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PLOS ONE

Long-Term Activation upon Brief Exposure to Xanomleline Is Unique to M_1 and M_4 Subtypes of Muscarinic Acetylcholine Receptors

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Abstract

Xanomeline is an agonist endowed with functional preference for M_1/M_4 muscarinic acetylcholine receptors. It also exhibits both reversible and wash-resistant binding to and activation of these receptors. So far the mechanisms of xanomeline selectivity remain unknown. To address this question we employed microfluorometric measurements of intracellular calcium levels and radioligand binding to investigate differences in the short- and long-term effects of xanomeline among muscarinic receptors expressed individually in Chinese hamster ovary cells. 1/One-min exposure of cells to xanomeline markedly increased intracellular calcium at hM_1 and hM_4, and to a lesser extent at hM_2 and hM_3 muscarinic receptors for more than 1 hour. 2/Unlike the classic agonists carbachol, oxotremorine, and pilocarpine 10-min exposure to xanomeline did not cause internalization of any receptor subtype. 3/Wash-resistant xanomeline selectively prevented further increase in intracellular calcium by carbachol at hM_1 and hM_4 receptors. 4/After transient activation xanomeline behaved as a long-term antagonist at hM_5 receptors. 5/The antagonist N-methylscopolamine (NMS) reversibly blocked activation of hM_1 through hM_4 receptors by xanomeline. 6/NMS prevented formation of xanomeline wash-resistant binding and activation at hM_2 and hM_4 receptors and slowed them at hM_1, hM_3 and hM_5 receptors. Our results show commonalities of xanomeline reversible and wash-resistant binding and short-time activation among the five muscarinic receptor subtypes. However long-term receptor activation takes place in full only at hM_1 and hM_4 receptors. Moreover xanomeline displays higher efficacy at hM_1 and hM_4 receptors in primary phasic intracellular calcium release. These findings suggest the existence of particular activation mechanisms specific to these two receptors.

Citation: Šantrůčková E, Doležal V, El-Fakahany EE, Jakubík J (2014) Long-Term Activation upon Brief Exposure to Xanomleline Is Unique to M_1 and M_4 Subtypes of Muscarinic Acetylcholine Receptors. PLoS ONE 9(2): e88910. doi:10.1371/journal.pone.0088910

Editor: Wolfgang Blenau, Goethe University Frankfurt, Germany

Received July 5, 2013; **Accepted** January 14, 2014; **Published** February 18, 2014

Funding: This work was supported by the Academy of Sciences of the Czech Republic project [AV0Z 50110509] and support [RVO:67985823], the Grant Agency of the Czech Republic grants [305/09/0681] and [P304/12/G069]. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Muscarinic receptors are members of the G protein coupled receptor (GPCR) family A. To date, five distinct subtypes of muscarinic acetylcholine receptors (M_1–M_5) have been cloned and sequenced [1]. Muscarinic acetylcholine receptors that are present both in the central and peripheral nervous systems are involved in numerous physiological and pathological processes and thus represent important pharmacological targets [2]. One of the most important roles of muscarinic receptor-mediated cholinergic neurotransmission in the CNS relates to cognitive functions, mainly through the activation of the M_1 subtype of muscarinic receptors. Its disruption is connected with psychiatric and neurologic disorders including Alzheimer's disease (AD), Parkinson's disease, schizophrenia, epilepsy, sleep disorders, neuropathic pain, and others. Specifically, muscarinic agonists or inhibitors of acetylcholine esterase have been shown to reverse cognitive deficits associated with disrupted cholinergic neurotransmission in patients with a clinical diagnosis of Alzheimer presenile dementia [3] and a variety of other pathological states [4,5].

However, subtype-selective muscarinic agonists are difficult to obtain due to high homology of the orthosteric agonist binding site among the five subtypes of muscarinic receptors. So far, one of the few known selective muscarinic agonists is xanomeline (3-hexoxy-4-(1-methyl-3,6-dihydro-2H-pyridin-5-yl)-1,2,5-thiadiazole) [6]. Xanomeline has been shown to stimulate phosphatidyl inositol hydrolysis in mice via M_1 receptors [7]. In clinical studies xanomeline significantly improved cognition and ameliorated hallucinations and delusions in patients with Alzheimer's disease [8]. However, it was withdrawn from clinical trials due to unacceptable side effects including bradycardia, gastrointestinal distress, excessive salivation, and sweating [9]. Later on xanomeline proved to be also a potent agonist at M_4 receptors [10,11]. These findings have led to interest in xanomeline as a potential therapy for schizophrenia [12–15]. Besides its M_1/M_4 preference, xanomeline binds to all muscarinic receptor subtypes in a way that is resistant to intensive washing and causes persistent receptor activation or antagonism [16–22].

Functional subtype preference of xanomeline among muscarinic receptors is rather puzzling. Its reversible binding and receptor activation occur with the same affinity and potency at all subtypes

of muscarinic receptors [20,23,24]. Also xanomeline wash-resistant binding occurs at all receptor subtypes with the same affinity [25]. So far, the only observed qualitative exception from uniform behavior of xanomeline at muscarinic receptors is functional antagonism by wash-resistant xanomeline at M_5 receptors [22]. There are also differences in kinetics of xanomeline binding and activation between M_1 and M_2 receptors [20] and in long-term effects and receptor regulation between M_1 and M_3 receptors [24,26].

In this study we investigated which property of xanomeline-receptor kinetics correlates with xanomeline functional preference for M_1/M_4 receptors observed *in vivo*. We focused on the differences among subtypes of muscarinic receptors in the formation of wash-resistant binding and persistent activation upon brief exposure to xanomeline followed by washing. To this end we employed radioligand binding and microfluorometric measurements of levels of intracellular calcium. Our results show commonalities of xanomeline reversible and wash-resistant binding and short-time activation but this commonality does not extend to long-term receptor activation. Wash-resistant xanomeline binding elicits full long-term receptor activation only at M_1 and M_4 receptors. Identification of this key difference is crucial for the design of future experiments aimed at unraveling the molecular mechanisms of xanomeline preference, with particular emphasis on identification of specific amino acid(s) or conformations associated with persistent activation by wash-resistant xanomeline unique to these two subtypes.

Materials and Methods

Cell culture

Chinese hamster ovary (CHO) cells stably expressing human variants of individual subtypes of muscarinic acetylcholine receptors were purchased from Missouri S&T cDNA Resource Center (Rolla, MO, USA). Cells were maintained in Dulbecco's modified Eagle medium enriched with 10% fetal bovine serum and 0,005% geneticin. For microfluorometry measurements about 250,000 cells were seeded on 24 mm diameter microscopic glasses (Karl Hecht KG, Sondheim, Germany) in 30 mm Petri dishes containing 3 ml DMEM and cultivated for 3 days. For binding experiments, 100,000 cells per well were seeded into 24-well plates in 2 ml of DMEM and grown for 4 days.

Chemicals

Plasmid containing cDNA for human G protein G_{16} for transient transfection was from Invitrogen (Carlsbad, CA, USA). Other reagents for transient transfection – Lipofectamine and OptiMEM – were purchased from GibcoBRL (Gaithesburgh, MD, USA). Fura 2-AM for microfluorometry measurements was purchased from Molecular Probes – Invitrogen (Carlsbad, CA, USA). Fura 2-AM was dissolved in dimethylsulfoxide (Sigma, St. Louis, MO, USA) at 2 mM concentration and mixed 1:1 with 20% pluronic P68 (Sigma). Krebs-HEPES buffer (KHB; final concentrations in mM: NaCl 138; KCl 4; $CaCl_2$ 1.3; MgCl2 1; NaH_2PO_4 1.2; Hepes 20; glucose 10; pH adjusted to 7.4) with or without probenecid (Serva Feinbiochemica, Heidelberg, Germany) was used for washing of cells. Forskolin and isomethylbutylxantine and muscarinic receptor ligands carbamoylcholine chloride (carbachol) and N-methylscopolamine bromide (NMS) were from Sigma (St. Louis, MO, USA) and xanomeline was from Eli Lilly & Company (Indianapolis, IN, USA). Radiolabeled muscarinic receptor antagonists methyl[^{3}H]scopolamine ([^{3}H]NMS) and quinuclidinylbenzilate ([^{3}H]QNB) were from Amersham (Little Chalfont, Buckinghamshire, UK), radiolabeled adenine was from American Radiolabeled Chemicals (St. Louis, MO). Drugs were diluted directly in Krebs-HEPES buffer unless stated otherwise.

Transient transfection

Using 6-well plates 5 μg of cDNA was diluted in 2.5 ml OptiMEM and 50 μl of Lipofectamine was diluted in 2.5 ml OptiMEM. After 5 mins of occasional stirring both solutions were combined (final concentration was 1 μg of cDNA and 10 μl of Lipofectamine per ml), stirred and then incubated 20 mins in room temperature and stirred occasionally. Meanwhile DMEM was removed from Petri dishes and cells were washed with 2 ml of sterile PBS. 0.8 ml of the mixture of cDNA-Lipofectamine was added to washed cells in each dish. After 6 hours incubation in 37°C 2 ml of warmed DMEM was added. After 48 hours cells were ready for the experiment.

Fast microfluorometry

Microfluorometry experiments were carried out on the CHO cells stably expressing individual subtypes of muscarinic receptors on the third and fourth day after seeding. In order to facilitate measurements of calcium responses, cells stably expressing M_2 and M_4 receptors were one day after seeding transiently transfected with cDNA encoding human G protein G_{16} as described above. On the day of the measurement cells were twice washed with KHB then pre-labeled with 5 μM Fura 2-AM in KHB enriched with 1 mM pluronic for one hour at 37°C. After pre-labeling cells were washed twice with KHB, mounted to a superfusion chamber, placed on a stage of Olympus IX-90 inverted fluorescent microscope, application capillary was positioned at the edge of the view-field and suction capillary was positioned at the opposite edge of the view field less than 2 mm apart and continuously superfused at a flow rate 0.5 ml/min. The maximum possible volume of droplet between capillaries was 2 mm^3. The measurements were conducted at room temperature air-conditioned to 27°C. The microscope was connected through a CCD camera to a computer equipped with Metafluor 2.0 software (Visitron Systems GmBH, Germany) for image acquisition and analysis. A cube with 330–385 nm excitation band pass and ≥420 nm emission wide band filter was used. Excitation wavelengths on Visitron monochromator were set to 340 nm and 380 nm. Acquisition time was 200 ms per image. Two acquisitions (pairs of images) were taken every second unless otherwise stated. During the measurements images of the whole visual field containing about 40 cells were saved and analyzed off-line after the measurements. Image darkest region devoid of cells was taken as the fluorescence background and was substracted from all values. Only cells responding to the first (control) carbachol stimulation were selected for further analysis. Eight to 12 cells with best response to first stimulation were selected (by exclusion of weakly and/or slow responding cells or cells with abnormal long-lasting response; the outliers in peak value, time to peak or fall time were identified by interquartile range (IQR) where data below Q1-1.5*IQR and above Q3+1.5*IQR were considered outliers) from every measurement and their calcium signals were averaged and normalized to basal calcium level. The average of initial 10-s period without agonist was taken as basal. Data were further analyzed by means of array oriented program Grace (plasma-gate.weizmann.ac.il/Grace/).

Four general schemes of calcium measurements were employed. In the first scheme differences among receptor subtypes in the long-term effects of brief exposure to xanomeline were tested. Initially, control stimulation with 300 nM carbachol lasting 5 s was performed. After 3 min of washing with KHB cells were stimulated with 10 μM xanomeline for 1, 3 or 10 min. Calcium

levels in the absence of xanomeline were measured for the subsequent hour. At the end of measurement the second control stimulation with 300 nM carbachol for 5 s was carried out. Additional experiments with a slightly modified scheme were performed in order to evidence the differences between effects of wash-resistant xanomeline and the classical agonists carbachol, oxotremorine, and pilocarpine. In these experiments carbachol, oxotremorine, or pilocarpine were applied for one hour three minutes after an initial control 5-s stimulation with 0.3 μM carbachol and then washed in drug-free KHB for 30 min. At the end of measurement the second control stimulation with 0.3 μM carbachol for 10 s was carried out.

In the second scheme, effects of the antagonist NMS on delayed response to xanomeline were measured. After 5-s control stimulation with 300 nM carbachol cells were washed for 5 min with KHB and then stimulated with 10 μM xanomeline for 20 s. After 2-min of washing the cells were exposed for two min to 10 μM NMS and then they were washed again for another 4 min.

In the third scheme, effects of antagonist NMS on immediate response and formation of xanomeline wash-resistant receptor activation were probed. After initial 10-s control stimulation with 300 nM carbachol cells were washed for 5 min with KHB and then exposed for 3 min to 10 μM NMS. 10 μM xanomeline was applied for 1 min together with NMS during the second min of NMS treatment. Cells were finally washed for 3 min using drug-free KHB.

In the forth scheme, effects of extracellular calcium on xanomeline-induced oscillations of intracellular calcium were probed. After 5-s control stimulation with 300 nM carbachol cells were washed for 6 min with KHB Cells expressing M_1 or M_4 receptors were exposed for 3 min to 10 μM xanomeline and then washed with calcium-free KHB for additional 7 min.

Binding experiments on membranes

For binding experiments 100,000 cells per well were seeded and grown in 3 ml of DMEM in 6-well plates. On day four after subculture cells stably expressing individual subtypes of muscarinic receptors from each well were detached by mild trypsinization, suspended in 1 ml of KHB, and then incubated at room temperature in KHB containing 10 μM xanomeline for 1, 3 or 10 min or in KHB containing 1 μM carbachol, 1 μM oxotremorine or 3 μM pilocarpine for 10 min. Control cells were sham treated with KHB. Subsequently, cells were spinned down and washed 3-times with 1 ml of ice cold KHB to remove free xanomeline and incubated in fresh KHB for another 10 min or one hour at room temperature. After incubation the cells were cooled on ice and membranes were prepared as follows. Treated cells were suspended in 1 ml of ice cold homogenization medium (100 mM NaCl, 10 mM $MgCl_2$, 10 mM EDTA, 20 mM Na-HEPES pH = 7.4) and homogenized by two 30–second strokes at maximum speed and 30-second pause between strokes while cooled in ice by Ultra-Thurrax homogenizer. Homogenates were centrifuged at 1,000 g for 5 min and the resulting supernatant was centrifuged at 30,000 g for 30 min. Pellets were re-suspended in 1 ml of KHB and centrifugation was repeated. The membranes (50 μg of proteins per sample) were labeled with [^{3}H]NMS in final concentration ranging from 60 pM to 4 nM at 30°C for 1 hour in 96-deep-well plates. Final incubation volume was 0.8 ml. Incubation was terminated by fast filtration through Whatman GF/C glass fiber filters on Brandel cell harvester. Non-specific binding was determined in the presence of 10 μM NMS. Filters were dried and then solid scintillator Meltilex A was applied using heating plate at 105°C for 75 s. After filters cooled radioactivity was measured in Microbeta scintillation counter (Wallac, Finland). Maximum binding capacity (B_{MAX}) was corrected according to protein amount determined colorimetrically [27] on Wallac Victor 2 plate reader (Wallac, Finland).

Assay of cyclic AMP formation

On day four after subculture cells stably expressing M_2 or M_4 subtypes of muscarinic receptors were suspended in KHB, preincubated for 1 h at 37°C with 0.25 μM [^{3}H]adenine (10 μCi/ml). Xanomeline in a final concentration 10 μM was added to a portion of the cells for last 3 min of incubation. Cells were quickly washed three-times by centrifugation, resuspended in KHB and washed either for 10 min or 1 hour, centrifuged and washed twice by centrifugation and resuspended in KHB buffer containing 1 mM isobutylmethylxanthine and divided into individual incubation tubes. Forskolin was added to the cells at a final concentration of 5 μM or 20 μM. The incubation was in a volume of 0.8 ml per tube, with 300,000–400,000 cells per tube. Cells were incubated for 20 min at 37°C. Incubation was stopped by addition of 0.2 ml per tube of 2.5 M HCl and the extract was applied on a column filled with 1.5 g alumina. The column was washed with a portion of 2 ml of 100 mM ammonium acetate (pH 7.0) and the retained [^{3}H]cAMP was eluted with the next portion of 4 ml of 100 mM ammonium acetate, collected in scintillation vials and quantified by liquid scintillation spectrometry. The synthesis of [^{3}H]cAMP was measured as the difference between the content of [^{3}H]cAMP in the samples at the end and in the beginning of the 20-min incubation period. Accumulation of [^{3}H]cAMP in xanomeline-treated and sham-treated cells corrected for content of protein was compared.

Data analysis

Data from binding experiments were pre-processed using Open Office (www.openoffice.org) and analyzed using Graph Pad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA). Data from microfluorometry experiments were analyzed using Grace (Weizmann Institute of Science, Rehovot, Israel; http://plasma-gate.weizmann.ac.il/Grace/). Statistical analysis was done with statistical package R (www.r-project.org).

Concentration response

$$y = 1 + \frac{(E_{MAX} - 1) * x}{EC_{50} + x} \qquad \text{(Eq.1)}$$

where y is maximum stimulation by agonist at concentration x, E_{MAX} is maximal response and EC_{50} is half-efficient concentration.

Saturation binding experiments

$$y = \frac{B_{MAX} * x}{K_D + x} \qquad \text{(Eq.2)}$$

where y is specific [^{3}H]NMS binding at free concentration of [^{3}H]NMS x, K_D is equilibrium dissociation constant and B_{MAX} is maximum binding capacity was fitted to the data from saturation binding experiments. Added radioligand was measured for each concentration by liquid scintillation and the initial concentration calculated based on specific radioactivity and final volume. Free radioligand concentration was calculated by subtraction of bound radioligand from initial radioligand concentration.

Signaling efficacy

Apparent affinity constant K_G of the G protein for the agonist-receptor complex was calculated according Lu and Hulme [28] using the following equation:

$$K_G = E_{MAX\ FR}/(1-E_{MAX\ FR})/B_{MAX} \qquad \text{(Eq.3)}$$

where $E_{MAX\ FR}$ is maximal response calculated according Eq. 1 and expressed as fraction of E_{MAX} of carbachol ($E_{MAX\ agonist}-1$)/($E_{MAX\ carbachol}-1$) and B_{MAX} is maximum binding capacity calculated according Eq. 2 from binding data on cell membranes.

Results

Preliminary experiments

CHO cell lines expressed individual subtypes of muscarinic receptor in similar levels (Table S1 in File S1). In cells expressing hM_2 or hM_4 receptors and not transfected with G_{16} G protein the calcium response to 1 μM carbachol was weak (increase by 8 to 11% above basal level) and slow (time to reach maximum level was 50 to 80 s) (data not shown). Preliminary control experiments of the stability of intracellular calcium signal measured by the probe FURA-2 showed that the signal is stable (no photobleaching occurred) for more than 1 hour under experimental conditions (2 exposures for 200 ms every 20 s) and the response to carbachol was the same at 3 consequent stimulations with 3 min interval between stimulations as well as the stimulation after 1-hour superfusion (data not shown). Basal level signal was more than twice above the background level and peak signals (application of agonists) were about 20% of assay maximum (application of ionomycin). Intracellular calcium response to agonist carbachol and the partial agonists oxotremorine and pilocarpine was uniform among receptor subtypes (Fig. S1 and Table S3 in File S1).

Potency and efficacy of brief exposure of cells to xanomeline on intracellular calcium level

Brief exposure (20 s) to xanomeline elicited a transient increase in intracellular calcium level (Fig. 1). At hM_2, hM_3 and hM_5 receptors intracellular calcium level returned to basal but remained elevated at hM_1 and hM_4 receptors (Fig. 1). E_{MAX} effect elicited by 10 μM xanomeline was close to the maximal at all subtypes (Table S2 in File S1). Xanomeline had the same potency at all five receptor subtypes (Table 1). However, there was marked difference in xanomeline E_{MAX} among receptor subtypes. Calculated E_{MAX} is highest at hM_1 and lowest at hM_5 receptors (Table 1). Order of E_{MAX} values taken as per cent of full agonists carbachol E_{MAX} is $M_1 > M_4 = M_3 > M_5 > M_2$ and ranges from 90% to 44%. In control experiments (Fig. S1 in File S1) selectivity in efficacy of agonists oxotremorine and pilocarpine was much smaller and ranged from 56% at hM_2 to 73% at hM_5 to and from 52% at hM_2 to 66% at hM_5, respectively (Fig. S2 in File S1, Table 1). The order of apparent affinity constants of G-protein for agonist-receptor complex (K_G) based on membrane expression level (Table 2) and calculated according Eq. 3 was $M_1 > M_4 > M_3 > M_5 > M_3$ (Table 1).

