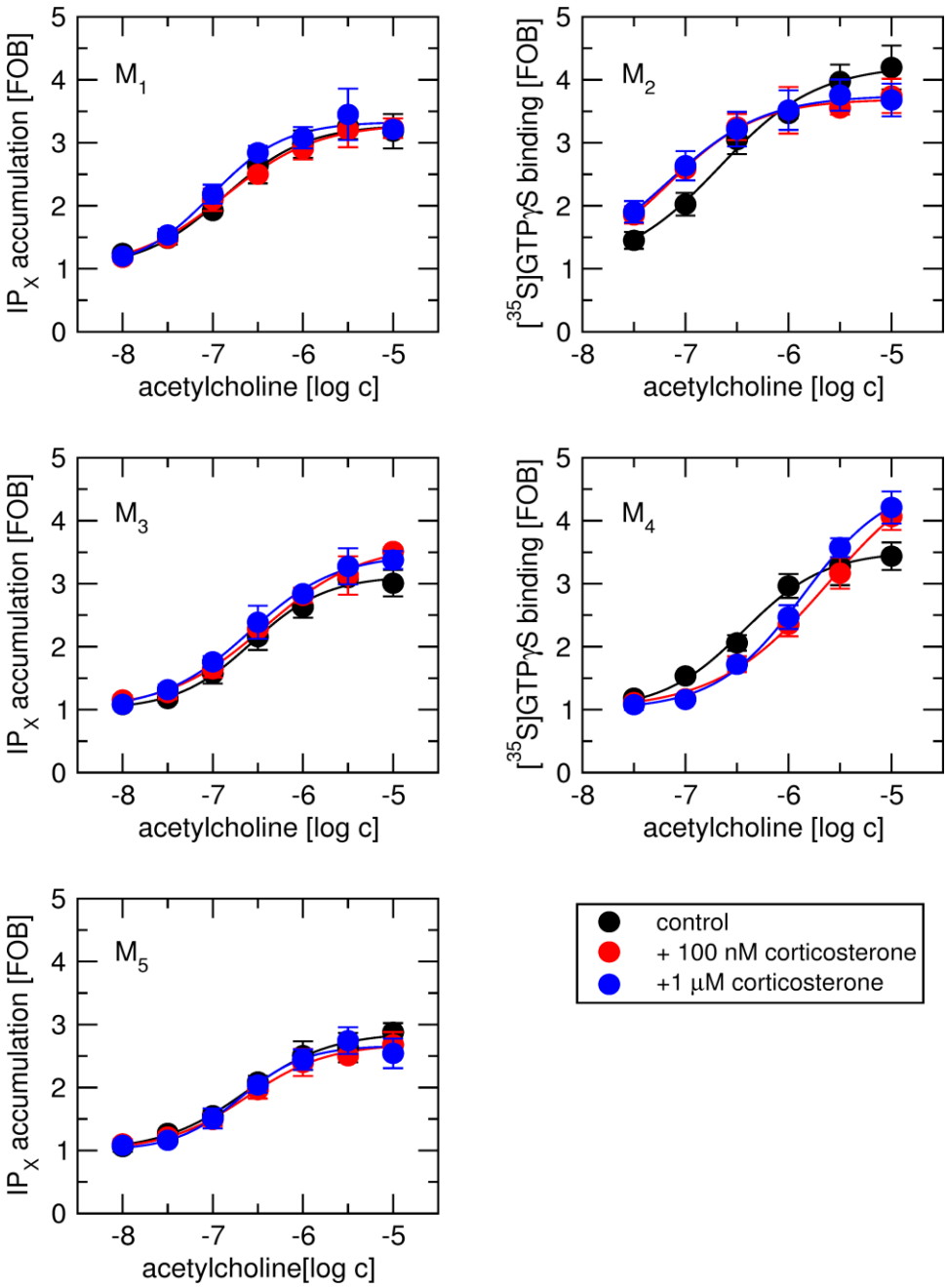


Fig. 2



640 *Fig. 3*

641

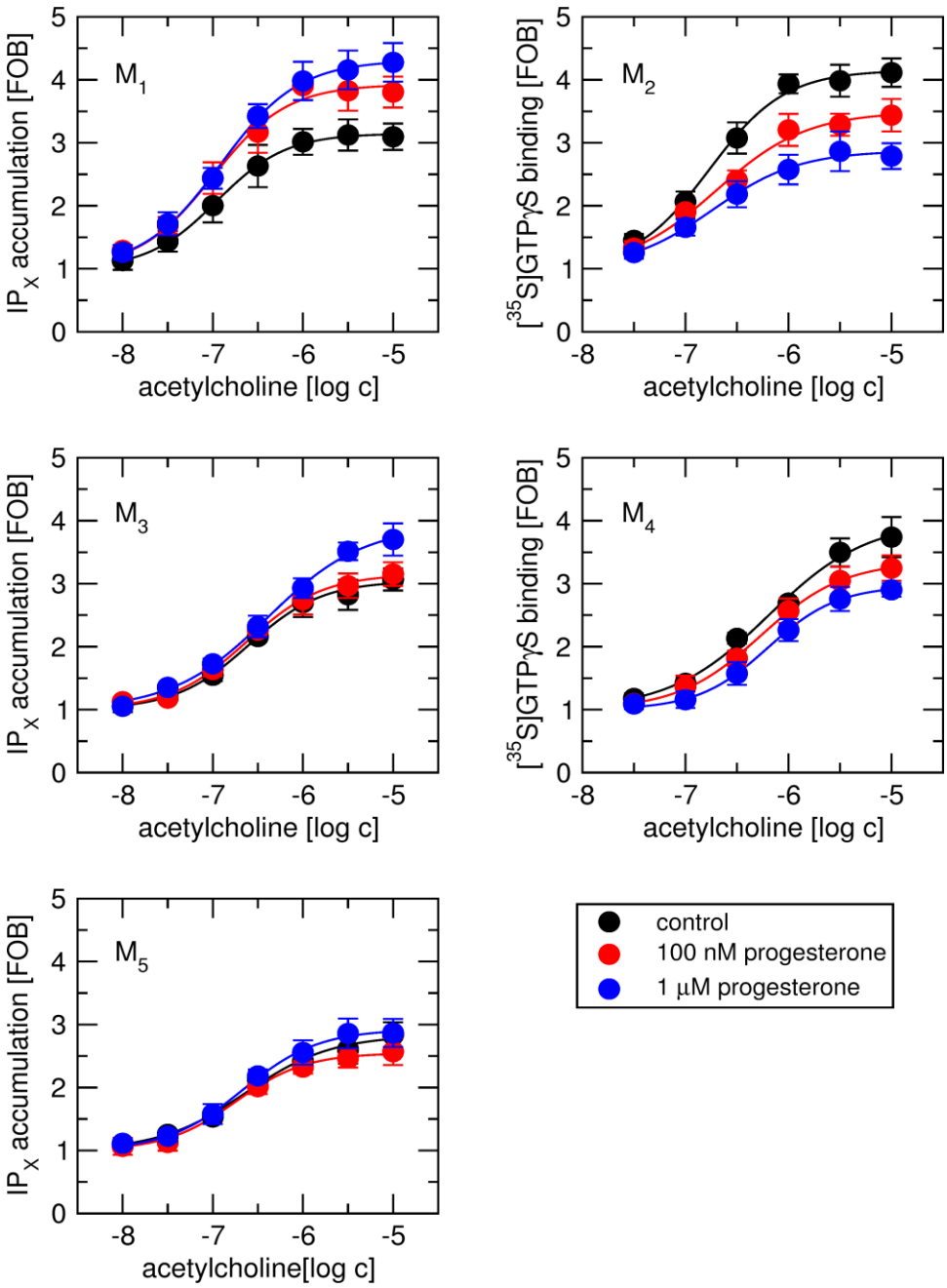


642

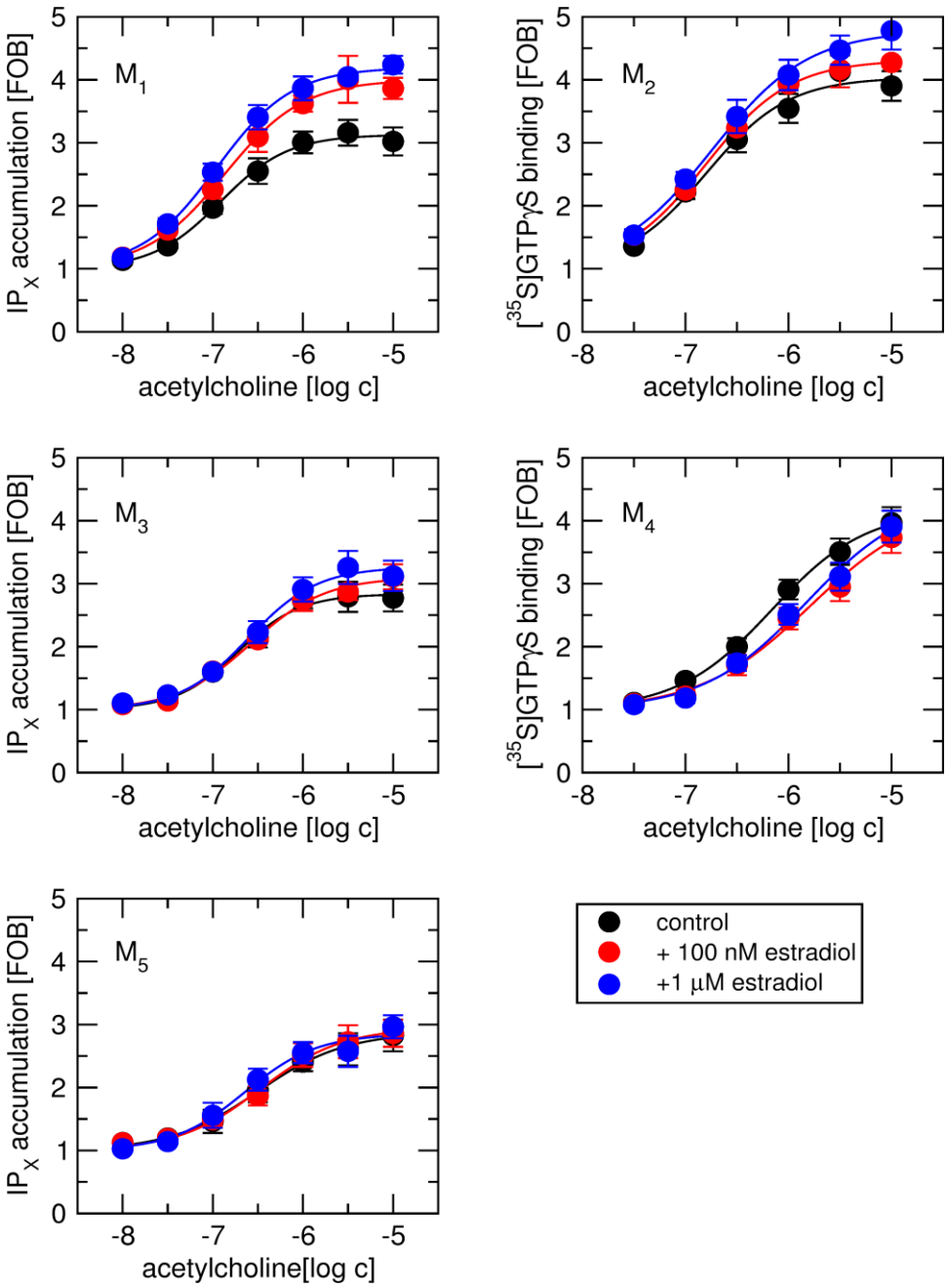
643

644

Fig. 4

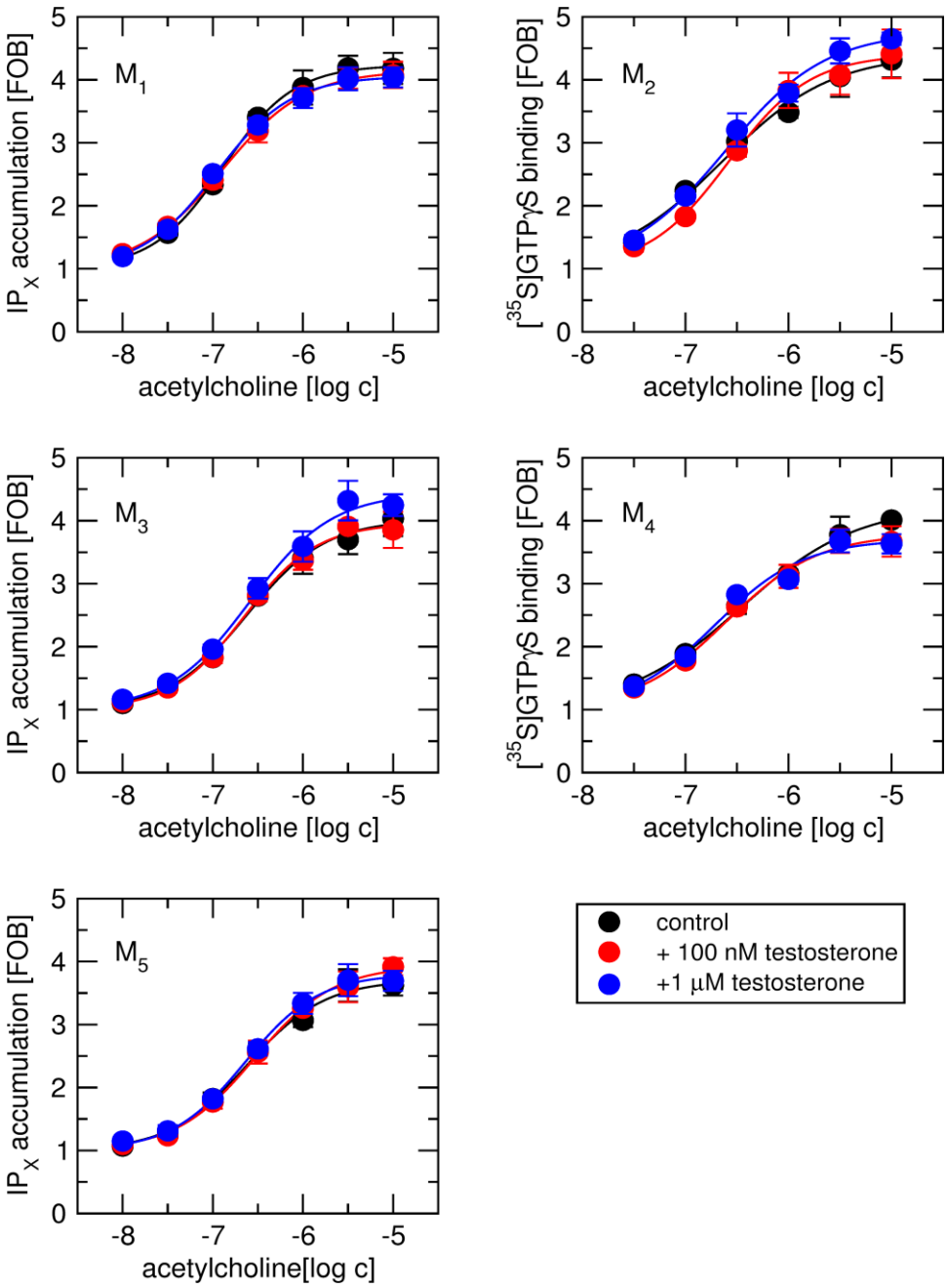

M1
M2
M3
M4
M5
IPX accumulation [FOB]
[35S]GTPγS binding [FOB]
acetylcholine [log c]
control
100 nM progesterone
1 µM progesterone

650 *Fig. 5*
651



652
653
654

Fig. 6


M1
M2
M3
M4
M5
IPX accumulation [FOB]
[35S]GTPγS binding [FOB]
acetylcholine [log c]
acetylcholine[log c]
control
+ 100 nM testosterone
+1 µM testosterone

Tables:

Table 1 Affinities – equilibrium binding
Affinities of tested compounds are expressed as the negative logarithm of the equilibrium dissociation constant K_A obtained by fitting Eq. 2 to data from equilibrium binding experiments. Values are means ± SD from 3 independent experiments performed in quadruplicates.

	M_1	M_2	M_3	M_4	M_5
11-Deoxycorticosterone	<6	<6	6.46 ± 0.11	<6	<6
Corticosterone	6.92 ± 0.1	7.27 ± 0.04	7.09 ± 0.04	6.63 ± 0.22	<6
5α-Dihydrodeoxycorticosterone	<6	<6	<6	6.68 ± 0.05	<6
17α-Hydroxyprogesterone	<6	6.69 ± 0.08	6.54 ± 0.09	<6	7.18 ± 0.09
Pregnenolone	7.35 ± 0.08	<6	<6	7.32 ± 0.06	<6
Progesterone	7.35 ± 0.11	7.35 ± 0.18	7.38 ± 0.12	6.52 ± 0.23	7.26 ± 0.08
5β-Dihydroprogesterone	6.07 ± 0.09	7.13 ± 0.12	<6	7.01 ± 0.11	6.07 ± 0.10
5α-Dihydroprogesterone	<6	<6	<6	<6	<6
Epipregnanolone	<6	<6	<6	6.7 ± 0.3	6.2 ± 0.3
Pregnanolone	6.6 ± 0.3	6.7 ± 0.3	<6	6.48 ± 0.10	7.35 ± 0.07
Isopregnanolone	<6	<6	<6	6.09 ± 0.06	<6
Allopregnanolone	<6	<6	<6	<6	<6
Estradiol	<6	<6	<6	6.21 ± 0.13	<6
DHEA	<6	6.3 ± 0.3	<6	6.2 ± 0.2	<6
Androstenediol	<6	<6	<6	<6	<6
Androstenedione	<6	7.09 ± 0.10	7.38 ± 0.15	<6	<6
Testosterone	6.63 ± 0.07	7.15 ± 0.06	<6	<6	<6
Dihydrotestosterone	<6	<6	<6	<6	<6
5α-androstenedione	<6	<6	<6	<6	<6
5β-androstenedione	<6	<6	<6	<6	<6

Table 2 Effects on the binding kinetics of [^{3}H]NMS

Effects of tested compounds on the kinetics of [^{3}H]NMS are expressed as ratios of the observed rate of [^{3}H]NMS dissociation in the presence of the tested compound to the dissociation rate in its absence obtained by fitting Eq. 3 to [^{3}H]NMS dissociation data. A value greater that than 1 denotes an acceleration of dissociation, lower than 1 denote a deceleration of dissociation. Values are means ± SD from 3 independent experiments performed in quadruplicates. *, different from 1 ($p<0.05$, one sample T-test).