Immediate and delayed effects of brief exposure to xanomeline on intracellular calcium levels

In microflourometric experiments of estimating the long-term effects of brief exposure to xanomeline on the level of intracellular calcium (Fig. 2) CHO cells expressing individual subtypes of muscarinic receptors were exposed to 10 μM xanomeline for 1, 3, or 10 min and intracellular calcium levels were measured for 1 hour under continuous superfusion with KHB to remove free xanomeline. Control 10-s stimulation with 300 nM carbachol was done before xanomeline application and at the end of measurements.

First (control) stimulation with 300 nM carbachol caused immediate mobilization of intracellular calcium at all subtypes of muscarinic receptors including hM_2 and hM_4 receptors (that were coupled to calcium response via transfection with the promiscuous G_{16} G protein α-subunit). After 4 mins of washing calcium levels returned to their basal values. Time needed to reach maximal response ranged from 6.2±0.3 s in case of M_2 receptors to 7.9±0.7 s at hM_5 receptors (Table S4 in File S1). The speed of calcium mobilization did not vary markedly among subtypes, but was slightly faster at hM_2 than hM_5 receptors. Thus, maximal calcium level elevation ranged from 1.47±0.04 to 1.68±0.09 fold of basal level at hM_5 and hM_3 receptors, respectively. It was the same at hM_1, hM_2 and hM_3 and was higher at these subtypes than at hM_4 and hM_5 subtypes.

Stimulation with 10 μM xanomeline (lasting 1, 3 or 10 min) led to a fast increase in intracellular calcium at all muscarinic receptor subtypes. Unlike carbachol (control) stimulation, the speed of calcium mobilization and maximum calcium level elevation varied among subtypes. The response was fastest at hM_1 receptors (time to reach maximum 9.6±1.7 s) and slowest at hM_5 receptors (time to reach maximum 39±6 s). Xanomeline caused the strongest response at the hM_1 receptor, increasing the calcium level to 118±3% of preceding control stimulation by carbachol. At hM_3 and hM_4 receptors the magnitude of response was the same as the response to carbachol (103±5 and 92±6% of response to carbachol, respectively). At hM_2 and hM_5 receptors the magnitude of xanomeline-induced calcium mobilization was about half of that induced by carbachol. After quickly reaching peak value intracellular calcium levels declined immediately despite ongoing xanomeline perfusion at all receptor subtypes. Cells expressing hM_1, hM_3 and hM_4 receptors treated with xanomeline for 1, 3 or 10 min followed by washing showed increased calcium level after 60 min washing with KHB. At hM_2 receptors, only 10-min xanomeline treatment increased calcium level after 60 min washing and at hM_5 receptors calcium level returned to its original values even after 10 min xanomeline treatment. Elevated calcium levels at hM_1 and hM_4 receptors showed oscillations that did not appear at hM_2 and hM_3 receptors (Fig. 2).

Application of 300 nM carbachol for 5 s after exposure to xanomeline and washing still caused fast mobilization of intracellular calcium at all subtypes except for M_1 (all treatments with xanomeline) and hM_4 (10-min treatment with xanomeline) where calcium levels remained markedly increased after xanomeline stimulation. Xanomeline pretreatment followed by washing slowed down the speed of calcium mobilization and decreased the magnitude of the calcium signal by carbachol (Fig. 2; parameters are summarized in Table S4 in File S1). These effects were most prominent at hM_3 receptors where time to reach maximum level was more than doubled and the maximal responses were close to half of the first stimulation.

Effects of 1-hour exposure to the agonists carbachol, oxotremorine and pilocarpine on intracellular calcium level

In microflourometric experiments measuring effects of long exposure to the agonists carbachol, oxotremorine and pilocarpine on the level of intracellular calcium (Fig. 3) CHO cells expressing individual subtypes of muscarinic receptors were exposed to 1 μM carbachol, 1 μM oxotremorine or 3 μM pilocarpine for 1 hour. Intracellular calcium levels were measured during agonist exposure

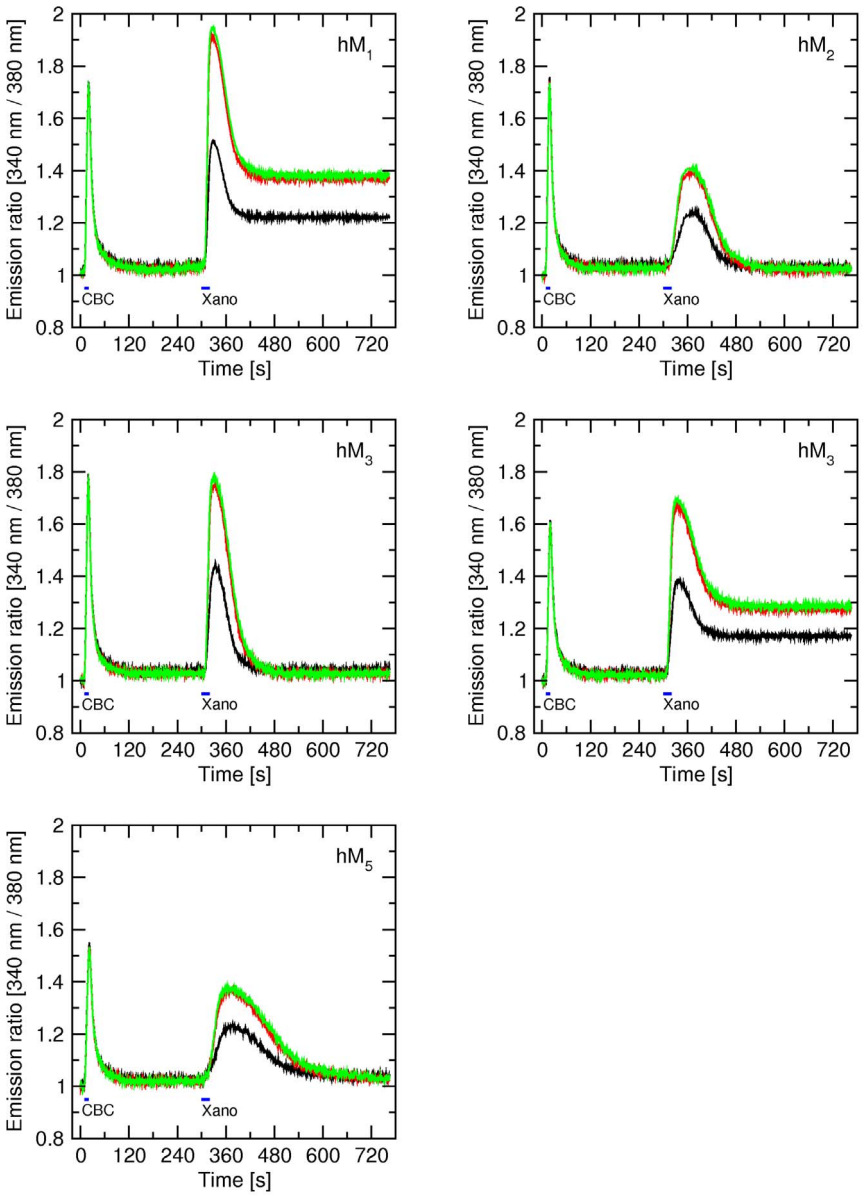

Figure 1. Concentration response to acute treatment with xanomeline. Cells were seeded, handled and loaded with Fura-2 as described in Methods. After an initial 10-s period cells were stimulated with 300 nM carbachol (CBC) for 5 s, washed with KHB for 5 min, then stimulated with 0.1 (black), 1 (red) or 10 μM (green) xanomeline (Xano) for 20 s and washed with KBH for 7 min. Traces are averages from 10 to 12 cells from representative experiment confirmed by 3 independent experiments. Signal variation (SD) among cells ranges from ±0.019 at the base line to ±0.035 at peaks. Parameters of calcium response are summarized in Table S2 in File S1. Calculated pEC_{50} and E_{MAX} of response to xanomeline are in Table 1.
doi:10.1371/journal.pone.0088910.g001

and following 30-min of continuous superfusion with KHB. Control 5-s stimulation with 300 nM carbachol was done before agonist application and at the end of measurements.

First (control) stimulation with 300 nM carbachol caused immediate mobilization of intracellular calcium, similar to the effects of xanomeline (Table S5 in File S1). One-hour stimulation with 1 μM carbachol, 1 μM oxotremorine or 3 μM pilocarpine caused transient increase in intracellular calcium level. During 1-hour carbachol stimulation (Fig. 3, black traces) a transient increase in intracellular calcium level lasted about 3 min and returned to the basal level at all receptor subtypes except M_1 where it remained slightly elevated (2.5% of peak value) until the end of carbachol stimulation. During oxotremorine stimulation (Fig. 2, red traces) intracellular calcium level transiently increased for about 4 min (hM_1 and hM_2), 5 min (hM_3) or 15 min (hM_4 and hM_5 receptors). After this transient increase intracellular calcium level remained elevated until the end of stimulation. Steady increased levels of intracellular calcium ranged from 8% at M_5 to 16% at M_1 receptors. During pilocarpine stimulation (Fig. 3, blue traces) a transient increase in intracellular calcium was observed that in about 3 min returned to basal level (hM_2 and hM_4) or elevated level (hM_1 and hM_3). Elevated level at hM_1 and hM_3 receptors represented 10% and 14% of peak value of initial transient increase, respectively. In case of hM_5 receptor the transient increase and return to the steady elevated level (16% of peak value) was slow and took about 30 min.

Immediately after 1-hour treatment with the agonists carbachol, oxotremorine and pilocarpine cells did not respond to 300 nM

Table 1. Potency and efficacy of agonists carbachol, oxotremorine, pilocarpine and xanomeline of intracellular calcium response.

	carbachol		oxotremorine		pilocarpine		xanomeline	
	pEC_{50}	E_{MAX} [Emission ratio]	pEC_{50}	E_{MAX} [Emission ratio]	pEC_{50}	E_{MAX} [Emission ratio]	pEC_{50}	E_{MAX} [Emission ratio]
hM_1	6.92±0.07	2.04±0.08	7.33±0.06	1.65±0.05	7.02±0.05	1.65±0.05	7.1±0.1	1.94±0.08
hM_2	6.71±0.05	2.26±0.09	7.19±0.05	1.71±0.06	6.95±0.07	1.66±0.05	7.1±0.1	1.42±0.04
hM_3	6.80±0.06	2.20±0.09	7.33±0.05	1.68±0.06	6.97±0.05	1.68±0.06	7.1±0.1	1.78±0.06
hM_4	6.65±0.07	2.06±0.08	7.17±0.06	1.64±0.05	7.15±0.07	1.60±0.05	7.1±0.1	1.70±0.06
hM_5	6.81±0.05	1.85±0.07	7.29±0.05	1.62±0.05	7.00±0.05	1.56±0.05	7.1±0.1	1.37±0.04
			E_{MAX} [% of carbachol E_{MAX}]	Efficacy [K_G]	E_{MAX} [% of carbachol E_{MAX}]	Efficacy [K_G]	E_{MAX} [% of carbachol E_{MAX}]	Efficacy [K_G]
hM_1			63	0.93	63	0.93	0.90	5.22
hM_2			56	1.00	52	0.85	0.33	0.69
hM_3			57	0.75	57	0.74	0.65	1.06
hM_4			60	1.64	57	1.41	0.66	2.09
hM_5			73	2.7	66	1.93	0.44	0.77

Potency is expressed as pEC_{50} (negative logarithm of half-efficient concentration), maximum increase E_{MAX} is expressed as ratio of emissions at 340 to 380 nm excitations and efficacy is expressed as per cent of carbachol E_{MAX} (maximum increase) and as G protein apparent affinity constant K_G. Parameters were obtained by fitting equation Eq. 1 to the data from individual experiments in Fig. 1 (xanomeline) and the Fig. S1 in File S1 (carbachol, pilocarpine and oxotremorine) and subsequently by Eq. 3 using expression data from Table 2. Including Hill coefficient (slope factor) in the Eq. 1 does not improve the fit. Data are means±S.E.M. from 3 independent experiments.

doi:10.1371/journal.pone.0088910.t001

Table 2. Maximum binding capacities (B_{MAX}) of [^{3}H]NMS binding to the membranes of the cells treated with xanomeline, carbachol, oxotremorine or pilocarpine are expressed as pmol of binding sites per mg of membrane protein.

	hM_1	hM_2	hM_3	hM_4	hM_5
10-min washing					
control	1.80±0.03	1.29±0.03	1.75±0.03	0.928±0.023	0.998±0.021
xano 1-min	1.73±0.12	1.29±0.03	1.78±0.05	0.890±0.023	0.969±0.005
xano 3-min	1.86±0.12	1.34±0.02	1.79±0.09	0.898±0.097	0.973±0.035
xano 10-min	1.76±0.09	1.29±0.03	1.69±0.05	0.979±0.055	0.966±0.047
carbachol	1.34±0.02*	0.746±0.036*	1.34±0.01*	0.561±0.016*	0.802±0.012*
oxotremorine	1.54±0.12*	0.931±0.029*	1.46±0.02*	0.672±0.038*	0.833±0.035*
pilocarpine	1.64±0.02*	1.11±0.03*	1.62±0.02*	0.815±0.035*	0.941±0.013*
1-hour washing					
control	1.58±0.03b	1.22±0.02b	1.55±0.03b	0.775±0.021b	0.857±0.012b
xano 1-min	1.58±0.07b	1.23±0.05b	1.60±0.03b	0.818±0.033	0.866±0.014b
xano 3-min	1.64±0.05b	1.22±0.05b	1.46±0.07b	0.760±0.057b	0.843±0.040b
xano 10-min	1.54±0.03b	1.20±0.02b	1.61±0.03b	0.766±0.009b	0.862±0.012b
carbachol	1.31±0.02*	0.696±0.009*b	1.21±0.02*b	0.460±0.007*b	0.661±0.007*b
oxotremorine	1.54±0.09	1.11±0.05*b	1.36±0.12*	0.722±0.039	0.827±0.021
pilocarpine	1.60±0.09	1.16±0.03*b	1.58±0.07	0.761±0.029	0.838±0.035

Intact cells were exposed to 10 μM xanomeline for 1, 3 or 10 min or for 10 min to 1 μM carbachol, 1 μM oxotremorine or 3 μM pilocarpine or sham-treated (control) and washed with KHB for 10 min or 1 hour and then membranes were prepared as described in Methods. *, different from control, [a], different from shorter treatment with xanomeline, [b], different from 10-min washing, $P<0.05$ by ANOVA and Tukey-Kramer post-test. Data are average values ± S.E.M. from 3 independent measurements performed in triplicates. Binding curves are in Fig. S3 in File S1.

doi:10.1371/journal.pone.0088910.t002

carbachol stimulation (data not shown). Response E_{MAX} to the third stimulation (300 nM carbachol) carried after 30-min washing with KHB (following 1-hour application of agonists) was diminished after carbachol treatment at all receptor subtypes (Fig. 3, Table S5 in File S1). Maximal response of the third stimulation was also diminished at hM_1 receptors after oxotremorine and pilocarpine treatment. Response of hM_5 receptors was completely abolished after pilocarpine treatment.

Effects of xanomeline treatment on the number of membrane receptors

The number of membrane receptors was determined in [^{3}H]NMS saturation binding of membranes prepared from cells treated with xanomeline for 1, 3 or 10 min (Fig. S3 A in File S1). To simulate conditions in microfluorometric experiments membranes were prepared 10 min or 1 hour after treatment of intact cells with xanomeline. Xanomeline treatment decreased the affinity of [^{3}H]NMS to all receptor subtypes under every condition (Table 3) but did not change the number of membrane receptors at any receptor subtype under any condition (Table 2). Xanomeline-induced decrease in the affinity of [^{3}H]NMS was largest at hM_4 (25-fold decrease after 10-min treatment) and smallest at hM_2 (2.5-fold decrease) receptors. In contrast, 10-min treatment of the cells with 1 μM carbachol, 1 μM oxotremorine or 3 μM pilocarpine (Fig. 3B) had no effect on [^{3}H]NMS affinity at any receptor subtype (Table 3) but decreased the number of membrane receptors (Table 2). Carbachol decreased the number of membrane receptors by 20% at hM_5, about 25% at hM_1 and hM_3 and about 40% at hM_2 and hM_4 receptors. In general, oxotremorine and pilocarpine decreased the number of membrane receptors to a lesser extent. Extension of cell washing in KHB from 10 min to 1 hour led to a decrease in the number of membrane receptors even under control conditions (sham treatment without agonist). There was no change in the number of any of the receptor subtypes as a result of xanomeline treatment followed by washing for 1 hour. Treatment with carbachol reduced the number of membrane receptors by the same extent at all receptor subtypes except hM_1 where 26% decrease in receptor number after 10-min washing fell to 17% after 1-hour washing. Similarly, the relative decrease in the number of membrane receptors (with respect to corresponding control) after oxotremorine treatment was smaller after 1-hour washing than after 10-min washing. There was no decrease in the number of membrane receptors after pilocarpine treatment followed by 1-hour washing. One-hour washing after treatment with carbachol, oxotremorine or pilocarpine had no effect on [^{3}H]NMS affinity. Reduction in [^{3}H]NMS affinity after 10-min treatment with xanomeline at hM_4 receptors was the same after 10-min and 1-hour washing. Reduction in [^{3}H]NMS affinity after 1-min and 3-min treatment with xanomeline at hM_4 receptors became stronger during 1-hour washing. At hM_3 receptors the reduction in [^{3}H]NMS affinity became stronger during 1-hour washing. In contrast, the reduction in [^{3}H]NMS affinity became weaker at the remaining receptor subtypes.

Effects of blockade of the receptor orthosteric binding site on calcium level elevated by xanomeline

Prior to actual measurement of the effects of NMS on calcium levels elevated by xanomeline (Fig. 4) control stimulation by 300 nM carbachol for 5 s was done. After 5 min of washing with KHB, 20-s stimulation with 10 μM xanomeline was done. Cells were washed for two mins and then 10 μM NMS was applied for 2 min followed by washing in drug-free buffer to visualize the effects of xanomeline bound in a wash-resistant manner. Characteristics of immediate effects of carbachol and xanomeline on calcium responses (Table S6 in File S1) served as internal controls and were similar to those described above.

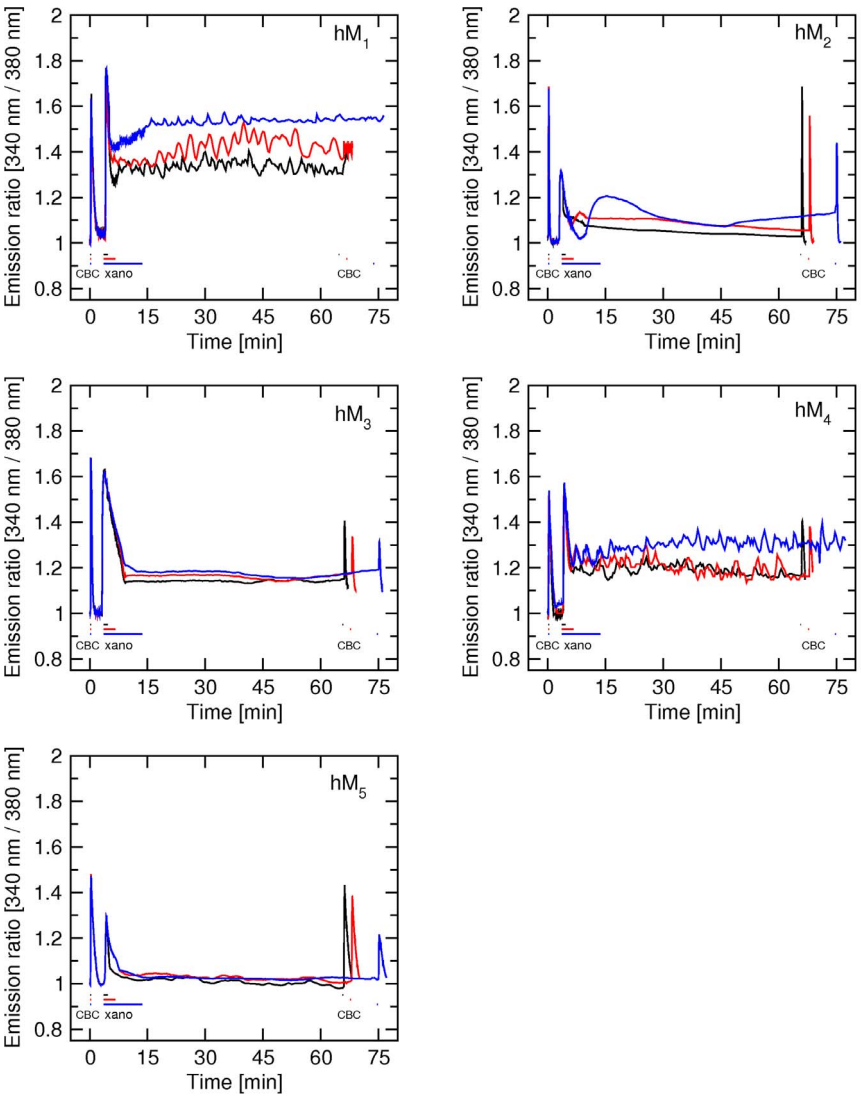

Figure 2. Effects of short-term application of xanomeline on the time-course of changes in intracellular calcium concentration in CHO cells expressing individual subtypes of muscarinic receptors. The time-course of intracellular calcium concentration (abscissa) after stimulation of hM_1 to hM_5 muscarinic receptor subtypes with the agonists carbachol (CBC) and xanomeline was measured as described in Methods. First stimulation: After 10 s of initial (resting) period 300 nM carbachol was applied for 10 s and then washed. Second stimulation: Three min after the first stimulation 10 μM xanomeline was applied for 1 min (black curve), 3 min (red curve) or 10 min (blue curve) followed by washing. Third stimulation: One hour after the second stimulation 300 nM carbachol was applied for 10 s followed by washing. Intracellular calcium concentration (ordinate) is expressed as fluorescence intensity (340 nm/380 nm) ratio normalized to basal calcium level. Representative traces are averages of 8 to 12 best responding cells from one experiment. Signal variation (SD) among cells ranges from ±0.017 at the base line to ±0.063 at peaks. Results were confirmed in 5 additional independent experiments. Parameters of xanomeline effects are summarized in Table S4 in File S1.
doi:10.1371/journal.pone.0088910.g002

Application of 10 μM NMS brought increased calcium levels persisting after xanomeline exposure and washout to their basal levels at all subtypes. After switching back to perfusion with KHB calcium levels rose again at hM_1 and hM_4 but not at hM_2 and hM_3 receptors. In case of hM_1 receptors an overshoot above steady state level appeared (Fig. 4, black trace, third peak). Time to reach maximum level after washing out NMS was several times shorter in case of the M_1 receptor than in case of the hM_4 receptor (Fig. 4, Table S6 in File S1). Increased steady state calcium levels after NMS withdrawal were similar at these two receptor subtypes and remained elevated during the following 1 hour of washing (not shown).

Effects of NMS on formation of xanomeline wash-resistant activation

In another set of experiments the effects of the antagonist NMS on the formation of xanomeline wash-resistant receptor activation were investigated. Five mins after 5-s control stimulation with 300 nM carbachol, cells were superfused for 3 min with 10 μM NMS. Xanomeline was applied for 1 min at 10 μM (together with NMS) during the second min of NMS superfusion (Fig. 5, Table S7 in File S1).

NMS decreased basal level of calcium signal by 4.5% at hM_1 receptors (Fig. 5, black trace) but did not cause any changes in intracellular calcium level at other receptor subtypes. Xanomeline applied concurrently with NMS had no immediate effect on

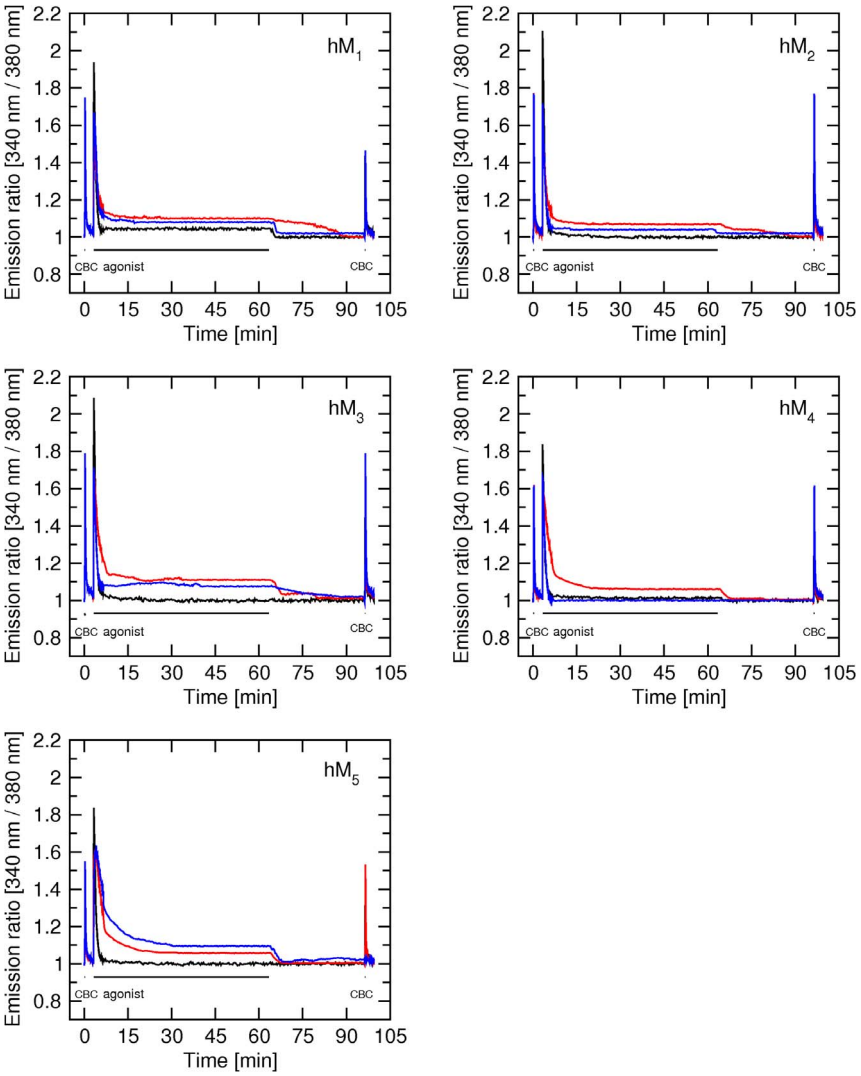

Figure 3. Effects of long-term application of classic agonists on the time-course of changes in intracellular calcium concentration in CHO cells expressing individual subtypes of muscarinic receptors. The time-course of changes in intracellular calcium concentration (abscissa) after stimulation of hM_1 to hM_5 muscarinic receptor subtypes with the agonists carbachol (CBC), oxotremorine and pilocarpine was measured as described in Methods. First stimulation: After 10 s of initial (resting) period 300 nM carbachol was applied for 10 s and then washed. Second stimulation: Three min after the first stimulation either 1 μM carbachol (black curve) or 1 μM oxotremorine (red curve) or 3 μM pilocarpine was applied for 1 hour followed by 30-min washing. Third stimulation: After washing following the second stimulation 300 nM carbachol was applied for 10 s followed by washing. Intracellular calcium concentration (ordinate) is expressed as fluorescence intensity (340 nm/380 nm) ratio normalized to basal calcium level. Representative traces are averages of 12 to 16 best responding cells from one experiment. Signal variation (SD) among cells ranges from ±0.018 at the base line to ±0.067 at peaks. Results were confirmed in 2 additional independent experiments. Parameters of agonist effects are summarized in Table S5 in File S1.
doi:10.1371/journal.pone.0088910.g003

calcium signal. However, removal of NMS during the final washing with fresh KHB (Fig. 5, from 480 s on) caused elevation of calcium level in cells expressing hM_1 and hM_3 receptors. Thus, NMS did not prevent formation of xanomeline wash-resistant binding at these subtypes and its removal unmasked activation by wash-resistant xanomeline. This unmasked activation persisted for the next 1 hour (not shown). A similar treatment protocol with xamomeline and NMS followed by washing did not restore activation of hM_2 and hM_4 receptors (Fig. 5, red and blue traces). Thus, NMS prevented the formation of xanomeline wash-resistant receptor activation at hM_2 and hM_4 receptor subtypes but not at hM_1 and hM_3 subtypes.

Effects of NMS on formation of xanomeline wash-resistant action at hM_5 receptors

Effects of the antagonist NMS on the formation of xanomeline wash-resistant binding were tested in a separate set of experiments at M_5 receptors since xanomeline did not produce long-term elevated calcium level at this receptor subtype under any experimental conditions. After control stimulation with 300 nM carbachol for 5 s and 5 min of washing with KHB cells expressing M_5 receptors were treated with NMS and xanomeline in the same way as in the previous set of experiments, except that exposure to the mixture of xanomeline and NMS was extended to 10 min. Cells were then perfused with KHB for 1 hour and stimulated with 300 nM carbachol for 5 s (Fig. 6). The latter second

Table 3. Equilibrium dissociation constants (K_D) of [^{3}H]NMS binding to the membranes of the cells treated with xanomeline, carbachol, oxotremorine or pilocarpine is expressed in nM.

	hM_1	hM_2	hM_3	hM_4	hM_5
10-min washing					
control	0.264±0.010	0.356±0.014	0.239±0.004	0.229±0.008	0.302±0.003
xano 1-min	2.11±0.10*	0.551±0.001*	0.717±0.021*	2.64±0.05*	1.44±0.02*
xano 3-min	2.44±0.18*	0.575±0.013*	0.863±0.037*a	3.67±0.36*a	1.79±0.04*a
xano 10-min	2.59±0.10*	0.894±0.030*a	0.899±0.017*	5.74±0.20*a	2.58±0.13*a
carbachol	0.255±0.006	0.348±0.015	0.226±0.003	0.235±0.004	0.294±0.008
oxotremorine	0.261±0.007	0.359±0.011	0.234±0.003	0.216±0.005	0.295±0.004
pilocarpine	0.248±0.006	0.384±0.014	0.236±0.004	0.217±0.004	0.288±0.011
1-hour washing					
control	0.248±0.006	0.367±0.009	0.232±0.003	0.220±0.011	0.312±0.006
xano 1-min	0.821±0.004*b	0.579±0.026*	1.34±0.01*b	3.45±0.15*b	1.08±0.01*b
xano 3-min	0.863±0.015*ab	0.589±0.019*	1.32±0.03*b	4.61±0.30*ab	1.45±0.02*ab
xano 10-min	0.915±0.014*ab	0.680±0.003*ab	1.67±0.09*ab	5.81±0.17*a	1.84±0.02*ab
carbachol	0.239±0.006	0.360±0.003	0.231±0.003	0.228±0.006	0.301±0.006
oxotremorine	0.241±0.004	0.365±0.007	0.226±0.009	0.227±0.005	0.297±0.009
pilocarpine	0.242±0.003	0.355±0.003	0.246±0.011	0.224±0.007	0.321±0.015

Intact cells were exposed to 10 μM xanomeline for 1, 3 or 10 min or for 10 min to 1 μM carbachol, 1 μM oxotremorine or 3 μM pilocarpine or sham-treated (control) and washed with KHB for 10 min or 1 hour and then membranes were prepared as described in Methods. *, different from control, [a], different from shorter treatment with xanomeline, [b], different from 10-min washing, $P<0.05$ by ANOVA and Tukey-Kramer post-test. Data are average values ± S.E.M. from 3 independent measurements performed in triplicates. Binding curves are in Fig. S3 in File S1.

doi:10.1371/journal.pone.0088910.t003

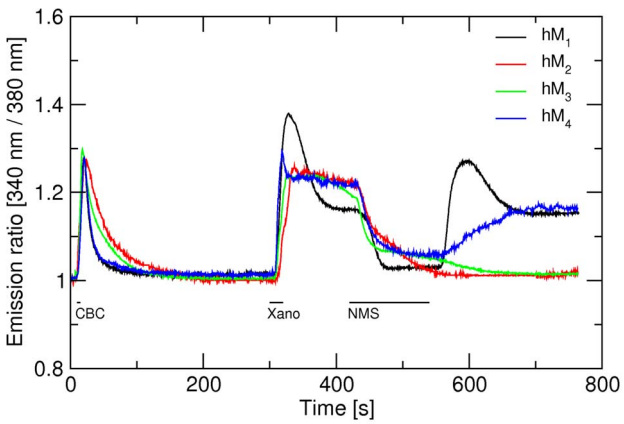

Figure 4. Effects of NMS on delayed elevation of intracellular calcium levels induced by short-term application of xanomeline at hM_1 through hM_4 receptors. Changes in the concentration of intracellular calcium (ordinate) are expressed as changes in fluorescence intensity (340 nm/380 nm) ratio normalized to basal calcium level. First (control) stimulation: 300 nM carbachol (CBC) for 5 s was applied. Second stimulation: At 300 s stimulation with 10 μM xanomeline (Xano) was applied for 20 s. After 2-min washing with KHB cells were superfused with 10 μM NMS for 2 min and then washed with KHB for additional 4 min. Traces are averages of 8 to 12 best responding cells from one experiment. Signal variation (SD) among cells ranges from ±0.015 at the base line to ±0.037 at peaks. Results were confirmed in 5 additional independent experiments. Parameters of xanomeline effects are summarized in Table S6 in File S1.

doi:10.1371/journal.pone.0088910.g004

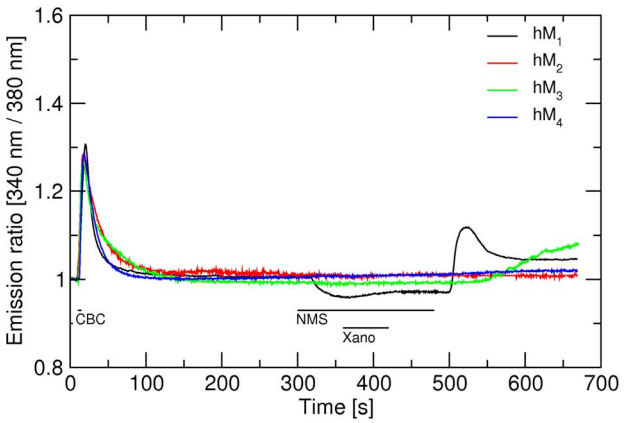

Figure 5. Effects of NMS on formation of xanomeline wash-resistant activation at hM_1 through hM_4 receptors. Changes in the concentration of intracellular calcium (ordinate) are expressed as changes in normalized fluorescence intensity (340 nm/380 nm) ratio normalized to basal calcium level. First (control) stimulation: Control 300 nM carbachol (CBC) was applied for 5 s. Second stimulation: At 5 min receptors were blocked by 10 μM of the antagonist NMS (1 min), then a mixture of 10 μM xanomeline (Xano) and 10 μM NMS was applied for 1 min and then 10 μM NMS was applied for an additional 1 min. Cells were then washed with KHB for additional 3 min. Representative traces are averages of 8 to 12 best responding cells from one experiment. Signal variation (SD) among cells ranges from ±0.015 at the base line to ±0.033 at peaks. Results were confirmed in 5 to 7 additional independent experiments. Parameters of xanomeline effects are summarized in Table S7 in File S1.

doi:10.1371/journal.pone.0088910.g005

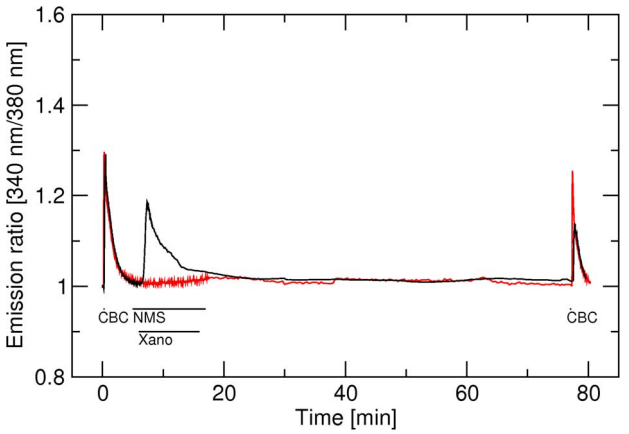

Figure 6. Effects of NMS on the formation of xanomeline wash-resistant action at hM_5 receptors. Changes in the concentration of intracellular calcium (ordinate) are expressed as changes in normalized fluorescence intensity (340 nm/380 nm) ratio normalized to basal calcium level. Red trace: First stimulation: Control 5-s stimulation with 300 nM carbachol (CBC) was performed. Second stimulation: At 5 min receptors were blocked by 10 μM of the antagonist NMS (1 min), then a mixture of 10 μM xanomeline (Xano) and 10 μM NMS was applied for 10 min and finally 10 μM NMS wash- applied for an additional 1 min. Third stimulation: After washing of the cells with KHB for 60-min 300 nM carbachol was applied for 5 s. Black trace: Control curve, same as red one but NMS was not applied. Representative traces are averages of 8 to 12 best responding cells from one experiment. Signal variation (SD) among cells ranges from ±0.015 at the base line to ±0.032 at peaks. Results were confirmed in 5 additional independent experiments.
doi:10.1371/journal.pone.0088910.g006

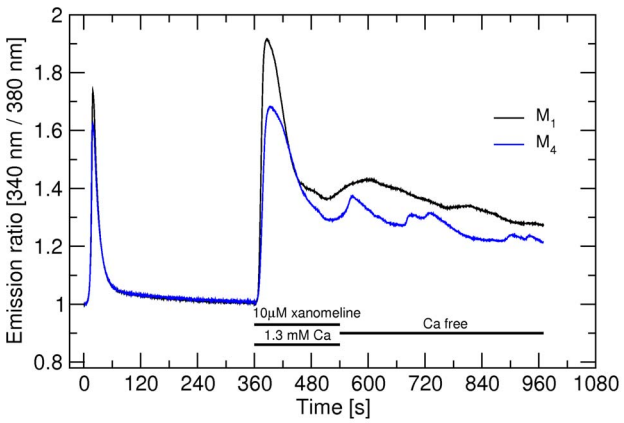

Figure 7. Lack of effects of changing extracellular calcium on calcium oscillations. Changes in the concentration of intracellular calcium (ordinate) are expressed as changes in normalized fluorescence intensity (340 nm/380 nm) ratio normalized to basal calcium level. Black trace: M_1 receptors, blue trace: M_4 receptors. First stimulation: Control 5-s stimulation with 300 nM carbachol (CBC) was performed. Second stimulation: After 6 min washing with KHB receptors were stimulated with 10 μM xanomeline for 3 min and then washed with calcium free KHB. Representative traces are averages of 12 best responding cells from one experiment. Signal variation (SD) among cells ranges from ±0.015 at the base line to ±0.063 at peaks. Results were confirmed in 2 additional independent experiments.
doi:10.1371/journal.pone.0088910.g007

stimulation led to slightly smaller and slower response compared to the control carbachol response ($P<0.05$ in paired t-test). This is in sharp contrast to the marked antagonism caused by wash-resistant xanomeline in the absence of NMS. These data indicate that NMS blocks the formation of xanomeline wash-resistant blockade of hM_5 receptors.

Lack of effects of changing extracellular calcium on calcium oscillations induced by xanomeline

Regardless of the expressed subtype of muscarinic receptor CHO cells responded to 1 μM carbachol even in KHB where the concentration of calcium was lowered to 0.65 mM and even in calcium-free KHB (Fig. S4 in File S1). In reduced calcium KHB intacellular calcium peaks were lower than at normal calcium KHB and were even lower in calcium-free medium. Basal level of intracellular calcium was also reduced at the end of 12-min measurements. These data indicate that upon stimulation by carbachol calcium is released principally from intracellular stores and the decrease in peaks is likely due to depletion of intracellular stores. To test the possible role of extracellular calcium in xanomeline-induced oscillation in intracellular calcium at M_1 and M_4 receptors cells were stimulated for 3 min with 10 μM xanomeline and then washed with calcium-free KHB (Fig. 7). Washing cells with calcium-free KHB did not prevented oscillations in the intracellular calcium.