	M_1	M_2	M_3	M_4	M_5
11-Deoxycorticosterone	1.03 ± 0.04	1.04 ± 0.07	1.05 ± 0.09	1.00 ± 0.08	0.65 ± 0.03*
Corticosterone	0.92 ± 0.09	1.05 ± 0.07	0.93 ± 0.07	1.09 ± 0.08	0.72 ± 0.02*
5α-Dihydrodeoxycorticosterone	1.00 ± 0.05	1.14 ± 0.07	1.08 ± 0.07	0.84 ± 0.06	0.87 ± 0.07
17α-Hydroxyprogesterone	0.89 ± 0.06	1.41 ± 0.05*	1.18 ± 0.07*	1.42 ± 0.09*	0.70 ± 0.06*
Pregnenolone	0.64 ± 0.07*	0.80 ± 0.05*	1.00 ± 0.03	1.51 ± 0.09*	1.01 ± 0.09
Progesterone	0.67 ± 0.08*	0.81 ± 0.09*	1.00 ± 0.03	1.42 ± 0.08*	1.02 ± 0.03
5β-Dihydroprogesterone	1.37 ± 0.09*	1.00 ± 0.05	1.92 ± 0.08*	1.00 ± 0.05	0.83 ± 0.07*
5α-Dihydroprogesterone	1.06 ± 0.06	0.83 ± 0.06*	1.27 ± 0.02*	1.16 ± 0.07*	0.52 ± 0.10*
Epipregnanolone	0.79 ± 0.08*	1.00 ± 0.05	0.98 ± 0.03	1.00 ± 0.05	1.42 ± 0.07*
Pregnanolone	1.49 ± 0.09*	1.22 ± 0.07*	1.53 ± 0.09*	1.92 ± 0.15*	0.76 ± 0.05*
Isopregnanolone	0.93 ± 0.05	1.19 ± 0.06*	1.06 ± 0.05	1.16 ± 0.07*	1.07 ± 0.06
Allopregnanolone	0.92 ± 0.06	1.26 ± 0.08*	1.12 ± 0.07	0.88 ± 0.06	1.05 ± 0.06
Estradiol	1.97 ± 0.07*	1.35 ± 0.05*	1.58 ± 0.08*	2.18 ± 0.08*	0.42 ± 0.06*
DHEA	1.06 ± 0.04	0.95 ± 0.05	0.82 ± 0.05*	0.88 ± 0.05*	0.95 ± 0.05
Androstenediol	1.01 ± 0.02	0.97 ± 0.04	0.71 ± 0.03*	0.95 ± 0.02	1.04 ± 0.09*
Androstenedione	0.73 ± 0.08*	1.38 ± 0.08*	1.00 ± 0.07	1.00 ± 0.05	1.18 ± 0.05*
Testosterone	0.57 ± 0.07*	1.40 ± 0.09*	1.06 ± 0.08	1.00 ± 0.06	1.07 ± 0.04
Dihydrotestosterone	1.90 ± 0.09*	1.35 ± 0.05*	0.95 ± 0.03	2.08 ± 0.05*	0.33 ± 0.08*
5α-androstenedione	1.18 ± 0.08*	1.10 ± 0.07	1.07 ± 0.05	1.11 ± 0.08	1.19 ± 0.07*
5β-androstenedione	1.18 ± 0.07*	1.04 ± 0.06	1.03 ± 0.05	0.87 ± 0.07	0.95 ± 0.06

Table 3 Effects of hormones on the functional response of muscarinic receptors

Effects of hormones at 100 nM and 1μM concentration on acetylcholine potency (ΔpEC_{50}) and maximal elicited response (% of control E_{MAX}) at muscarinic receptors. Positive ΔpEC_{50} means an increase in acetylcholine potency. Values are means ± SD from 3 independent experiments performed in quadruplicates. *, different from 0 (ΔpEC_{50}), 100 % (E_{MAX}) and 1 (β, α) ($p<0.05$, one sample T-test).

Table 3A – Effects of corticosterone

	100 nM		1 μM			
	ΔpEC_{50}	% of control E_{MAX}	ΔpEC_{50}	% of control E_{MAX}	β	α
M_1	-0.01 ± 0.06	101 ± 9	0.13 ± 0.07	107 ± 3	1.02 ± 0.02	1.33 ± 0.09*
M_2	0.49 ± 0.05*	85 ± 6*	0.54 ± 0.06*	85 ± 6*	0.80 ± 0.08*	3.52 ± 0.13*
M_3	0.01 ± 0.04	111 ± 4*	0.02 ± 0.04	117 ± 5*	1.24 ± 0.07*	0.94 ± 0.08
M_4	-0.49 ± 0.09*	120 ± 8*	-0.43 ± 0.07*	121 ± 6*	1.73 ± 0.10*	0.20 ± 0.10*
M_5	0.07 ± 0.03	97 ± 7	0.06 ± 0.06	96 ± 6	0.88 ± 0.07	1.24 ± 0.09

Table 3B – Effects of progesterone

	100 nM		1 μM			
	ΔpEC_{50}	% of control E_{MAX}	ΔpEC_{50}	% of control E_{MAX}	β	α
M_1	-0.03 ± 0.06	127 ± 6*	0.00 ± 0.05	133 ± 9*	2.09 ± 0.03*	0.58 ± 0.03*
M_2	0.01 ± 0.04	81 ± 9	0.02 ± 0.02	68 ± 8*	0.53 ± 0.05*	1.21 ± 0.07*
M_3	-0.01 ± 0.06	104 ± 5*	0.03 ± 0.05	120 ± 8*	1.71 ± 0.08*	0.46 ± 0.08*
M_4	0.13 ± 0.11	92 ± 5	0.23 ± 0.07*	84 ± 6*	0.60 ± 0.09*	1.27 ± 0.12*
M_5	-0.08 ± 0.06	92 ± 7	-0.09 ± 0.04	104 ± 6	1.06 ± 0.06	1.04 ± 0.08

Table 3C – Effects of estradiol

	100 nM		1 μM			
	ΔpEC_{50}	% of control E_{MAX}	ΔpEC_{50}	% of control E_{MAX}	β	α
M_1	-0.02 ± 0.04	124 ± 5*	0.04 ± 0.04	130 ± 5*	1.97 ± 0.06*	0.75 ± 0.07*
M_2	0.06 ± 0.06	101 ± 5	0.01 ± 0.09	115 ± 9	1.42 ± 0.12*	0.73 ± 0.11*
M_3	-0.07 ± 0.08	104 ± 5	0.02 ± 0.04	111 ± 5	1.29 ± 0.06*	0.69 ± 0.07*
M_4	-0.33 ± 0.08*	103 ± 7	-0.39 ± 0.09*	110 ± 7	1.12 ± 0.15	0.44 ± 0.17*
M_5	-0.11 ± 0.04	102 ± 4	0.01 ± 0.04	103 ± 5	0.98 ± 0.08	1.42 ± 0.11*