Effects of xanomeline on accumulation of cAMP

Accumulation of [^{3}H]cAMP stimulated by 5 or 20 μM forskolin in cells expressing M_2 or M_4 receptors was measured after treatment of the cells with 10 μM xanomeline for 3 min followed by 10-min or 1-hour washing (Fig. 8). Xanomeline treatment had minimal effects on accumulation of [^{3}H]cAMP in cells expressing M_2 receptors under this experimental setup. After 10 min of washing xanomeline slightly (8%) inhibited [^{3}H]cAMP accumulation (stimulated by 20 μM forskolin) but it had no effect on [^{3}H]cAMP accumulation after 1-hour washing. In cells expressing M_4 receptors xanomeline inhibited [^{3}H]cAMP accumulation by almost 40% after 10-min washing and by more than 20% after 1-hour washing (Fig. 8).

Discussion

The major finding of this study is that xanomeline functional preference for M_1 and M_4 muscarinic receptors originates at the receptor level. Xanomeline is one of few muscarinic agonits that is functionally preferring for M_1 and M_4 muscarinic acetylcholine receptors [7,10]. Xanomeline exerts unusual pharmacological properties. Besides the reversible binding to and activation of muscarinic receptors it also binds to these receptors in a way that is resistant to intensive washing and is associated with persistent receptor activation [16]. Despite growing experimental data on the molecular mechanisms [19] and kinetics [20] of xanomeline binding and receptor activation, the basis of xanomeline functional preference remains enigmatic. Only indirect evidence from *in vivo* and behavioral experiments supports xanomeline selectivity [7,10]. In contrast, xanomeline activates all subtypes of muscarinic receptors with the same potency [20,23,24] (Fig. 1 and Table 1), and the affinity of xanomeline reversible as well as wash-resistant binding is the same at all receptor subtypes [25]. So far, the only observed qualitative exception from uniform behavior of xanomeline at muscarinic receptors is its wash-resistant functional antagonism at M_5 receptors [22]. The fundamental question where xanomeline selectivity *in vivo* comes from remains unanswered. Three possibilities may be considered, where xanomeline functional selectivity may be based on: a) pharmacodynamics (receptor level); b) differential receptor regulation (cell level) [24,26]; c) pharmacokinetics (system level).

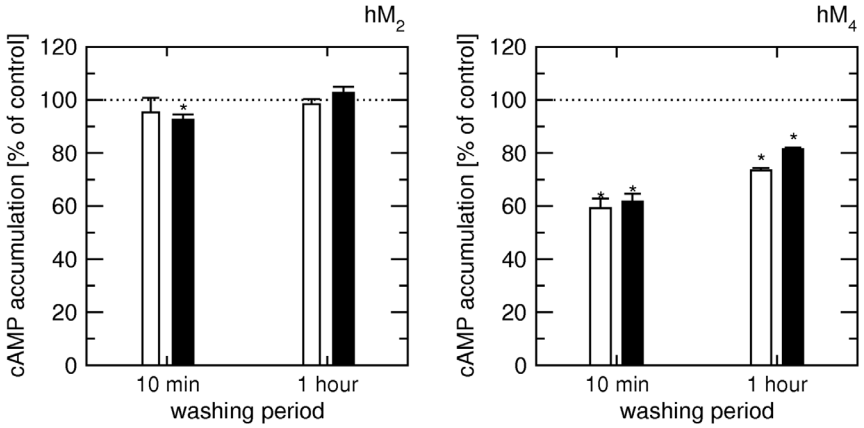

Figure 8. Effects of xanomeline on the accumulation of cyclic AMP. Cells expressing hM_2 (left) or hM_4 (right) receptors were treated with 10 μM xanomeline for 3 min followed by washing for 10 min or 1 hour (coordinate) prior to 20-min incubation with 5 μM (white bars) or 20 μM (black bars) forskolin. Accumulation of [^{3}H]cAMP (ordinate) is expressed as per cent of control accumulation of [^{3}H]cAMP in xanomeline sham treated cells (corrected for content of protein). Data are means ± S.E.M. from 3 experiments performed in triplicates. *, different from control (sham treated) cells by t-test, $P<0.05$.
doi:10.1371/journal.pone.0088910.g008

Experimental setup

We employed fast microfluorimetric measurements of intracellular calcium levels that, unlike measurements of accumulation of second messengers (e.g. cyclic nucleotides or inositol phosphates), enabled us to observe potential fast short-term differences in the kinetics of receptor activation as well as long-term changes (both increase and decrease) in calcium signal reflecting potential differences in receptor activation and signal regulation. Only odd-numbered subtypes of muscarinic receptors directly elevate intracellular calcium levels via the $G_{q/11}$ G proteins, phospholipase Cβ and 1,4,5-inositoltrisphosphate pathway. Even-numbered muscarinic receptors preferentially inhibit cAMP formation via $G_{i/o}$ G proteins and changes induced in calcium level are slow and weak. To facilitate coupling of even-numbered receptors to the calcium-generating pathway we transiently transfected CHO cells with G_{16} G protein that links G protein coupled receptors to activation of phospholipase Cβ [29]. The coupling of hM_2 and hM_4 receptors was successful as evidenced by fast calcium response to carbachol that is similar to the response in odd-numbered subtypes (Fig. 1, Fig. S1, Table S3 in File S1). All five receptor systems responded to full non-selective agonist carbachol and partial agonists oxotremorine and pilocarpine in the same or very similar way proving the method to be applicable for detection of potential subtypes differences (Fig. S1, Table S3 in File S1). Moreover, xanomeline has the same affinity for all subtypes of muscarinic receptors and has similar potency at all these systems indicating the same coupling efficiency and no bias for xanomeline signal (Fig. 1, Table 1).

Effects of acute exposure to xanomeline

Exposure to xanomeline for 20 s elicits a transient response in intracellular calcium (Fig. 1). The observed similar potency of xanomeline to release intracellular calcium at all receptor subtypes (Table 1) is in accordance with uniform xanomeline affinity for all receptor subtypes [25] and previous findings on functional responses to xanomeline [23]. However, xanomeline maximal response and coupling efficacy varied among subtypes. When maximal responses are expressed as per cents of the maximal response of the full agonist carbachol the rank order of maximal values follows putative xanomeline functional selectivity, being highest at hM_1, intermediate at hM_3 and hM_4 and lowest at hM_5 and hM_2 receptors (Table 1). When receptor expression levels are taken into account and apparent affinity of G protein for agonist receptor complex K_G is calculated variations in xanomeline coupling efficacy become even more apparent (Table 1). In addition to higher maximal responses to xanomeline at hM_1 and hM_4 receptors, the calcium signal was longer lasting at these receptors compared to other subtypes (Fig. 1).Subtype differences in the coupling efficiency of xanomeline may thus be the basis of xanomeline functional selectivity. Coupling efficacy of oxotremorine and pilocarpine exhinits a different pattern from xanomeline and is highest at hM_5 and lowest at hM_3 receptors (Table 1). This excludes the possibility that coupling of hM_1 and hM_4 receptors to calcium signal is generally better in an agonist-independent manner.

Sustained activation of M_1 and M_4 receptors

At the hM_1, hM_3, and hM_4 subtypes, treatment with xanomeline as briefly as 1 min markedly elevated intracellular calcium, an effect that persisted for more than 1 hr after washing xanomeline (Fig. 2, black traces). In case of hM_1 and hM_4 receptors elevated calcium levels showed significant oscillation. Extended periods of calcium levels oscillating at levels higher than resting values indicate that these receptors are kept in an active conformation that overcome the efficiency of intracellular mechanisms responsible for sequestering free calcium. Lack of decrease in calcium level over extended period of time indicates that these receptors are not desensitized. Longer treatment with xanomeline was required to induce sustained elevated levels of intracellular calcium at hM_2 receptors. At hM_5 receptors xanomeline application induced only a transient increase in intracellular calcium concentration that depended on the length of treatment. The effects of the second application of carbachol were blocked by xanomeline treatment and washing at hM_1, hM_4 and hM_5 receptors. While at M_1 and hM_4 receptors xanomeline behaved as a competitive agonist (no decrease in elevated calcium level) it behaved as competitive antagonist at hM_5 receptors (no increase in calcium basal level). These data are in perfect fit with the observed functional preference of xanomeline for M_1 and M_4 receptors [7,10], with delayed action of wash-resistant xanomeline at M_2 receptors [20,21] and functional antagonism by wash-resistant xanomeline at M_5 receptors [22].

Possible signal bias

Although bias of individual agonists towards different signaling pathway has been described at muscarinic receptors [30] it cannot be fully accountable for observed effects as M_1 receptors couple to phospholipase Cβ via $G_{q/11}$ G proteins while M_4 receptors in our experiments couple via G_{16} G proteins. Importantly, intracellular calcium level during 1-hour treatment with carbachol is not substantially elevated at any receptor subtype but it is elevated during 1-hour treatment with the partial agonists oxotremorine and pilocarpine (Fig. 3). In contrast to the effects of xanomeline, the level of intracellular calcium upon treatment with these partial agonists was not significantly oscillating and was highest at M_5 and M_3 receptor. These observations rule out the possibility that high and oscillating levels of intracellular calcium after brief exposure to xanomeline is an artifact of M_1 and M_4 systems.

Role of receptor regulation

Recent data suggest that xanomeline functional preference could be based on differential regulation of muscarinic receptor subtypes [24,26]. It has been shown repeatedly that regulation of muscarinic receptors differs among receptor subtypes [31–33] and is agonist dependent [34]. Presumably, weaker and/or slower down-regulation of the signaling induced by xanomeline at one subtype could result in stronger signaling via this subtype over a prolonged period of time. Data in Tables 2 and 3 Fig. S3 in File S1, however, show that xanomeline (under our experimental conditions) forms wash-resistant binding and allosterically decreases affinity of NMS but does not cause internalization of any muscarinic receptors, unlike the full agonist carbachol and the partial agonists oxotremorine and pilocarpine (Tables 2 and 3 and Fig. S3 in File S1). Thus, sustained elevation of intracellular calcium level at only hM_1 and hM_4 receptors cannot be explained by different degrees of receptor internalization (to reduce xanomeline signal) and recycling (to gain responsiveness to carbachol). Sustained elevation of intracellular calcium level at only hM_1 and hM_4 receptors can neither be explained by higher degree of receptor desensitization at hM_2 and hM_3 as these receptors respond to agonist carbachol after activation by xanomeline better than hM_1 and hM_4 receptors.

Role of kinetics

Our previous studies [20] showed that the kinetics of formation of xanomeline wash-resistant activation of hM_2 receptors is much slower than that at hM_1 receptors and suggested that differences in kinetics of wash-resistant binding and subsequent receptor activation may be involved in xanomeline functional preference. However, the kinetics of xanomeline wash-resistant binding does not correlate with the functional preference of xanomeline for M_1 and M_4 receptors. Although kinetics of wash-resistant binding is fastest at M_1 receptors, it was equally fast at non-preferred M_5 receptors and preferred M_4 receptors (Table 2 and Fig. S3 A in File S1). Xanomeline wash-resistant binding further develops during 1-hour washing (Table 2 and Fig. S3 A (left vs. right) in File S1). Inhibition of NMS binding becomes weaker during 1-hour washing at preferred hM_1 receptor and becomes stronger at non-preferred hM_3 receptors (Table 3). Thus differential kinetics of xanomeline wash-resistant binding and activation cannot explain xanomeline preference for M_1 and M_4 activation.

Agonist specific interactions

Other possible explanations of xanomeline functional preference include a differential mode of interaction with the receptor, interaction with different domains on the receptor or a different mode of receptor activation. For this purpose we tested whether xanomeline wash-resistant activation can be blocked by the orthosteric antagonist NMS (Fig. 4) and whether formation of xanomeline wash-resistant activation (Fig. 5) or wash-resistant functional antagonism (Fig. 6) can be blocked by NMS. As shown in Fig. 4, elevated calcium level in the continued presence of xanomeline was diminished by NMS at all subtypes (decrease at time 430 to 550 s). While intracellular calcium rises again after washing of NMS at hM_1 and hM_4 receptors it remains at basal level at hM_2 and hM_3 receptors (Fig. 4; Table S6 in File S1). Among these 4 receptor subtypes NMS has the slowest binding kinetics at hM_3 receptors and the fastest at hM_2 receptors [35]. Although slow binding kinetics of NMS at hM_3 receptors can explain lack of increase in intracellular calcium after withdrawal of NMS at this receptor it contradicts with the fact that the decrease in calcium signal at this receptor after application of NMS is faster than at other subtypes, especially at hM_2 where the kinetics of NMS is fastest. Lack of rise in intracellular calcium level after NMS withdrawal at hM_2 receptors cannot be explained by binding kinetics of NMS (as NMS dissociation from hM_2 is faster than from hM_1 or hM_4 receptors) and in agreement with Fig. 1 and Fig. 2 demonstrate that 20-s exposure of M_2 receptors to 10 μM xanomeline is not sufficient for development of xanomeline wash-resistant activation.

When applied to receptors blocked by NMS xanomeline wash-resistant activation was reduced at hM_1 and hM_3 receptors (Fig. 5 black and green traces vs. Fig. 2 black traces; Table S7 vs. Table S4 in File S1) and completely blocked at hM_2 and hM_4 receptors (Fig. 5, red and blue traces). At hM_5 receptor wash-resistant antagonism of xanomeline on activation by carbachol was diminished (Fig. 6). Thus, although to a different extent, NMS slows down the formation of xanomeline wash-resistant action at all receptors.

The role of extracellular calcium

Absence of extracellular calcium does not affect muscarinic signaling indicating that persistent activation and oscillations observed at hM_1 and hM_4 receptors are not due to differential coupling to extracellular calcium influx at these subtypes. All cells responded well to carbachol even in calcium-free medium (Fig. S4 in File S1) demonstrating that the primary response to carbachol stimulation is independent from extracellular calcium. Similarly, washing the cells expressing hM_1 or hM_4 receptors with calcium-free KHB after xanomeline stimulation had no immediate effect on the prolonged increase in intracellular calcium and neither prevented calcium oscillations (Fig. 7). If this effect was due to extracellular (transmenbrane) calcium influx then removal of extracellular calcium would have immediate effects in reducing the calcium signal. Thus calcium oscillations observed only at hM_1 and hM_4 receptors are not due to coupling to extracellular calcium source. Taken together all five subtypes appear to couple to the same signaling pathway.

Non-selective properties of xanomeline

In contrast with previous findings of uniform (non-selective) properties of xanomeline (i.e. the same affinity of both reversible and wash-resistant xanomeline binding at the various receptor subtypes [25] and potency of reversible xanomeline to activate all receptor subtypes (Fig. 1), numerous differences in xanomeline short and long-term effects on muscarinic receptors were found in the present study. They include differences in kinetics of xanomeline action, differences in NMS obliteration of xanomeline wash-resistant action and differences in interaction between xanomeline and NMS. However, none of these differences correlates with the

observed functional preference of xanomeline for M_1 and M_4 receptors and thus cannot constitute the basis of xanomeline selectivity. The only principal difference among muscarinic receptor subtypes identified in this study that correlates with functional preference is variation in xanomeline efficacy at calcium signaling and the ability of wash-resistant xanomeline to keep M_1 and M_4 receptors in an active conformation over time. This is evidenced by persistent increase in intracellular calcium and, unlike at M_3 receptors, inability of carbachol to induce further increase in calcium level. The physiological relevance of sustained hM_4 receptor activation is supported by prolonged inhibition of accumulation of its natural second messenger cAMP that is absent at hM_2 receptors (Fig. 8).

Conclusions

Our results show uniform xanomeline potency in releasing intracellular calcium. In contrast, data demonstrate higher efficacy of xanomeline in calcium signaling and longer lasting responses at hM_1 and hM_4 receptors over the rest of the subtypes. Together, our data suggest the existence of a distinct activation mechanism at the hM_1 and hM_4 receptor subtypes.Taken together, the data presented herein answer the fundamental question of the origin of xanomeline selectivity observed *in vivo* and provide evidence that such preference is based on subtype differences in efficacy and long term activation and that is not due to differential receptor regulation at the cell level or in pharmacokinetic at a system level. However, further experiments are needed to delineate detailed molecular basis of xanomeline functional selectivity, most importantly the receptor domains involved.

Supporting Information

File S1 Portable document file containing results from control experiments and analytical data of Fig. 1 through 5 of the main manuscript.
(PDF)

Author Contributions

Conceived and designed the experiments: ES VD EEE JJ. Performed the experiments: ES JJ. Analyzed the data: ES JJ. Wrote the paper: ES VD EEE JJ.

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Supporting information:

Long-term activation upon brief exposure to xanomleline is unique to M_1 and M_4 subtypes of muscarinic acetylcholine receptors

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Summary

This supplementary information shows that all five cell lines used express muscarinic receptors at similar levels and that their response to the full agonist carbachol as well as the partial agonists oxotremorine and pilocarpine in elevation of intracellular calcium is uniform across receptor subtypes. Activation of all five subtypes leads to extracellular calcium independent release of calcium from intracellular stores. Thus the the calcium signal in this cellular model may serve for detection of potential subtype specific agonists. Xanomeline has the same binding affinity for all subtypes of muscarinic receptors and brief exposure to this drug has similar potency at all subtypes, indicating the same coupling efficiency and lack of system bias for the xanomeline signal. On the other hand different efficacies in elevation of intracellular calcium level at these cell lines indicates xanomeline functional selectivity among muscarinic receptor subtypes.

Methods

Methods were the same as in the main manuscript unless otherwise indicated.

Binding experiments on whole cells

Cells were seeded and grown in 24-well plates for each binding experiment independently. Subconfluent cells were washed twice with 1 ml of KHB and then incubated in KHB containing 10 µM xanomeline for 1, 3 or 10 min. Control cells were sham treated with KHB. Subsequently, cells were washed 3-times with 1 ml of KHB to remove free xanomeline and incubated in fresh KHB for another

10 min or one hour. After incubation the cells were cooled on ice and washed 3-times with 1 ml of KHB and then were labeled with 1 nM [^{3}H]NMS or [^{3}H]QNB. [^{3}H]NMS labeling lasted 20 min whereas labeling with [^{3}H]QNB was carried out overnight at 4°C. Non-specific binding was determined in the presence of 10 µM NMS or 10 µM QNB, respectively. After labeling cells were quickly washed 3-times with 1 ml of KHB and solubilized in 0.5 ml of 1M NaOH. Aliquots of 0.25 ml were mixed with 3 ml of Rotiszint liquid scintillator and radioactivity was measured in Microbeta scintillation counter (Wallac, Finland). Radioactivity was corrected according to protein amount determined colorimetrically on Wallac Victor 2 plate reader (Wallac, Finland).

Results

Receptor expression levels

Expression levels of muscarinic acetylcholine receptors were determined by binding of the radiolabeled antagonists [^{3}H]N-methylscopolamine ([^{3}H]NMS) and [^{3}H]-quinuclidinyl bezilate ([^{3}H]QNB). [^{3}H]NMS has a positive charge therefore does not penetrate membranes and in intact cells labels only receptors in the cell membrane. On the other hand, [^{3}H]QNB has no charge and is hydrophobic, therefore is able to penetrate membranes and labels extra- and intracellular receptors even in intact cells.

CHO cell lines used express muscarinic receptors at levels ranging from 29 to 111 fmol per mg of protein in membranes ([^{3}H]NMS binding) and from 24 to 184 fmol per mg of protein in total ([^{3}H]QNB binding (Table S1). One-hour washing with KHB (simulating conditions during microfluorometry) decreased the number of membrane as well as total receptors at all receptor subtypes, except for M_5 membrane receptors that remained unchanged.

Potency and efficacy of carbachol, oxotremorine and pilocarpine in changing intracellular calcium levels

CHO cells expressing individual subtypes of muscarinic receptors were stimulated with increasing concentrations of the full agonist carbachol or the partial agonists oxotremorine and pilocarpine and the level of intracellular calcium was measured by microflourometry. Potency (EC_{50}) and efficacy (E_{MAX}) were estimated by fitting Eq. 1 to the data from individual experiments. Numbers are means ± S.E.M. from 3 experiments.

Carbachol

The concentration of carbachol used was from 100 nM to 3 µM, stimulation lasted 5 s and cells were

washed for 6 min between stimulations (Fig. S1 A). The response to 3 µM carbachol was the same as to 1 µM carbachol indicating signal saturation (Table S3 A). Stimulation with 3 µM carbachol was repeated. The response to the second stimulation with 3 µM carbachol was smaller than the response to the first stimulation with the same concentration at all receptor subtypes indicating desensitization. To determine carbachol potency and efficacy the intracellular calcium response was measured for 3, 10 and 30 nM and 10 µM carbachol in similar experimental setup (Fig. S2 top). Carbachol potency was similar at all receptor subtypes (pEC_{50} from 6.92 ± 0.07 at M_1 receptors to 6.65 ± 0.07 at M_4 receptors) and efficacy (E_{MAX}) ranged from 2.26 ±0.09 at M_2 receptors to 1.85 ±0.07 at M_5 receptors (Table 1 in the main manusript).

Oxotremorine

The concentration of oxotremorine used was from 30 nM to 1 µM, stimulation lasted 10 s and cells were washed for 6 min between stimulations (Fig. S1 B). Prior to the first and after the last oxotremorine stimulation cells were stimulated with 300 nM carbachol for 5 s (non-desensitizing stimulation). The response to the last carbachol stimulation was the same as to the first indicating lack of desensitization to stimulation by oxotremorine (Table S3 B). The response to 300 nM and 1 µM oxotremorine was the same at M_1, M_3 and M_5 receptor subtypes indicating signal saturation at these subtypes. To determine oxotremorine potency and efficacy intracellular calcium response was measured for 1, 3 and 10 nM and 3 µM oxotremorine in similar experimental setup (Fig. S2 middle). Oxotremorine potency (pEC_{50}) ranged from 7.33 ± 0.06 at M_1 receptors to 7.17 ± 0.06 at M_4 receptors and efficacy was the same at all subtypes (E_{MAX} from 1.71 ±0.06 at M_2 receptors to 1.62 ±0.05 at M_5 receptors) (Table 1 in the main manuscript).

Pilocarpine

The concentration of pilocarpine used was from 100 nM to 3 µM, stimulation lasted 20 s and cells were washed for 8 min between stimulations (Fig. S1 C). Prior to the first and after the last pilocarpine stimulation cells were stimulated with 300 nM carbachol for 5 s (non-desensitizing stimulation). The response to the last carbachol stimulation was the same as to the first indicating lack of desensitization to stimulation by pilocarpine (Table S3 C). Response to 1 µM and 3 µM pilocarpine was the same at all receptor subtypes indicating signal saturation. To determine pilocarpine potency and efficacy intracellular calcium response was measured for 3, 10 and 30 nM and 10 µM pilocarpine in similar experimental setup (Fig. S2 bottom). Pilocarpine potency (pEC_{50} from 7.15 ± 0.07 at M_4 receptors to 6.95 ± 0.07 at M_2 receptors) and efficacy (E_{MAX} from 1.68 ±0.06 at M_3 receptors to 1.56 ±0.05 at M_5 receptors) were same at all subtypes.

Effect of agonists on [^{3}H]NMS binding to membranes

Exposure of the intact cells to 10 µM xanomeline for 1, 3 or 10 min does not cause receptor internalization as maximum binding capacity for [^{3}H]NMS remains unchanged (Fig. S3 A and Table 2 in the main manuscript). Only affinity of [^{3}H]NMS is decreased that may be explained by allosteric action of wash-resistant xanomeline on [^{3}H]NMS binding. Unlike xanomeline, 10 min exposure of the intact cells to 1 µM carbachol, 1 µM oxotremorine and 3 µM pilocarpine caused intermalization of all receptor subtypes as evidenced by decrease in maximum binding capacity for [^{3}H]NMS without change in affinity of [^{3}H]NMS (Fig. S3 A and Table 2 in the main manuscript).

Effect of changing the concentration of extracellular calcium

Cells were stimulated with 1 µM carbachol consecutively three-times for 5-s (Fig. S4). The first stimulation and subsequent washing were done under normal concentration of calcium (1.3 mM), the second one under reduced concentration of calcium (0.65 mM) and the third one in calcium-free medium. Cells responded in reduced calcium as well as calcium-free KHB at all receptor subtypes. This impliies that the source of receptor-induced elevation of cellular calcium is independent of extracellular sources, and is rather due to calcium release from the intracellular stores. Maxima of responses were slightly attenuated in reduced and calcium-free medium. This may be attributed to partial depletion of intracellular stores that cannot be replenished during and after stimulation at reduced calcium and calcium free conditions.

Tables

Table S1. Expression levels of individual subtypes of muscarinic receptors after 10-min and 1-hour incubation of the cells in KHB at room temperature.

	10 min		1 hour	
	^{3}H-NMS bindig [fmol / mg prot.]	^{3}H-QNB binding [fmol / mg prot.]	^{3}H-NMS binding [fmol / mg prot.]	^{3}H-QNB binding [fmol / mg prot.]
hM$_1$	37.0 ± 1.9	143 ± 7	28.8 ± 1.3*	107 ± 2*
hM$_2$	39.9 ± 4.8	48.1 ± 2.4	30.9 ± 0.9*	37.0 ± 3.3*
hM$_3$	111 ± 13	184 ± 7	89.9 ± 6.3*	162 ± 6*
hM$_4$	12.5 ± 0.6	23.9 ± 1.4	8.15 ± 1.20*	18.2 ± 0.7*
hM$_5$	29.5 ± 1.7	79.5 ± 4.8	19.6 ± 0.5	59.7 ± 1.8*

Expression level of muscarinic receptors is determined as ^{3}H-NMS (membrane receptors) and ^{3}H-QNB (all receptors) binding to intact cells and expressed in fmol of specifically bound radioligand per mg of protein. Data are averages ± S.E.M. from 4 to 8 independent experiments performed in triplicates. *, significantly different from 10-min treatment, $P<0.05$ by t-test, data from individual experiments paired.

Table S2. Parameters of calcium level changes upon acute exposure to xanomeline in Fig. 1 in the main manuscript.

	0.1 µM xanomeline		1 µM xanomeline		10 µM xanomeline	
	TTM [s]	E_{MAX} [Emission ratio]	TTM [s]	E_{MAX} [Emission ratio]	TTM [s]	E_{MAX} [Emission ratio]
hM_1	12 ± 1*	1.52 ± 0.04*	9.5 ± 0.8*	1.91 ± 0.06	9.1 ± 0.8	1.94 ± 0.07
hM_2	39 ± 8*	1.24 ± 0.03*	29 ± 7	1.39 ± 0.04	29 ± 6	1.41 ± 0.04
hM_3	15 ± 4*	1.44 ± 0.04*	11 ± 3	1.75 ± 0.06	11 ± 3	1.78 ± 0.06
hM_4	16 ± 3*	1.38 ± 0.03*	14 ± 2	1.67 ± 0.05	13 ± 2	1.70 ± 0.06
hM_5	47 ± 8*	1.23 ± 0.02*	39 ± 7	1.37 ± 0.03	35 ± 7	1.37 ± 0.03

Parameters are derived from experiments shown in Fig. 1 of the main manuscript. Data are means ± S.E.M. from 4 independent experiments; TTM, time to reach the maximum; *, different from 10 µM xanomeline by Dunnett's test, $P<0.05$.

Table S3. Parameters of calcium level changes upon exposure to agonists in Fig. S1.

Carbachol

	100 nM		300 nM		1 µM		3 µM (1st)		3 µM (2nd)	
	TTP [s]	E_{MAX}	TTP [s]	E_{MAX}	TTP [s]	E_{MAX}	TTP [s]	E_{MAX}	TTP [s]	E_{MAX}
M_1	5.9 ± 0.5	1.45 ± 0.03	6.0 ± 0.5	1.75 ± 0.03	6.2 ± 0.5	1.95 ± 0.04[a]	6.4 ± 0.5	2.02 ± 0.04	6.3 ± 0.6	1.92 ± 0.04*
M_2	4.0 ± 0.4	1.40 ± 0.03	4.1 ± 0.5	1.77 ± 0.03	4.2 ± 0.4	2.12 ± 0.04*	4.2 ± 0.5	2.20 ± 0.04	4.2 ± 0.4	2.06 ± 0.04*
M_3	4.4 ± 0.5	1.46 ± 0.03	4.5 ± 0.5	1.79 ± 0.04	4.6 ± 0.5	2.07 ± 0.04*	4.6 ± 0.5	2.14 ± 0.04	4.5 ± 0.4	2.07 ± 0.04*
M_4	4.5 ± 0.5	1.32 ± 0.03	4.5 ± 0.5	1.62 ± 0.03	4.7 ± 0.5	1.91 ± 0.04*	4.9 ± 0.5	1.97 ± 0.04	4.8 ± 0.5	1.82 ± 0.04*
M_5	4.4 ± 0.5	1.33 ± 0.03	4.5 ± 0.5	1.55 ± 0.03	4.6 ± 0.5	1.73 ± 0.03*	4.8 ± 0.5	1.82 ± 0.04	4.8 ± 0.5	1.73 ± 0.03*

Oxotremorine

	300 nM CBC (1st)		30 nM		100 nM		300 nM		1 µM		300 nM CBC (2nd)	
	TTP [s]	E_{MAX}	TTP [s]	E_{MAX}	TTP [s]	E_{MAX}	TTP [s]	E_{MAX}	TTP [s]	E_{MAX}	TTP [s]	E_{MAX}
M_1	6.2 ± 0.5	1.75 ± 0.03	10 ± 1	1.27 ± 0.03	9.9 ± 0.7	1.46 ± 0.03*	10 ± 1	1.56 ± 0.03*	10 ± 1	1.62 ± 0.03	6.2 ± 0.5	1.74 ± 0.04
M_2	4.2 ± 0.4	1.76 ± 0.03	7.8 ± 0.6	1.23 ± 0.03	8.1 ± 0.6	1.42 ± 0.03*	8.2 ± 0.7	1.58 ± 0.03*	8.3 ± 0.7	1.66 ± 0.03*	4.1 ± 0.4	1.76 ± 0.03
M_3	4.6 ± 0.5	1.79 ± 0.03	9.1 ± 0.7	1.27 ± 0.03	9.3 ± 0.7	1.46 ± 0.03*	9.3 ± 0.7	1.57 ± 0.03*	9.4 ± 0.7	1.65 ± 0.03	4.6 ± 0.5	1.80 ± 0.04
M_4	4.4 ± 0.5	1.63 ± 0.03	8.8 ± 0.6	1.21 ± 0.03	8.8 ± 0.7	1.37 ± 0.03*	9.0 ± 0.7	1.50 ± 0.03*	9.0 ± 0.7	1.59 ± 0.03*	4.3 ± 0.5	1.63 ± 0.03
M_5	4.6 ± 0.5	1.55 ± 0.03	9.2 ± 0.7	1.22 ± 0.03	9.3 ± 0.7	1.37 ± 0.03*	9.3 ± 0.7	1.48 ± 0.03*	9.3 ± 0.7	1.55 ± 0.03	4.6 ± 0.5	1.54 ± 0.03

Pilocarpine

	300 nM CBC (1st)		100 nM		300 nM		1 µM		3 µM		300 nM CBC (2nd)	
	TTP [s]	E_{MAX}	TTP [s]	E_{MAX}	TTP [s]	E_{MAX}	TTP [s]	E_{MAX}	TTP [s]	E_{MAX}	TTP [s]	E_{MAX}
M_1	6.1 ± 0.5	1.74 ± 0.03	13 ± 1	1.35 ± 0.03	13 ± 1	1.49 ± 0.03*	14 ± 1	1.60 ± 0.03*	14 ± 1	1.64 ± 0.03	6.3 ± 0.5	1.74 ± 0.03
M_2	4.3 ± 0.4	1.76 ± 0.03	10 ± 1	1.34 ± 0.03	9.8 ± 0.7	1.47 ± 0.03*	10 ± 1	1.59 ± 0.04*	10 ±1	1.64 ± 0.03	4.5 ± 0.4	1.77 ± 0.03
M_3	4.6 ± 0.4	1.79 ± 0.03	12 ± 1	1.34 ± 0.03	12 ± 1	1.50 ± 0.03*	12 ± 1	1.61 ± 0.03*	13 ± 1	1.66 ± 0.03	4.8 ± 0.4	1.79 ± 0.03
M_4	4.6 ± 0.4	1.62 ± 0.03	12 ± 1	1.35 ± 0.03	12 ± 1	1.47 ± 0.03*	13 ± 1	1.56 ± 0.03*	13 ± 1	1.59 ± 0.03	4.7 ± 0.4	1.63 ± 0.03
M_5	4.5 ± 0.4	1.54 ± 0.03	12 ± 1	1.29 ± 0.03	12 ± 1	1.41 ± 0.03*	13 ± 1	1.50 ± 0.03*	12 ± 1	1.53 ± 0.03	4.8 ± 0.4	1.54 ± 0.03

Parameters are derived from experiments shown in Fig. S1. Data are means ± S.E.M. from 3 independent experiments; TTM, time to reach the maximum; *, different from the first (1st) stimulation by carbachol of the same concentration; a, different from lower concentration by ANOVA and Tukey-Kramer post-test, $P<0.05$.

Table S4. Parameters of calcium level changes upon activation of individual muscarinic receptor subtypes by the agonists carbachol and xanomeline in Fig. 2 in the main manuscript.

	hM_1	**hM_2**	**hM_3**	**hM_4**	**hM_5**
1st stimulation (300 nM carbachol)					
Time needed to reach the maximum [s]	6.8 ± 0.7	6.2 ± 0.3	6.3 ± 0.7	6.9 ± 0.6	7.9 ± 0.7
Maximum (max_1) [Em ratio]	1.64 ± 0.05	1.67 ± 0.07	1.68 ± 0.09	1.53 ± 0.05	1.47 ± 0.04
2nd stimulation (10 µM xanomeline)					
Time needed to reach the maximum [s]	9.6 ± 1.7	29 ± 6	13 ± 6	17 ± 3	39 ± 3
Maximum [% max_1]	118 ± 3	45 ± 2	92 ± 6	103 ± 5	59 ± 4
Average value of steady signal in the time period of 30–60 min. [Em ratio]					
After 1 min xano	1.34 ± 0.06*	1.04 ± 0.03	1.12 ± 0.06*	1.18 ± 0.06*	1.01 ± 0.03
After 3 min xano	1.44 ± 0.12*	1.08 ± 0.05	1.16 ± 0.07*	1.19 ± 0.08*	1.02 ± 0.03
After 10 min xano	1.53 ± 0.09*	1.09 ± 0.05	1.18 ± 0.05*	1.31 ± 0.13*	1.03 ± 0.03
3rd stimulation (300 nM carbachol)					
Time needed to reach the maximum [s]					
After 1 min xano	n.a.	6.4 ± 0.3	14 ± 1[a]	7.1 ± 0.6	13 ± 1[a]
After 3 min xano	n.a.	7.4 ± 1.0	14 ± 1[a]	8.6 ± 0.9	13 ± 1[a]
After 10 min xano	n.a.	7.6 ± 0.3	15 ± 1[a]	n.a.	16 ± 1[a]
Maximum [% max_1]					
After 1 min xano	n.a.	99 ± 5	65 ± 4[a]	74 ± 3[a]	89 ± 5
After 3 min xano	n.a.	79 ± 4[a]	59 ± 4[a]	70 ± 3[a]	79 ± 4[a]
After 10 min xano	n.a.	61 ± 3[a]	55 ± 3[a]	n.a.	43 ± 2[a]

n.a., not applicable; *, different from basal level), [a], different from first stimulation, $P<0.05$ by Dunnett's test; Em ratio, fluorescence emission ratio. Data are average values ± S.E.M. from 6 independent measurements as those shown in Fig. 2 in the main manuscript.

Table S5. Parameters of calcium level changes upon activation of individual muscarinic receptor subtypes by the agonists carbachol, oxotremorine and pilocarpine in Fig. 3 in the main manuscript.

	hM_1	**hM_2**	**hM_3**	**hM_4**	**hM_5**
1st stimulation (300 nM carbachol)					
Time needed to reach the maximum [s]	6.4 ± 0.6	5.3 ± 0.5	5.5 ± 0.7	5.7 ± 0.6	5.9 ± 0.7
Maximum (max_1) [Em ratio]	1.73 ± 0.05	1.75 ± 0.07	1.77 ± 0.06	1.59 ± 0.05	1.53 ± 0.05
2nd stimulation					
Time needed to reach the maximum [s]					
Carbachol	6.4 ± 0.5	5.4 ± 0.5	5.6 ± 0.6	5.9 ± 0.6	6.0 ± 0.6
Oxotremorine	12 ± 1	9.6 ± 0.5	11 ± 1	10 ± 1	11 ± 1
Pilocarpine	16 ± 1	12 ± 1	15 ± 1	13 ± 1	15 ± 2
Maximum [Em ratio]					
Carbachol	1.92 ± 0.06	2.09 ± 0.05	2.08 ± 0.05	1.84 ±0.05	1.82 ± 0.05
Oxotremorine	1.65 ± 0.04	1.69 ± 0.03	1.68 ± 0.04	1.57 ± 0.04	1.60 ± 0.04
Pilocarpine	1.66 ± 0.04	1.68 ± 0.04	1.69 ± 0.04	1.64 ± 0.04	1.61 ± 0.05
Average value of steady signal in the time period of 30–60 min. [Em ratio]					
Carbachol	1.04 ± 0.01*	1.00 ± 0.01	1.00 ± 0.01	1.01 ± 0.01	1.00 ± 0.01
Oxotremorine	1.08 ± 0.02*	1.06 ± 0.01*	1.11 ± 0.02*	1.06 ± 0.02*	1.05 ± 0.02*
Pilocarpine	1.07 ± 0.02*	1.04 ± 0.01*	1.07 ± 0.03*	0.99 ± 0.01	1.09 ± 0.02*
3rd stimulation (300 nM carbachol)					
Time needed to reach the maximum [s]					
After carbachol	9.2 ± 0.5[a]	7.5 ±0.5[a]	6.1 ± 0.5	8.3 ± 0.5[a]	8.5 ± 0.5[a]
After oxotremorine	9.3 ± 0.5[a]	5.8 ± 0.5	5.8 ± 0.5	6.2 ± 0.5	6.3 ± 0.5
After pilocarpine	8.5 ± 0.5[a]	6.0 ± 0.5	5.6 ± 0.5	5.9 ± 0.5	n.a.[a]
Maximum [Em ratio]					
After carbachol	1.43 ± 0.03[a]	1.39 ± 0.04[a]	1.45 ± 0.03[a]	1.31 ± 0.03[a]	1.32 ± 0.03[a]
After oxotremorine	1.39 ± 0.03[a]	1.64 ± 0.03	1.60 ± 0.04	1.53 ± 0.04	1.52 ± 0.04
After pilocarpine	1.45 ± 0.04[a]	1.72 ± 0.05	1.76 ± 0.05	1.61 ±0.04	1.01 ± 0.01[a]

n.a., not applicable; *, different from basal level, [a], different from first stimulation, $P<0.05$ by Dunnett's test; Em ratio, fluorescence emission ratio. Data are average values ± S.E.M. from 3 independent measurements as those shown in Fig. 3 in the main manuscript.

Table S6. Parameters of calcium level changes induced by xanomeline at hM_1 through hM_4 receptors and effects of the antagonist NMS on delayed receptor activation in Fig. 4 in the main manuscript.

	hM_1	**hM_2**	**hM_3**	**hM_4**
	1st stimulation (300 nM carbachol)			
Time needed to reach the maximum [s]	4.2 ± 1.1	6.2 ± 0.8	4.0 ± 1.1	5.8 ± 0.9
Maximum (max1) [Em ratio]	1.28 ± 0.05	1.27 ± 0.07	1.29 ± 0.05	1.27±0.12
	2nd stimulation (10 µM xano)			
Time needed to reach the maximum [s]	14 ± 5	29 ± 7	15 ± 6	14 ± 3
Maximum (% max1) [Em ratio]	128 ± 9	98 ± 2	99 ± 3	96 ± 6
	Application of 10 µM NMS			
Inhibition effect (%)	98 ± 2	99 ± 1	100 ± 3	92 ± 3
Rise time after NMS application [s]	46 ± 8	n.a.	n.a.	136 ± 7
Maximum (max2) [Em ratio]	1.24 ± 0.05*	1.05 ± 0.06	1.00 ± 0.02	1.12 ± 0.02*

n.a., not applicable; *, different from basal level, $P<0.05$ by t-test, data from individual experiments paired; Em ratio, fluorescence emission ratio. Data are average values ± SEM from 6 independent measurements as those shown in Fig. 4 in the main manuscript.