Table 3D – Effects of testosterone

	100 nM		1 μM			
	ΔpEC_{50}	% of control E_{MAX}	ΔpEC_{50}	% of control E_{MAX}	β	α
M_1	-0.02 ± 0.03	97 ± 4	-0.02 ± 0.01	95 ± 4	0.89 ± 0.06	1.12 ± 0.06
M_2	-0.12 ± 0.04	104 ± 6	-0.06 ± 0.04	110 ± 7	1.19 ± 0.13	0.85 ± 0.15
M_3	-0.01 ± 0.04	101 ± 5	0.04 ± 0.04	107 ± 4	1.22 ± 0.12	0.88 ± 0.13
M_4	-0.01 ± 0.09	96 ± 5	0.08 ± 0.09	88 ± 7	0.92 ± 0.11	1.22 ± 0.16
M_5	-0.02 ± 0.03	103 ± 6	0.00 ± 0.02	100 ± 5	1.06 ± 0.09	1.01 ± 0.10

691

Figures

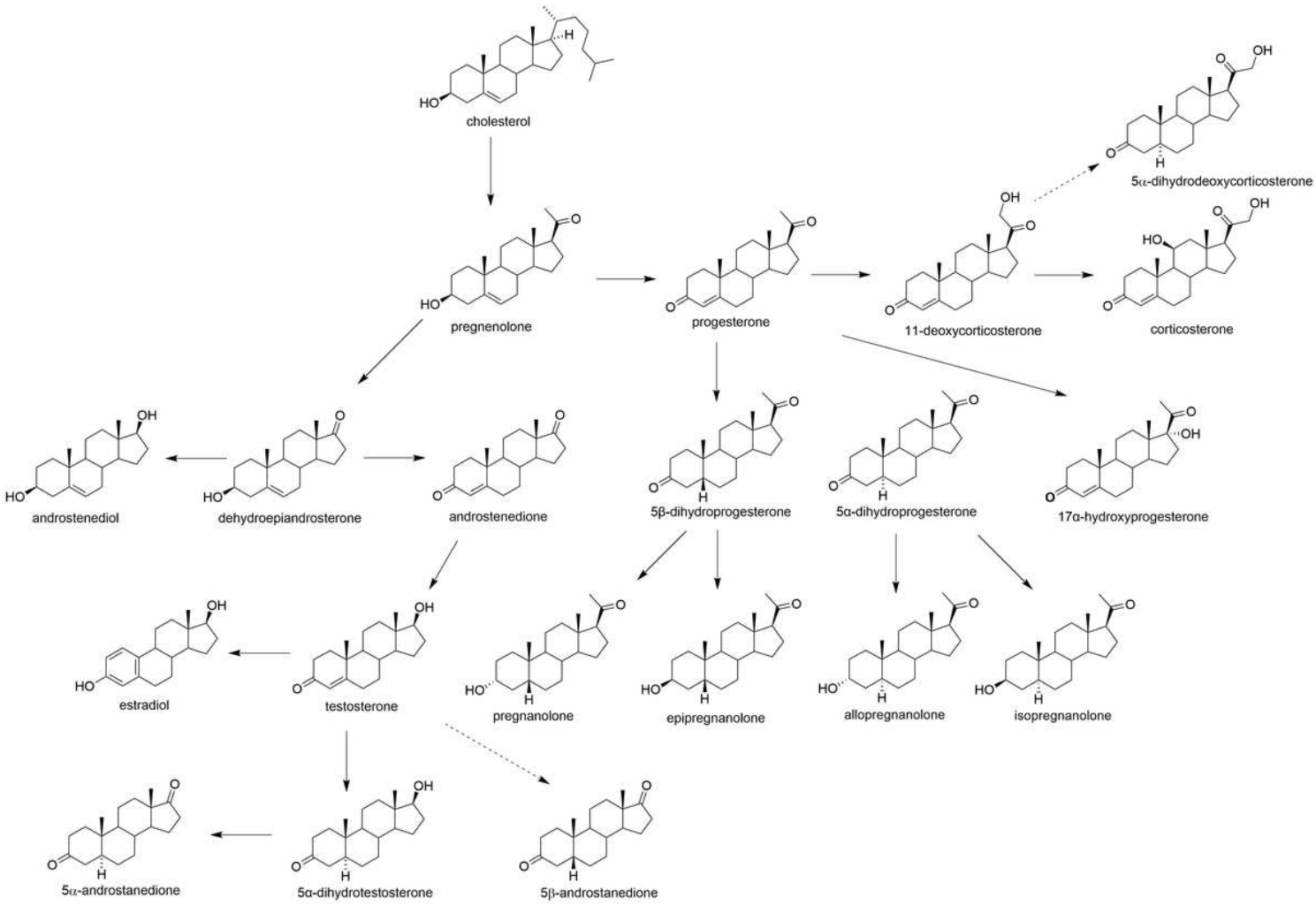


Figure 1

Structures and biosynthesis of tested compounds Structures of tested steroid compounds and their biosynthesis from cholesterol. Dashed arrows, multi-step reaction.