Table S7. Parameters of calcium level changes induced by xanomeline in the presence of the antagonist NMS at M_1 through M_4 receptors in Fig. 5 in the main manuscript.

	hM_1	**hM_2**	**hM_3**	**hM_4**
1st stimulation (300 nM carbachol)				
Time needed to reach the maximum [s]	4.4 ± 0.3	5.6 ± 1.1	4.5 ± 0.6	5.8 ± 1.3
Maximum (max1) [Em ratio]	1.29 ± 0.05	1.28 ± 0.07	1.29 ± 0.09	1.34 ± 0.09
2nd stimulation (10 µM xanomeline +10 µM NMS)				
Inhibition effect of NMS [%]	4.5 ± 0.3*	n.a.	n.a.	n.a.
Rise time after NMS withdrawal [s]	27 ± 3	n.a.	240 ± 38	n.a.
Maximum (max2) [Em ratio]	1.12 ± 0.03*	1.03 ± 0.01	1.09 ± 0.02*	1.04 ± 0.02

n.a., not applicable; *, different from basal level, $P<0.05$ by Dunnett's test; Em ratio, fluorescence emission ratio. Data are average values ± S.E.M. From 6 to 8 independent measurements as those shown in Fig. 5 in the main manuscript.

Figures

Fig. S1 A

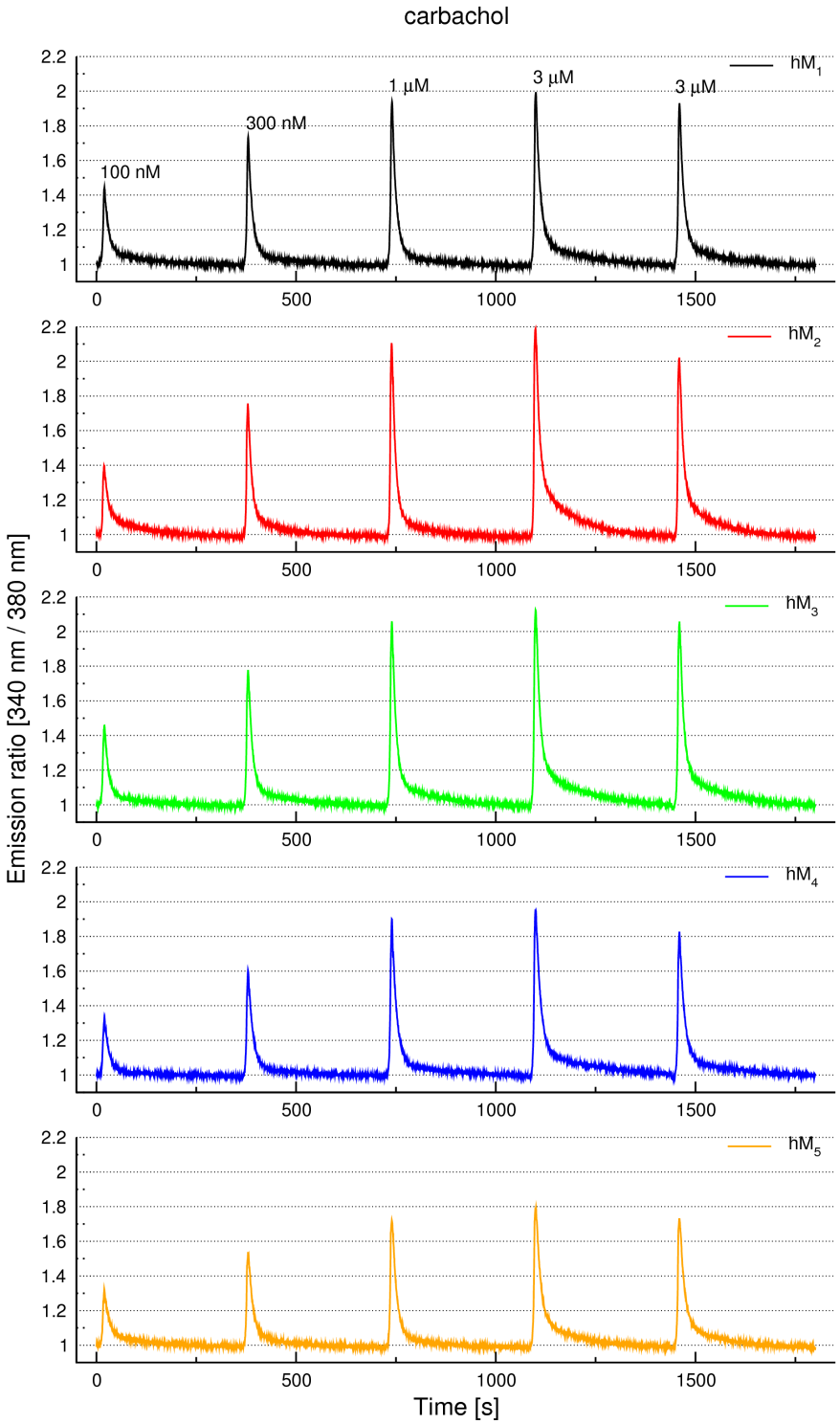
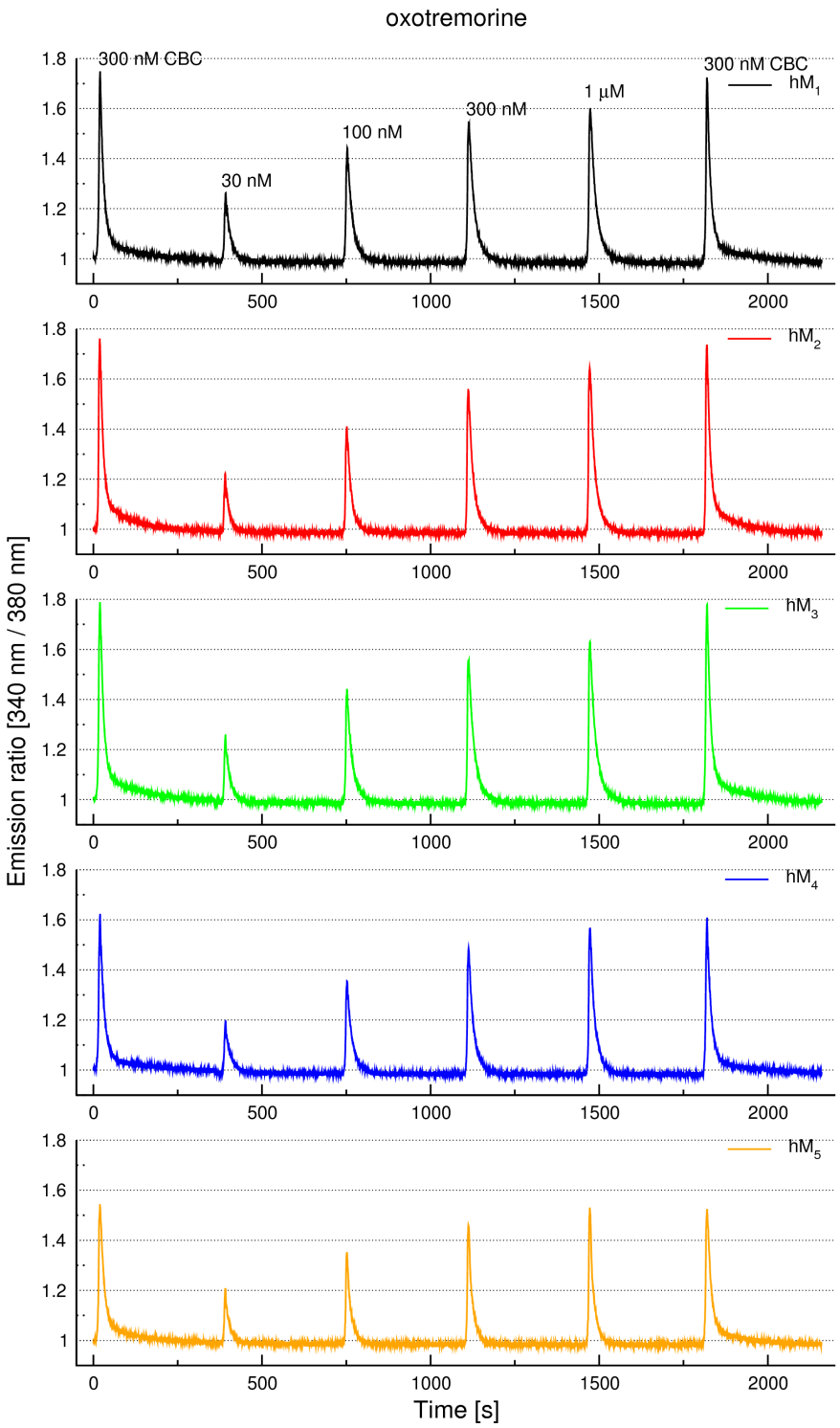


Fig. S1 B


oxotremorine
300 nM CBC
30 nM
100 nM
300 nM
1 µM
300 nM CBC
hM1
hM2
hM3
hM4
hM5
Emission ratio [340 nm / 380 nm]
Time [s]

SI Fig. 1C

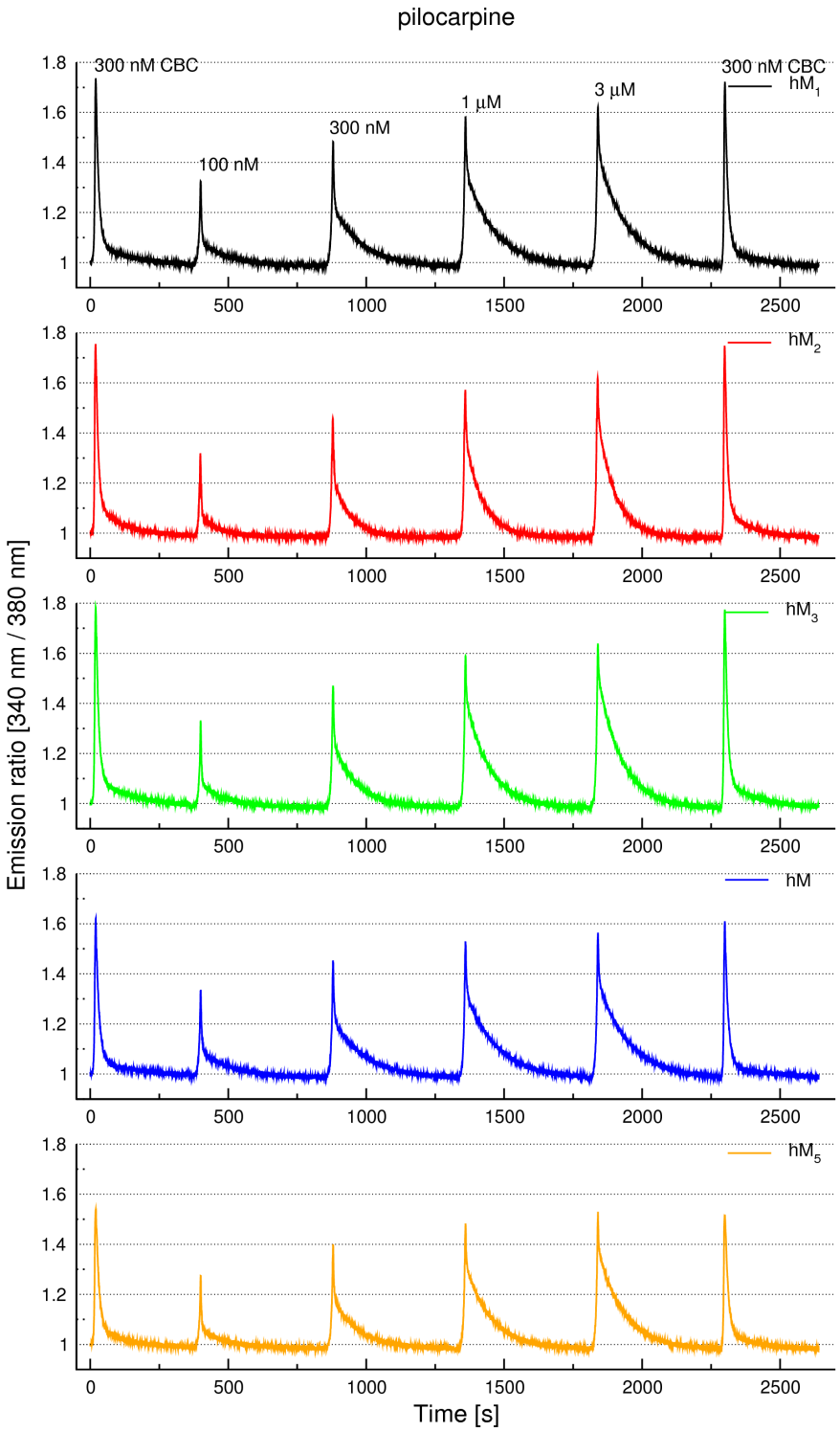

pilocarpine
300 nM CBC
100 nM
300 nM
1 µM
3 µM
300 nM CBC
hM1
hM2
hM3
hM
hM5
Emission ratio [340 nm / 380 nm]
Time [s]

Fig S1. Time courses of intracellular calcium response to acute treatment with the agonists carbachol, oxotremotine and pilocarpine

Cells were seeded, handled and loaded with Fura-2 as described in the main manuscript. Cells were repeatedly stimulated with increasing concentrations of carbachol (A), oxotremorine (B) or pilocarpine (C). **A:** After an initial 10-s period cells were stimulated with increasing concentrations of carbachol (100 nM, 300 nM, 1 µM and 3 µM) for 5 s. Then stimulation with 3 µM carbachol was repeated. Cells were perfused for 6 min between stimulations with KHB. **B:** After an initial 10-s period cells were stimulated with 300 nM carbachol (CBC) for 5 s then stimulated with increasing concentrations (30 nM, 100 nM, 300 nM and 1 µM) of oxotremorine for 10 s and then again with 300 nM carbachol for 5 s. Cells were perfused for 6 min between stimulations with KHB. **C:** After an initial 10-s period cells were stimulated with 300 nM carbachol (CBC) for 5 s then stimulated with increasing concetrations (100 nM, 300 nM, 1 µM and 3 µM) of pilocarpine for 20 s and then again with 300 nM carbachol for 5 s. Cells were perfused for 8 min between stimulations with KHB. Traces are averages from 8 cells from the representative experiment confirmed by 2 independent experiments. Signal variation (SD) among cells ranges from ± 0.021 at the base line to ± 0.088 at peaks. Parameters are summarized in Table S3.

Fig. S2

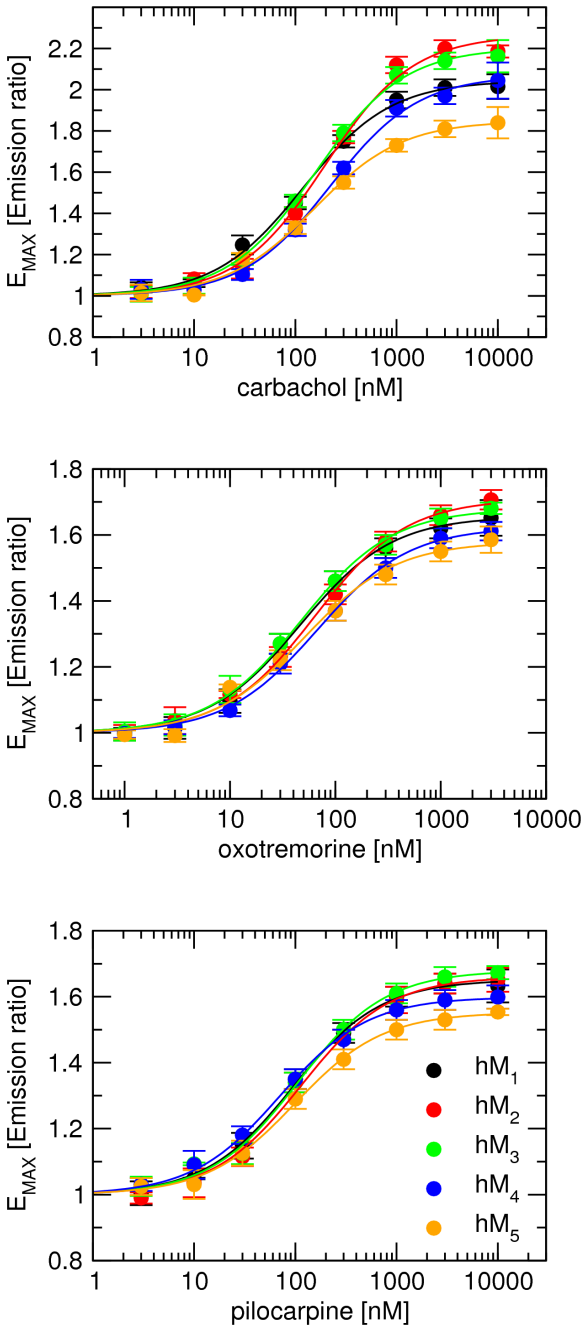

E_MAX [Emission ratio]
carbachol [nM]
oxotremorine [nM]
pilocarpine [nM]
hM1
hM2
hM3
hM4
hM5

Fig. S2 Concentration response to acute treatment with the agonists carbachol, oxotremotine and pilocarpine.

Maximal effects (E_{MAX}) reached in intracellular calcium response were measured in the same experimental setup as in Fig. S1. E_{MAX} values are plotted against concentration of agonist carbachol (top), oxotremorine (middle) or pilocarpine (bottom). Data are means ± S.E.M. from 3 independent experiments. Eq. 1 of the main manuscript was fitted to the data. Parameters are summarized in Table 1 in the main manuscript.

Fig. S3 A

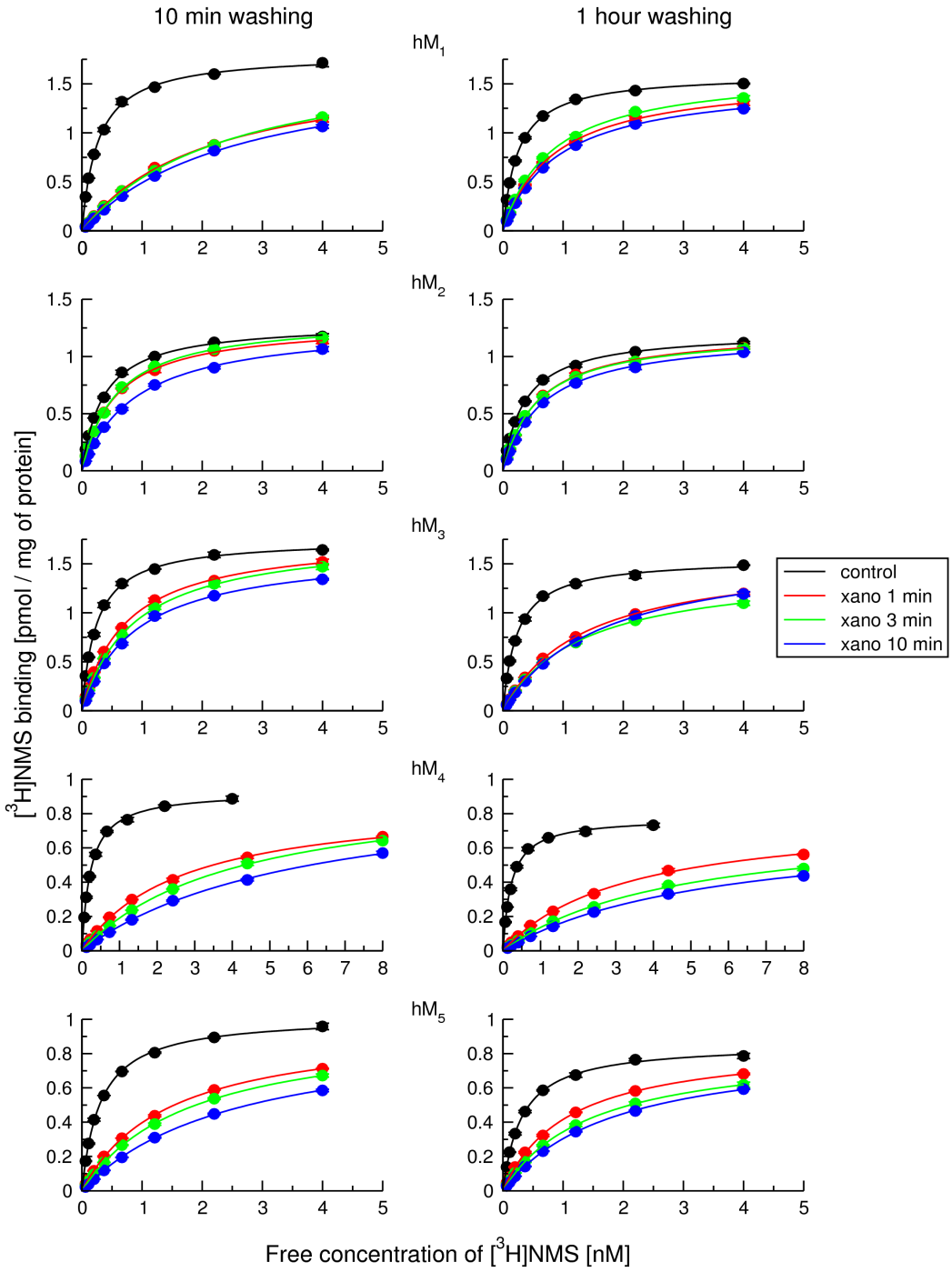

10 min washing
1 hour washing
hM1
hM2
hM3
hM4
hM5
[3H]NMS binding [pmol / mg of protein]
Free concentration of [3H]NMS [nM]
control
xano 1 min
xano 3 min
xano 10 min

Fig. S3 B

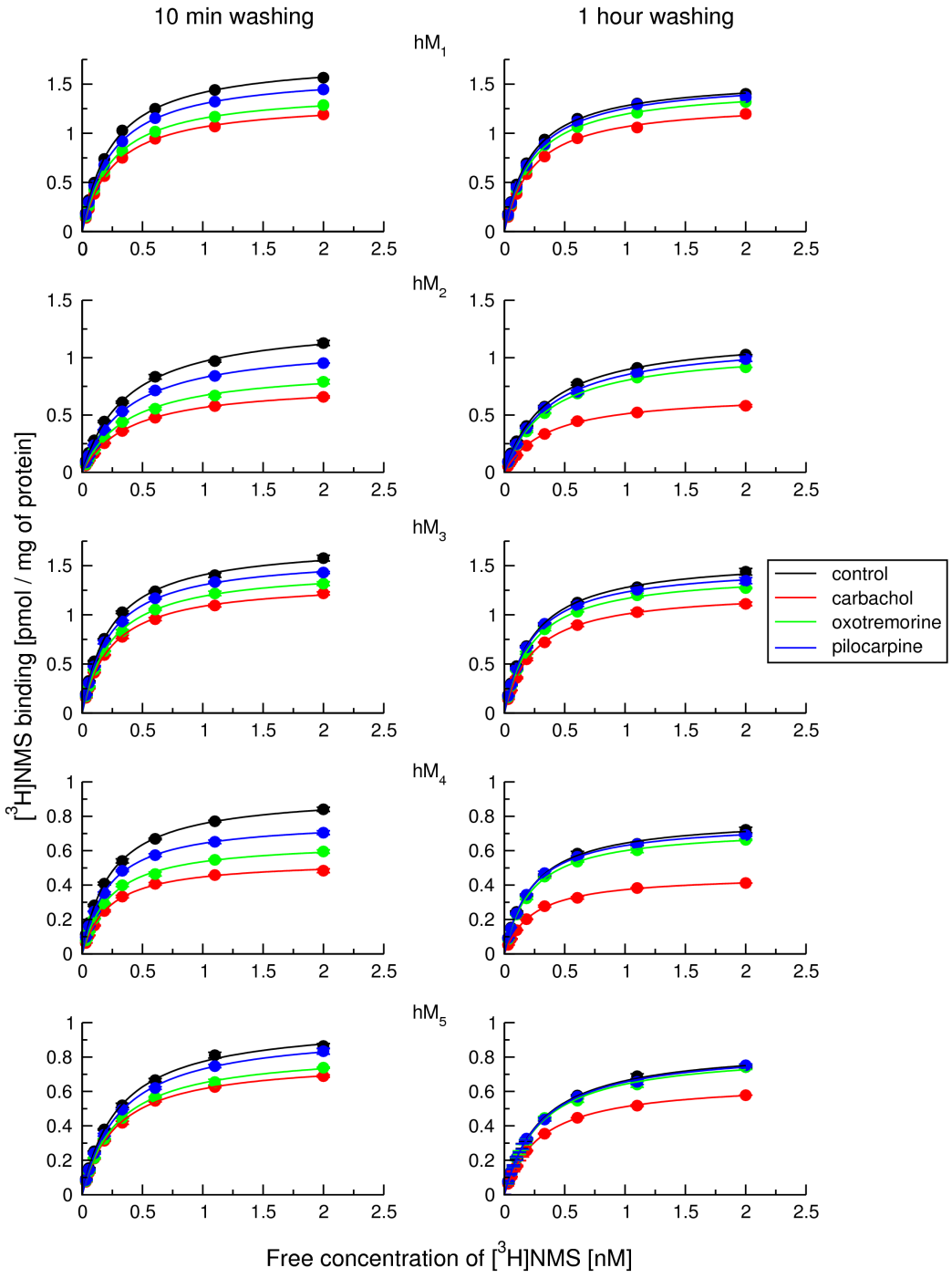

10 min washing
1 hour washing
hM1
hM2
hM3
hM4
hM5
[3H]NMS binding [pmol / mg of protein]
Free concentration of [3H]NMS [nM]
control
carbachol
oxotremorine
pilocarpine

Fig. S3. Saturation binding of [^{3}H]NMS to membranes from the cells treated with xanomeline.
Intact cells expressing hM_1 to hM_5 receptors were exposed to 10 μM xanomeline (panel A) for 1 (red), 3 (green) or 10 min (blue) or for 10 min to agonists (panel B) 1 μM carbachol (red), 1 μM oxotremorine (green) and 3 μM pilocarpine (blue) or sham-treated (black) and washed with KHB for 10 min (left column) or 1 hour (right column) and membranes were prepared as described in Methods in main manuscript. Binding of the radiolabeled antagonist [^{3}H]NMS in concentrations ranging from 60 pM to 8 nM (panel A) or from 30 pm to 2 nM (panel B) to the membranes is plotted as pmol per mg of protein of specifically bound [^{3}H]NMS (ordinate) against concentration of free [^{3}H]NMS in nM (abscissa). Data are averages ± S.E.M. of 3 independent experiments performed in triplicates. Parameters of [^{3}H]NMS binding are summarized in Tables 2 and 3 in the main manuscript.

Fig. S4

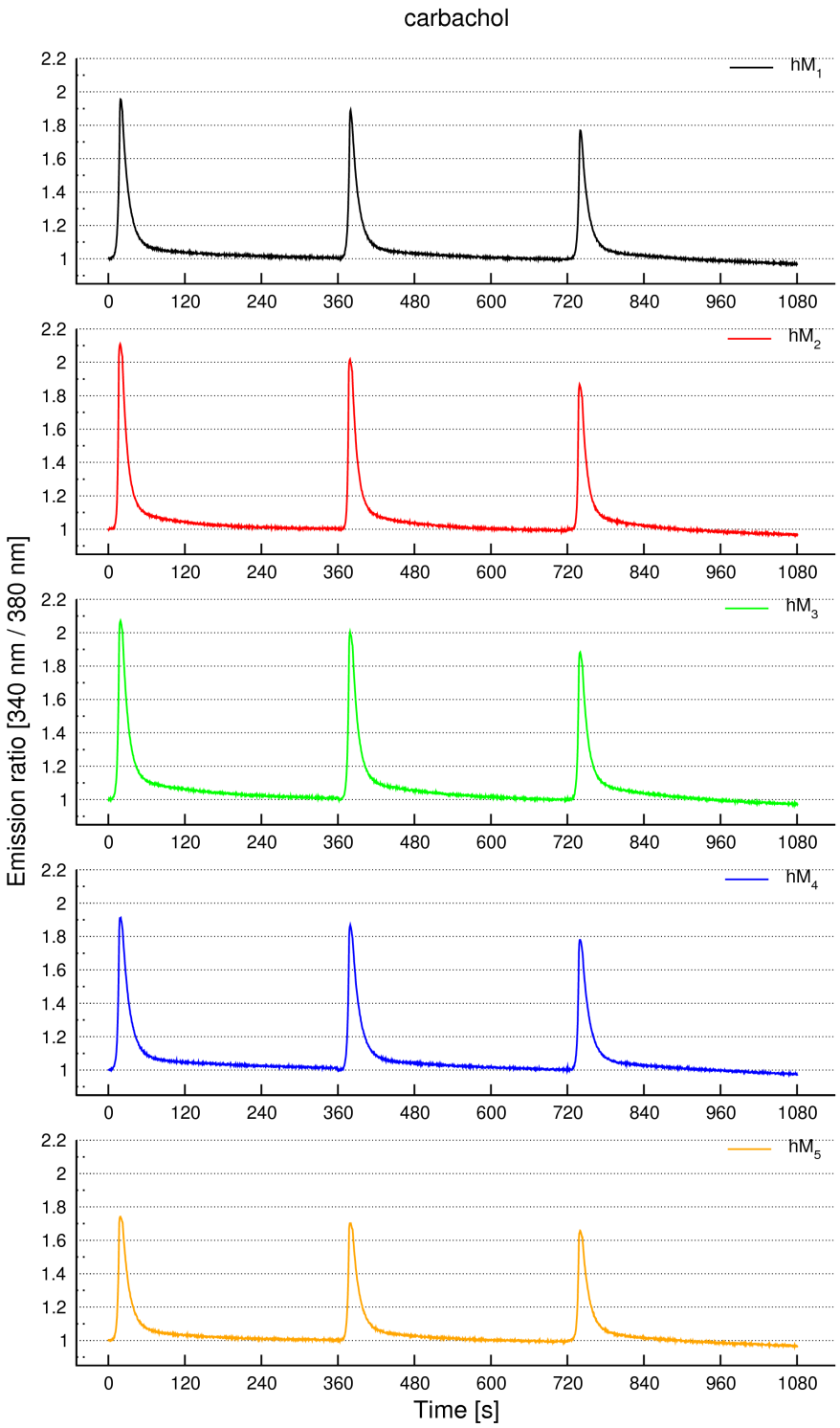

carbachol
hM1
hM2
hM3
hM4
hM5
Emission ratio [340 nm / 380 nm]
Time [s]
2.2
2
1.8
1.6
1.4
1.2
1
0
120
240
360
480
600
720
840
960
1080

Fig S4. Effects of changing the concentration of extracellular calcium.

Cells were seeded, handled and loaded with Fura-2 as described in the main manuscript. After an initial 10-s period cells were stimulated with 1 µM carbachol for 5 s, washed with KHB containing 1.3 µM $CaCl_2$ for 6 min, then washing was switched to KHB with $CaCl_2$ reduced to 0.65 µM then stimulated again with 1 µM carbachol for 5 s, washed with KHB containing 0.65 µM $CaCl_2$ for 6 min and then switched to calcium free KHB then stimulated again with 1 µM carbachol for 5 s and washed with calcium free KHB for final 6 min. Traces are averages from 10 to 12 cells from representative experiment confirmed by 2 independent experiments. Signal variation (SD) among cells ranges from ± 0.013 at the base line to ± 0.057 at peaks.

Physiol. Res. 63 (Suppl. 1): S177-S189, 2014

REVIEW

Outline of Therapeutic Interventions With Muscarinic Receptor-Mediated Transmission

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Received October 1, 2013
Accepted October 18, 2013

Summary

Muscarinc receptor-mediated signaling takes part in many physiological functions ranging from complex higher nervous activity to vegetative responses. Specificity of action of the natural muscarinic agonist acetylcholine is effected by action on five muscarinic receptor subtypes with particular tissue and cellular localization, and coupling preference with different G-proteins and their signaling pathways. In addition to physiological roles it is also implicated in pathologic events like promotion of carcinoma cells growth, early pathogenesis of neurodegenerative diseases in the central nervous system like Alzheimer´s disease and Parkinson´s disease, schizophrenia, intoxications resulting in drug addiction, or overactive bladder in the periphery. All of these disturbances demonstrate involvement of specific muscarinic receptor subtypes and point to the importance to develop selective pharmacotherapeutic interventions. Because of the high homology of the orthosteric binding site of muscarinic receptor subtypes there is virtually no subtype selective agonist that binds to this site. Activation of specific receptor subtypes may be achieved by developing allosteric modulators of acetylcholine binding, since ectopic binding domains on the receptor are less conserved compared to the orthosteric site. Potentiation of the effects of acetylcholine by allosteric modulators would be beneficial in cases where acetylcholine release is reduced due to pathological conditions. When presynaptic function is severly compromised, the utilization of ectopic agonists can be a thinkable solution.

Key words

Muscarinic receptors • G-proteins • Allosteric modulators • Ectopic agonists • Selectivity

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Physiology of muscarinic receptors

Muscarinic receptors belong to the family of G-protein coupled receptors (GPCR) that are the most abundant and pharmacologically targeted plasma membrane receptors (Lander *et al.* 2001, Fredriksson *et al.* 2003). A common structural feature of GPCR is the extracellular N-terminus, seven membrane spanning domains, three extracellular and three intracellular loops, and an intracellular C-terminus. Stimulation of various GPCRs leads to activation of particular G-proteins and their intracellular signaling pathways that play important regulatory roles in virtually all physiological functions. In addition to these well-established pathways, it has also been demonstrated that receptors also transduce non-G-protein-mediated signaling *via* arrestins and G-protein receptor kinases (Lefkowitz 1998, Lefkowitz and Shenoy 2005, Reiter and Lefkowitz 2006).

To date five subtypes of muscarinic receptors denoted as M_1-M_5 and encoded by five different genes have been discovered (Kubo *et al.* 1986a,b, Bonner *et al.* 1987, 1988, Peralta *et al.* 1987, Bonner 1989a,b). Muscarinic receptors are widely expressed in both the central and peripheral nervous system, with distinct cellular as well as tissue localization of individual subtypes. They mediate various physiological functions

of their natural agonist acetylcholine ranging from complex higher nervous functions such as arousal, memory and alertness to vegetative processes such as regulation of heart rate and cardiac output, blood pressure, temperature regulation, perspiration, secretion of exocrine and endocrine glands, and motility of the gastrointestinal tract (Eglen 2006, 2012). In addition to these functions mediated by neuronal acetylcholine, muscarinic receptors also play a role in mediating local responses of non-neuronally derived acetylcholine, e.g. modulation of immune responses or regulation of local circulation (Kawashima and Fujii 2004, 2008, Wessler and Kirkpatrick 2012). Non-neuronal acetylcholine has also been implicated in paracrine control influencing lung cancer growth through both nicotinic and muscarinic receptors signaling (Song *et al.* 2003a,b, Proskocil *et al.* 2004, Song *et al.* 2007, Schuller 2009).

Pharmacology of muscarinic receptors

Individual muscarinic receptor subtypes share a high degree of homology in the transmembrane domains while extracellular and intracellular loops are less well conserved (Hulme *et al.* 1990, 1991, 2003). The intracellular C-terminus may form the fourth intracellular loop by means of a glycosyl anchor. The N-terminal part of the third intracellular loop represents the contact domain for interaction with G-proteins (Wess *et al.* 1995, Hu *et al.* 2010). Higher variability of this domain enables selectivity of interaction with different G-proteins. The M_1, M_3, and M_5 receptor subtypes preferentially activate $G_{q/11}$ G-protein intracellular signaling while the M_2 and M_4 subtypes prefer $G_{i/o}$ G-proteins and activate their signaling pathways (Jones *et al.* 1991).

Muscarinic receptors have a classical (orthosteric) binding site for natural or exogenous agonists located deep in a pocket created by the transmembrane segments of the protein that are highly conserved among individual receptor subtypes (Hulme *et al.* 2003). Due to high conservation of the orthosteric site there are virtually no known selective orthosteric agonists. It is thus of prime importance to find out a way to influence selectively signaling pathways of individual muscarinic receptors. Apart from the orthosteric binding site that is naturally occupied by the endogenous agonist acetylcholine muscarinic receptors have allosteric binding sites located on less conserved extracellular loops. Allosteric ligands bind to an allosteric site on the receptor and may either activate the receptor by themselves or modulate receptor activation by acetylcholine. They exhibit subtype selectivity because they bind to less conserved receptor domains. Binding of allosteric ligands results in remarkable subtype selective influencing of orthosteric ligand binding that depends on the receptor subtype and the specific pair of orthosteric-allosteric ligands (Jakubik *et al.* 1995, 1997, 2005). Allosteric ligands (modulators) change receptor conformation and in this way increase, decrease, or have no influence (positive, negative, or neutral cooperativity) on the binding affinity of given orthosteric agonists, including the natural agonist acetylcholine (Jakubik and El-Fakahany 2010). The advantage of allosteric modulators is that their effect, with respect to the specific receptor-activated pathway, is given by the factor of cooperativity with orthosteric ligand that dictates a maximal degree of interaction of binding of both agents. This results in eliminating a danger of overdosing.

There are also so called ectopic ligands (Fig. 1 and 2) that attach to more distal parts of the receptor binding site pocket that is less conserved. Unlike allosteric modulators they prevent binding of orthosteric ligands to the orthosteric site. However, the selectivity of known ectopic ligands in terms of binding affinity to different receptor subtypes is generally poor. On the other hand, some of these compounds exhibit significant functional selectivity (e.g. N-desmethylclozapine, AC-42), which makes them good candidates for pharmacotherapy.

The next type of compounds that bind to muscarinic receptors are so called bitopic ligands. These agents can bind to two sites on a single receptor. An example is 77-LH-28-1 that was identified from a series of AC-42 analogs (Langmead *et al.* 2008) and shown to have selectivity for M1 receptors (Heinrich *et al.* 2009). *In vitro* studies indicated competitive interaction between the orthosteric antagonist scopolamine and 77-LH-28-1 (Langmead *et al.* 2008). Further functional and site-directed mutagenesis studies have demonstrated an allosteric mode of agonist action for this ligand. Another example of ligand that binds both to orthosteric and allosteric sites and can be labeled as bitopic is xanomeline (Jakubík *et al.* 2002). Xanomeline is one of few functionally selective muscarinic agonists. It preferentially activates M_1 and M_4 receptors while it has long-term antagonistic effects at M_5 receptors (Grant and El-Fakahany 2005, Grant *et al.* 2010). In addition, part of xanomeline binding that depends on the O-hexyl group of the molecule (Jakubik *et al.* 2004) is resistant to washing

(Christopoulos *et al.* 1998, Jakubik *et al.* 2002, 2006). Interestingly, wash-resistant xanomeline itself acts on the receptor both competitively and allosterically (Jakubik *et al.* 2002, Machová *et al.* 2007).

There is accumulating evidence that muscarinic receptors can be activated *via* several different allosteric sites (Jakubík *et al.* 1996, Lebois *et al.* 2010) and ectopic sites (Langmead *et al.* 2008). Thus regardless of the binding mode (orthosteric, ectopic, allosteric or bitopic; Fig. 1 and 2) ligands can act as agonists (induce response like natural neurotransmitter) or neutral antagonists (produce no response on their own but block activation by agonists) or inverse agonists (induce response opposite to the natural neurotransmitter).

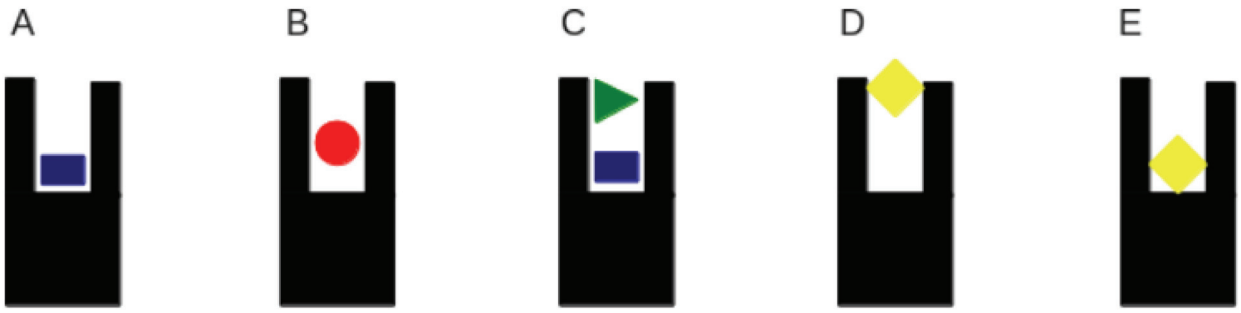

Fig. 1. Schematic representation of ligand binding modes. Binding of the orthosteric ligand (blue rectangle) to the othosteric site **(A)**, binding of the ectopic ligand (red circle) to the ectopic site that is different from the orthosteric site but prevents binding of the othosteric ligands **(B)**, allosteric ligand (green triangle) binds to the allosteric binding site concurrently with the orthosteric ligand **(C)**, bitopic ligand (yellow diamod) can bind to the allosteric binding site **(D)** as well as to the orthosteric binding site **(E)**.