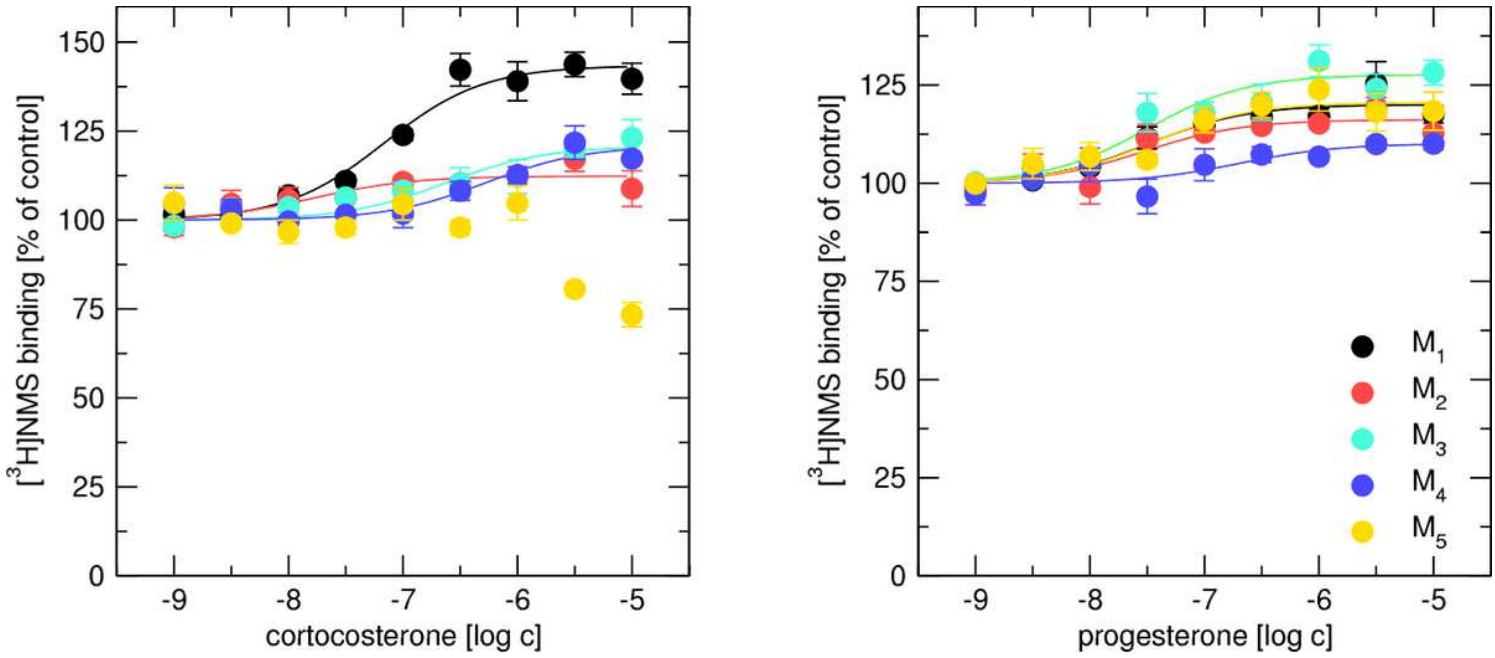


Figure 2

Allosteric modulation of [3H]NMS binding by corticosterone and progesterone Allosteric modulation of 100 pM [3H]NMS binding to individual subtypes of muscarinic receptors (indicated in legend) by corticosterone (left) and progesterone (right). Data are means ± SD from 3 independent experiments performed in quadruplicates.

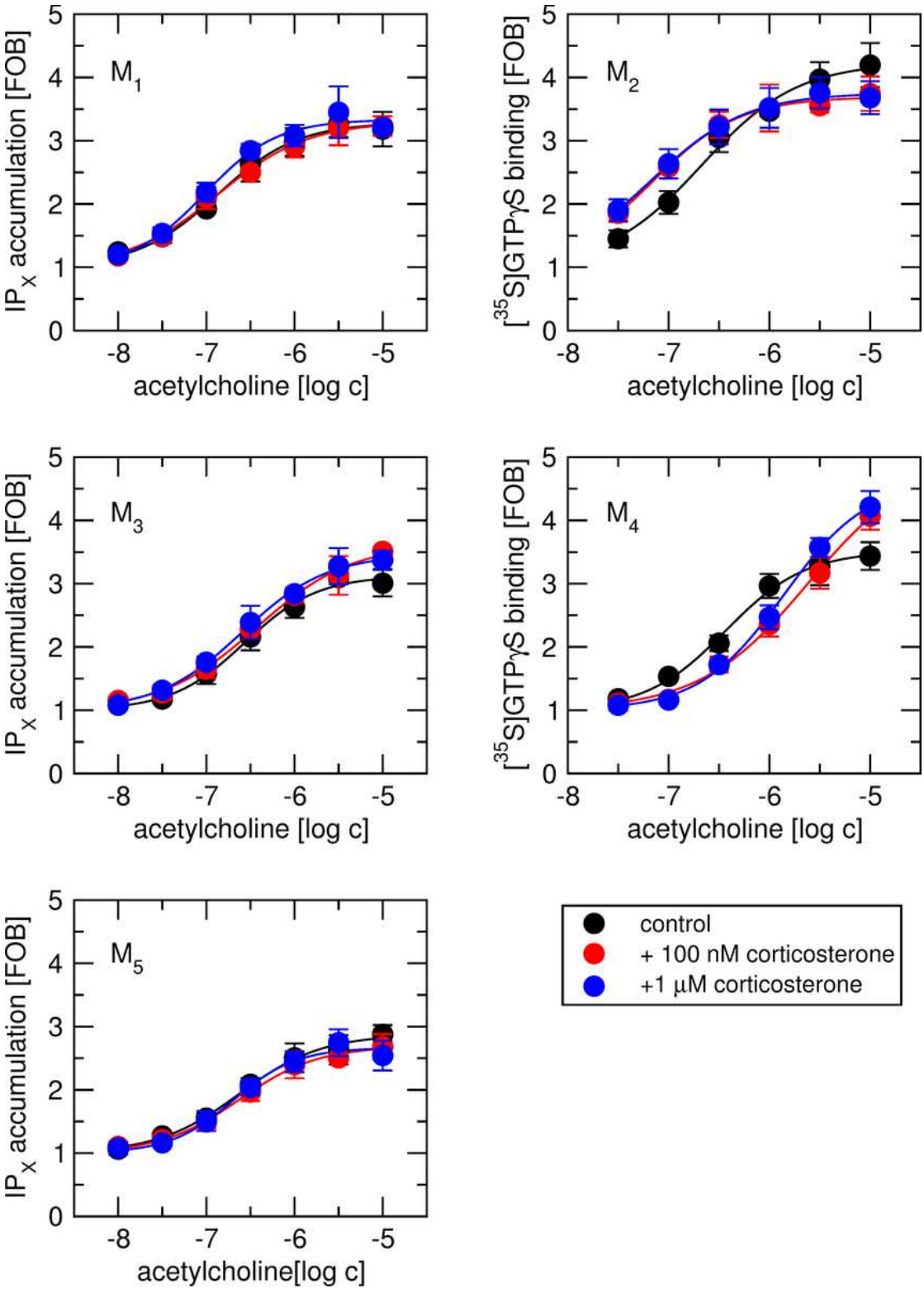


Figure 3

Modulation of functional response to acetylcholine by corticosterone The functional response of muscarinic receptors to acetylcholine was measured as an increase in the intracellular level of inositol phosphates (M1, M3 and M5) or increase in [35S]GTPγS binding to membranes (M2 and M4). Controls and concentrations of corticosterone are indicated in the legend. Functional response is expressed as fold-over basal level. Data are means ± SD from 3 independent experiments performed in quadruplicates.

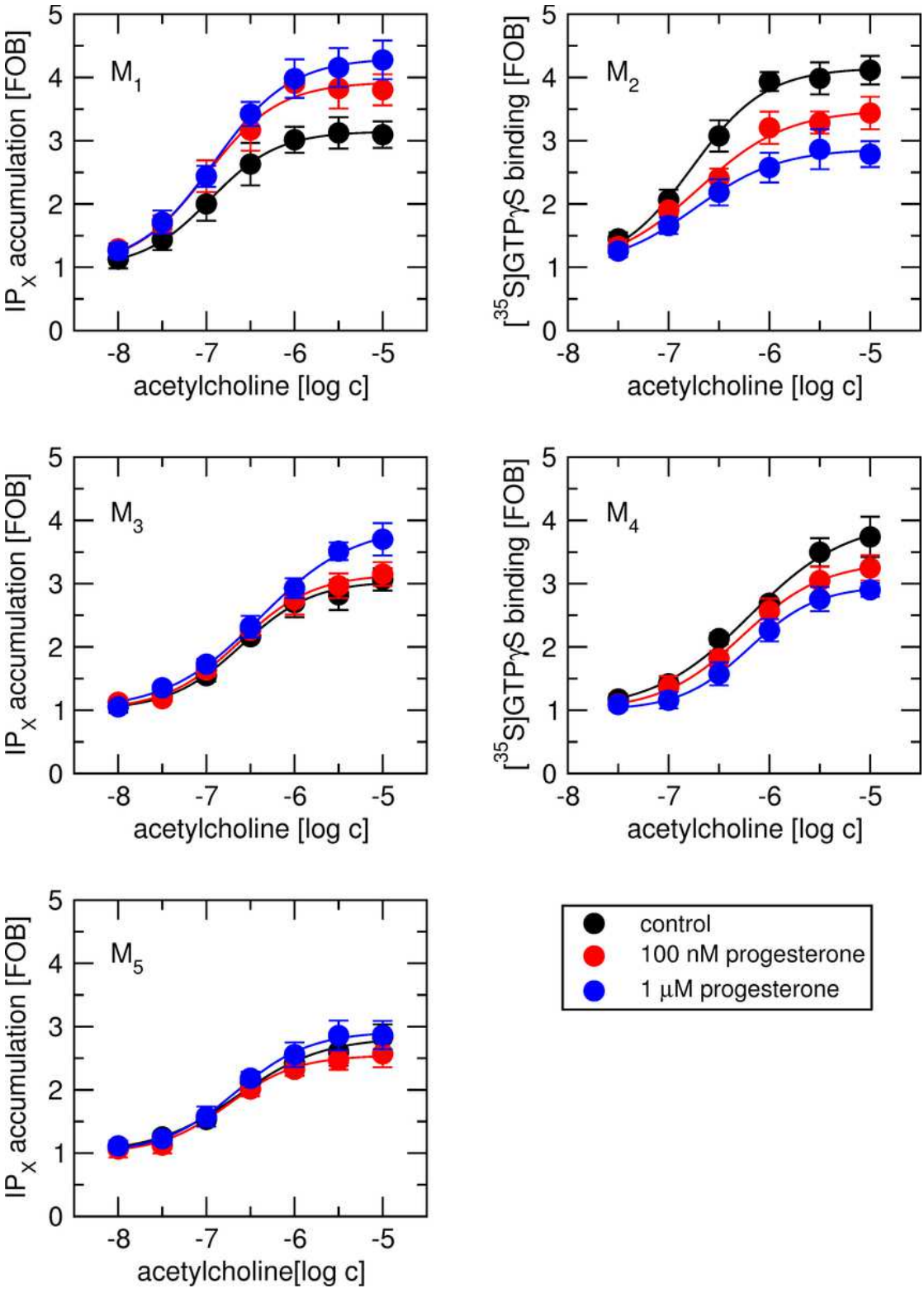


Figure 4

Modulation of functional response to acetylcholine by progesterone The functional response of muscarinic receptors to acetylcholine was measured as an increase in the intracellular level of inositol phosphates (M1, M3 and M5) or increase in [35S]GTPγS binding to membranes (M2 and M4). Controls and concentrations of corticosterone are indicated in the legend. Functional response is expressed as fold-over basal level. Data are means ± SD from 3 independent experiments performed in quadruplicates.

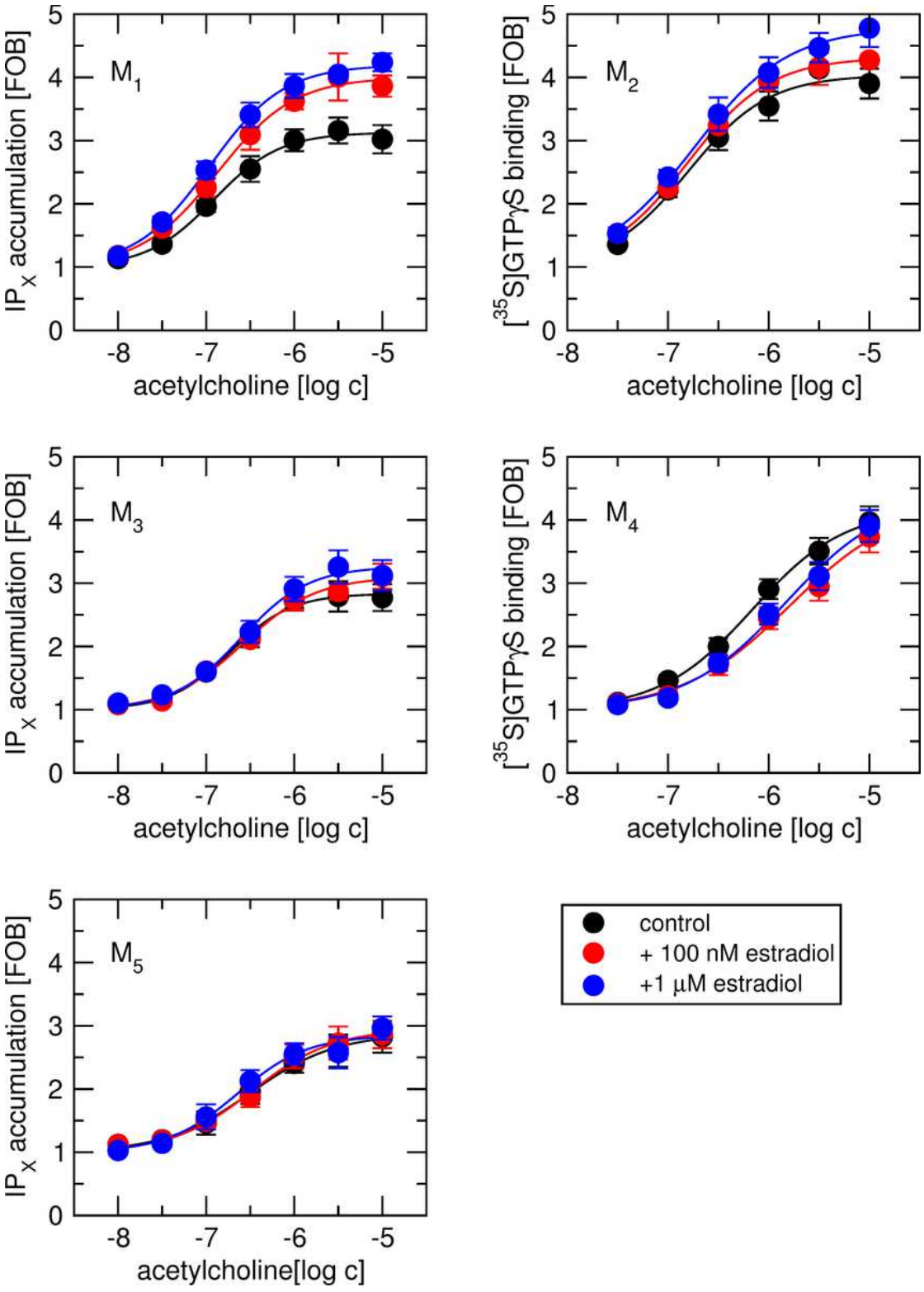


Figure 5

Modulation of functional response to acetylcholine by estradiol The functional response of muscarinic receptors to acetylcholine was measured as an increase in the intracellular level of inositol phosphates (M1, M3 and M5) or increase in [35S]GTPγS binding to membranes (M2 and M4). Controls and concentrations of corticosterone are indicated in the legend. Functional response is expressed as fold-over basal level. Data are means ± SD from 3 independent experiments performed in quadruplicates.

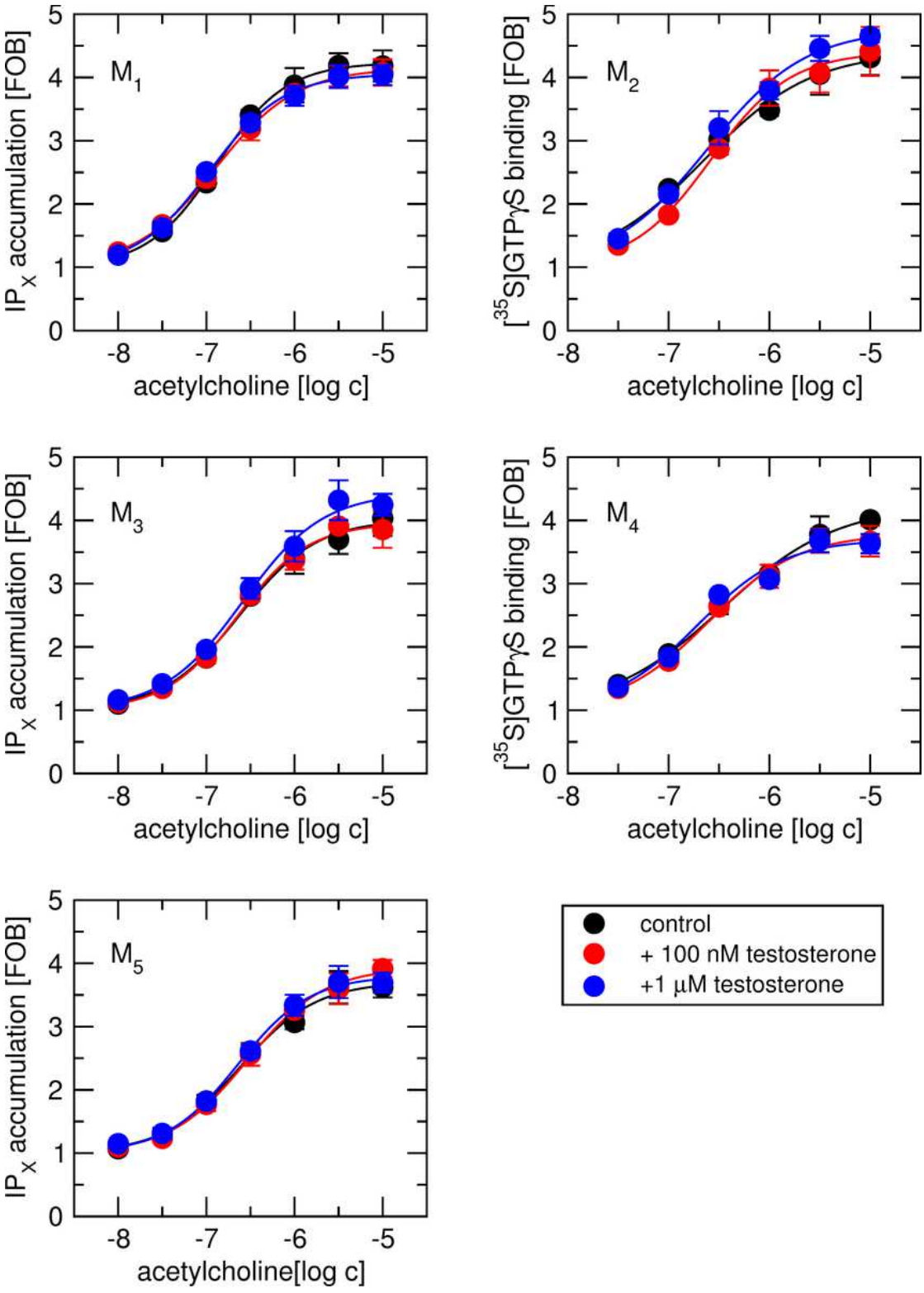


Figure 6

Modulation of functional response to acetylcholine by testosterone The functional response of muscarinic receptors to acetylcholine was measured as an increase in the intracellular level of inositol phosphates (M1, M3 and M5) or increase in [35S]GTPγS binding to membranes (M2 and M4). Controls and concentrations of corticosterone are indicated in the legend. Functional response is expressed as

fold-over basal level. Data are means ± SD from 3 independent experiments performed in quadruplicates.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SImanuscriptcholesterolMARNatCommun.docx