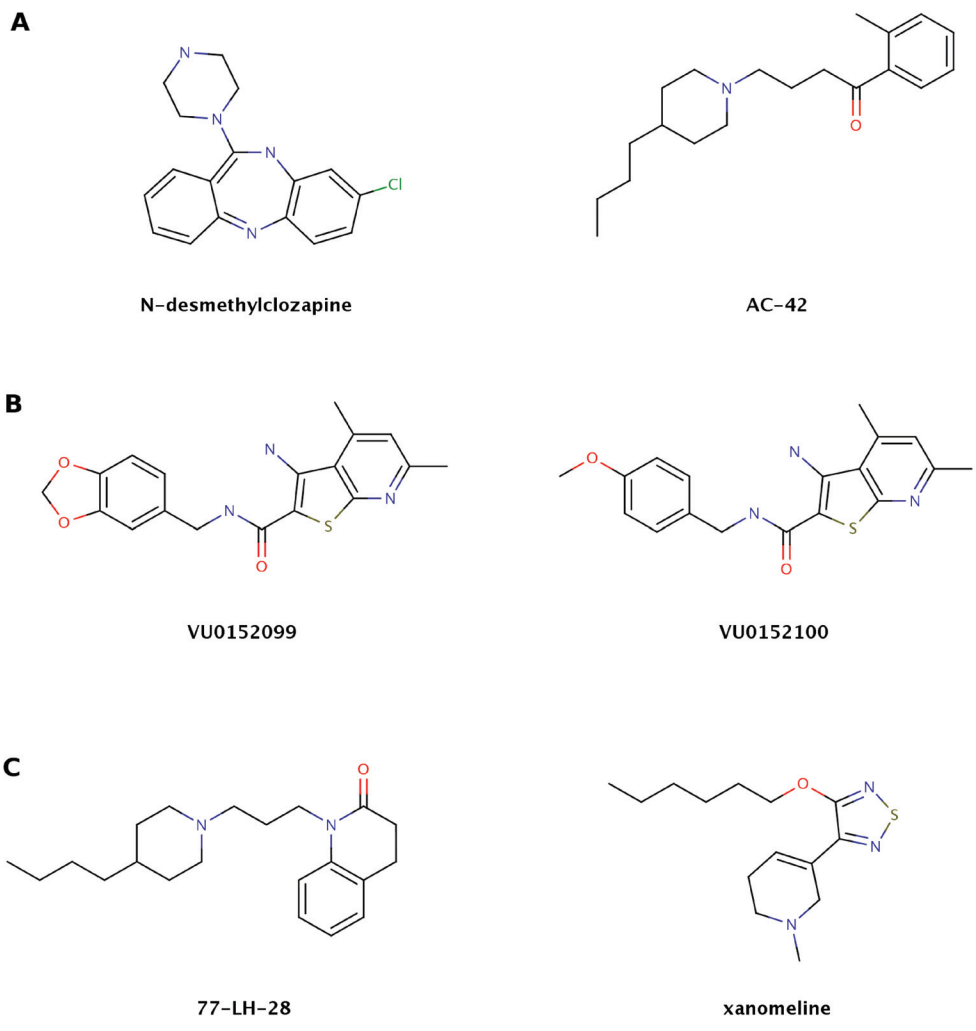

Fig. 2. Structures of atypical muscarinic ligands. **A**, ectopic ligands N-desmethylclozapine and AC-42; **B**, allosteric agonists VU0152099 and VU0152100; **C**, bitopic agonists 77-LH-28-1 and xanomeline.

Alzheimer's disease

Alzheimer´s disease (AD) is the most widespread dementing neurodegenerative disease. It was described in 1907 by Alois Alzheimer and since then enormous efforts have been exerted to find out how it originates and explore possibilities of an efficient treatment. Original pathological findings of amyloid plaques, neurofibrillary tangles, and impairments of the brain cholinergic system led to the formulation of the "cholinergic hypothesis" of AD (Bartus *et al.* 1982). Later on fragments of the amyloid precursor protein (APP), a major protein isolated from amyloid plaques (Masters *et al.* 1985a,b), were discovered (Kang *et al.* 1987). Proof of increased accumulation of these fragments in Alzheimer's brains gave rise to the "amyloid cascade hypothesis" (Hardy and Higgins 1992). Overproduction of Aβ fragments in hereditary cases of the disease is due to known defects of genes for APP localized on chromosome 21, presenilin 1 on chromosome 14 (Sherrington *et al.* 1995), and presenilin 2 on chromosome 1 (Levy-Lahad *et al.* 1995a,b). However, the reason for their increased production in sporadic cases representing the majority (up to 98 %) of cases is largely unknown. Allelic polymorphism of the ApoE gene is a major genetic risk factor in sporadic early onset AD that can nonetheless account for no more than 5-15 % of cases. By far the major risk factor of the disease is increasing age yet it is not known how it contributes to development of the disease. It has been suggested that exposure to a variety of insults during life cycle may lead to the gradual accumulation of native β-amyloid (Aβ) fragments and finally to the common clinical and pathological picture of Alzheimer´s disease (Mesulam 1999, Selkoe 2001, 2002, Kukar *et al.* 2005, Turner and Nalivaeva 2007, Karran *et al.* 2011).

The amyloid cascade hypothesis postulates that the primary event in the pathogenesis of AD is the overproduction of Aβ fragments as a result of known genetic defects in hereditary cases of the disease (Hardy 1997). It is now generally accepted that the causal agent that triggers and drives the disease progression is increased concentration of small soluble oligomers of Aβ, mainly fragment $A\beta_{1-42}$ (Selkoe 2002, Lesne *et al.* 2006, 2008, Maezawa *et al.* 2011, Shankar *et al.* 2011). However, familial AD disease represents only about 1 % of all cases. This has urged for investigations of the physiological function of Aβ that should help to explain the high prevalence of the disease in sporadic cases. The fragments of Aβ that are generated by sequential cleavage at the β and γ sites of APP have been reported to have both neuroprotective and neurotoxic effects (Whitson *et al.* 1989, 1990, Yankner *et al.* 1990, Pike *et al.* 1991). More recently, the specific physiological role of major Aβ fragments connecting APP and lipid metabolism has been demonstrated. Fragment $A\beta_{1-40}$ downregulates cholesterol synthesis by inhibiting hydroxymethylglutaryl-CoA synthase whereas fragment $A\beta_{1-42}$ decreases sphingomyeline levels by activating neutral sphingomyelinase (Grimm *et al.* 2005, 2007). In turn, changes in membrane lipid composition influence APP processing (Kojro *et al.* 2001, Grimm *et al.* 2008, 2011). The amyloid precursor protein is a receptor-like membrane protein. Tuning of proteolytic amyloidogenic/ nonamyloidogenic processing depends on plasma membrane properties and localization in membrane domains (Schneider *et al.* 2006, 2008, Hicks *et al.* 2012) and the same may be true for other transmembrane proteins including G-protein-coupled receptors (Rudajev *et al.* 2005, Michal *et al.* 2007, 2009).

Original neurochemical findings in Alzheimer´s disease brains pointed out disturbances of acetylcholine metabolism (Bowen *et al.* 1976, Davies and Maloney 1976, Perry *et al.* 1977a,b, Sims *et al.* 1981, Francis *et al.* 1985, 1999). Since then a large body of evidence supporting as well as questioning this hypothesis has accumulated (Bartus and Emerich 1999, Bartus 2000). Several lines of evidence argue for viability of the cholinergic hypothesis. Cholinergic muscarinic transmission plays an important role in mental functions like attention, learning, and memory (Peralta *et al.* 1988, Ehlert *et al.* 1994, Lahiri *et al.* 2004, Koch *et al.* 2005). These functions decline in the course of natural aging and more so in AD. In primates such a decline correlates with a decrease in the number of cholinergic neurons in the basal forebrain and treatments that rescue these neurons lead to improvement of cognitive performance (Smith *et al.* 1999, Conner *et al.* 2001). Cholinergic neurons are very sensitive to changes in homeostasis and disturbances of cognitive performance also accompany various insults like head trauma, intoxications, and hypoxia. Up to now the major therapeutic interventions that demonstrate certain benefits target the cholinergic system (e.g. clinically approved cholinesterase inhibitors). Conversely, it has been shown that application of antimuscarinic treatment in patients with Parkinson´s disease results in a significant increase in the probability to develop Alzheimer´s disease (Perry *et al.* 2003). In line

with this finding is an enhancement of amyloid pathology in transgenic APPswe/ind mice that express low levels of M_1 muscarinic acetylcholine receptors (Davis *et al.* 2010).

Aging is by far the most imporatant risk factor in sporadic Alzheimer´s disease. A decline of cholinergic transmission naturally occurring during aging is dramatically accentuated in Alzheimer´s disease and underlies cognitive symptoms of this devastating disorder. Up to now the only treatment of this disease that shows certain benefit is the use of cholinesterase inhibitors (Wilkinson *et al.* 2004). These drugs prevent hydrolysis of the endogenous muscarinic agonist acetylcholine and can thus be effective only when the presynaptic component of cholinergic synapses is operating. This is often not the case in clinically manifested stages of Alzheimer's disease. Moreover, preservation of synaptic acetylcholine by these compounds results not only in beneficial memory enhancing effects (through M_1 muscarinic receptors), but also significant side effects (mediated by other subtypes of muscarinic receptors). Muscarinic receptors are rather well preserved even in the late state of the disease although their activation appears somewhat compromised in the course of healthy aging and more so during disease progression (Tsang *et al.* 2006, Machová *et al.* 2008, 2010, Janickova *et al.* 2013). Thus M_1 selective agonists bear therapeutic potential for treatment of Alzheimer's disease. Recently, systemically active M_1 allosteric agonists VU0152099 and VU0152100, were synthesized at the Vanderbilt Center for Neuroscience Drug Discovery (Lebois *et al.* 2010).

The cholinergic and amyloid hypotheses are not mutually exclusive (Isacson *et al.* 2002). As mentioned above, the increase in Aβ concentration in hereditary cases is due to known gene defects. The link between cholinergic neurotransmission and increase in Aβ concentration has been demonstrated *in vitro*. Stimulation of $G_{q/11}$ G-protein coupled M_1 and M_3 muscarinic receptors increases non-amyloidogenic cleavage of APP at the α site by α-secretase and in this way prevents amyloidogenic processing of APP (Buxbaum *et al.* 1992, Nitsch *et al.* 1992). Weakening of cholinergic muscarinic signal transduction may thus lead to an increase in Aβ production and consequently to the acceleration of disease progression (Doležal and Kašparová 2003). Indeed, inhibition of $G_{q/11}$ G-protein function has been demonstrated in rodent primary cultures as a reduction of muscarinic receptor-induced GTPase activity (Kelly *et al.* 1996), and as a decrease in $G_{q/11}$ G-protein concentration (Kelly *et al.* 2005) and attenuation of muscarinic receptor-stimulated phosphatidylinositol hydrolysis in plasma membranes prepared from *post mortem* brain samples of Alzheimer's patients (Jope *et al.* 1997, Thathiah and De Strooper 2009).

Schizophrenia

Schizophrenia is a diagnosis that covers a set of disorders of different etiologies with the same symptoms. This disorder can be divided based on the presence or absence of negative symptoms or according to DSM-IV (The Diagnostic and Statistical Manual of Mental Disorders) to paranoid, disorganized, catatonic, undifferentiated, and residual types. Schizophrenia is characterized by faint pathology and has both sporadic and hereditary forms. The common pathologic aspect of schizophrenia is excessive dopaminergic transmission in striatal and mesolimbic areas that can be abated by dopamine D_2 receptor antagonists, and deficit of dopamine signaling in prefrontal cortex (Karam *et al.* 2010). An alternative hypothesis for the development of schizophrenia symptoms involves muscarinic receptors. Clinical trials provided evidence that muscarinic agonists are moderately effective as antipsychotic agents (Biel *et al.* 1962, Mego *et al.* 1988). Moreover, it has been shown that the levels of both M_1 and M_4 receptors are reduced in the prefrontal cortex, hippocampus, caudate and putamen in *post mortem* samples from schizophrenic patients (Dean *et al.* 1999, 2002, Crook *et al.* 1999, 2000, 2001). From studies in knockout mice, the M_1 receptor subtype has been viewed as the most likely candidate for mediating effects on cognition, attention mechanisms, and sensory processing so reduction in M_1 receptors may be the cause of cognitive symptoms of schizophrenia. The M_4 receptor is localized in dopamine rich brain regions (the mesolimbic dopaminergic pathway), and regulates dopamine levels in this region (Tzavara *et al.* 2004). Thus the "dopamine hyperfunction hypothesis" and the "cholinergic hypothesis" of schizophrenia are compatible.

The importance of the cholinergic system in schizophrenia has been further validated clinically by the use of clozapine, one of the most clinically useful atypical antipsychotics (Kane *et al.* 1988, Hagger *et al.* 1993, Goldberg and Winberger 1994). Numerous studies suggest that the unique efficacy of clozapine is due to its major circulating metabolite, N-desmethylclozapine (NDMC) acting as an M_1 ectopic agonist (Weiner *et al.*

2004, Burstein *et al.* 2005, Davies *et al.* 2005) in combination with its inhibition of D_2 receptors. Taken together M_1 and M_4 selective agonists have a potential to alleviate cognitive deficits and positive symptoms of schizophrenia. The studies with positive allosteric modulators of acetylcholine at M_4 receptors VU0152099 and VU0152100 (Brady *et al.* 2008, Shierey *et al.* 2008, Byun *et al.* 2011) provide further support for the "cholinergic hypothesis" of shizophrenia.

Overactive bladder

Current therapy of overactive bladder relies on inhibition of M_3 (and M_2) receptors of lower urinary tract smooth muscles by long acting muscarinic antagonists (LAMAs) (Smith and Wein 2010). LAMAs produce symptomatic improvement by decreasing detrusor overactivity, increasing bladder capacity, and reducing urgency and urge of urinary incontinence and frequency (Smith and Wein 2010). LAMAs, however, exert adverse effects, mainly dry mouth and constipation, probably due to the lack of binding selectivity. Their effect is primarily based on slower kinetics at M_3 receptors (Hegde 2006, Sykes *et al.* 2012). Thus, there is room for improvement of LAMAs in binding selectivity that would be beneficial in dose lowering and diminution of side effects. Importantly, currently available LAMAs do not possess the O-hexyl group that is responsible for xanomeline wash-resistant binding (Jakubík *et al.* 2004). Combination of potential M_3 selective antagonists with O-hexyl groups may thus open an avenue to synthesize new classes of LAMAs.

Drug addiction

Drug addiction is a disease that is not primarily caused by cell damage. Addictive drugs impact regular learning to reinforce their own intake. In general, addictive drugs increase dopaminergic transmission in the striatum (Sulzer 2011). Blocking of M_5 receptors has been shown to reduce reinforcement and withdrawal symptoms of morphine (Basile *et al.* 2002) as well as cocaine addiction (Lester *et al.* 2010). Occurrence of M_5 receptors in the body is limited to cerebral blood vessels (Yamada *et al.* 2001) and neurons of specific regions of brain-ventral tegmental area of substantia nigra, hippocampus, and striatum (Yamada *et al.* 2003, Raffa 2009). In the striatum M_5 receptors located on dopaminergic nerve terminals facilitate muscarinic agonist-induced dopamine release, a key process of drug addiction events of reward, reinforcement and withdrawal (Koob and Volkow 2010, Morales and Pickel 2012). Moreover, striatum innervating dopaminergic neurons almost exclusively express the M_5 receptor subtype (Yamada *et al.* 2001). Therefore M_5 antagonists have potential therapeutic use for treatment of drug addiction and abuse with minimum side effects. No M_5 selective antagonists are known so far (Eglen *et al.* 2006, Raffa 2009, Stahl *et al.* 2010). Search for ectopic antagonists that bind to the less conserved parts of the receptor but still effectively block the receptor by interaction with the orthosteric site may be a way to obtain potent M_5 selective antagonists.

Conclusions

The major problem of muscarinic pharmacotherapy is the paucity of targets influencing of muscarinic neurotransmission. The use of anticholinesterases to strengthen transmission, e.g. in treatment of Alzheimer´s disease, by prolonging the presence of the natural agonist acetylcholine in the synaptic cleft does not discriminate among various signaling pathways activated by various muscarinic receptor subtypes and consequently suffers of many side-effects and a peril of overdosing. Despite this disadvantage cholinesterases inhibitors are up to now the only approved drugs for Alzheimer´s disease that demonstrate marked therapeutic benefits. Provided that presynaptic function is at least partially preserved, allosteric modulators of acetylcholine binding provide unusual selectivity and may serve as a drug for selective activation (e.g. in Alzheimer´s disease) or attenuation (e.g. in Parkinson´s disease) of neurotransmission mediated by different muscarinic receptors. When presynaptic function is severly compromised, the utilization of ectopic agonists can be a thinkable solution. Unfortunately, in either case, no clinically exploitable drugs have been generated yet.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

This work was supported by the Academy of Sciences of the Czech Republic project AV0Z 50110509 and support RVO:67985823, the Grant Agency of the Czech Republic grants 305/09/0681 and P304/12/G069, EU FP7 project LipiDiDiet (Grant Agreement No 211696).

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PERSPECTIVES FOR DESIGN OF SELECTIVE MUSCARINIC AGONISTS

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Abstract

In this lecture we reviewed the therapeutic potential of muscarinic agonists in Alzheimer's disease and schizophrenia, summarized current status of the development of muscarinic subtype-selective agonists and discussed difficulties in design of these agents due to conservation of the orthosteric acetylcholine binding site among subtypes of muscarinic receptors. In the end we analyzed allosteric properties of selective muscarinic agonists and compared their therapeutic potential with allosteric modulators / potentiators of acetylcholine binding and action. Both laboratory experiments and current clinical studies demonstrate usefulness of selective reinforcement of muscarinic transmission in the treatment of Alzheimer's disease and schizophrenia.

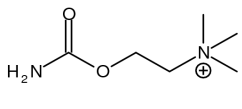
Physiological role of muscarinic acetylcholine receptors

Acetylcholine (ACh) is an important neurotransmitter in both the central (CNS) and peripheral nervous system acting through muscarinic and nicotinic acetylcholine receptors. Evidence suggests that cholinergic neurotransmission in the forebrain regions and cholinergic involvement in learning and memory are mediated primarily by muscarinic acetylcholine receptors (mAChRs) (Levey 1993). The muscarinic acetylcholine receptors are members of the G-protein-coupled receptor (GPCR) family A and mediate the metabotropic actions of the neurotransmitter ACh. To date, five distinct subtypes of mAChRs (M_1-M_5) have been cloned and sequenced (Bonner et al. 1987). M_1, M_3 and M_5 subtypes preferentially activate phospholipase C and calcium mobilization through $G_{q/11}$ whereas M_2 and M_4 receptors inhibit the activity of adenylyl cyclase or modulate conductance of ion channels through $G_{i/o}$ family of G-proteins (Caulfield 1993). mAChR-regulated cholinergic signaling plays a critical role in a wide variety of CNS and peripheral functions including learning, memory and attention mechanisms, motor control, nociception, regulation of sleep-wake cycles, cardiovascular function, renal and gastrointestinal functions, and many others. A wide variety of CNS disorders including Alzheimer's disease (AD), Parkinson's disease, schizophrenia, epilepsy, sleep disorders, neuropathic pain, and others involve malfunction of muscarinic transmission. This implies that agents selectively modulating the activity of specific mAChRs may have therapeutic potential in multiple pathological states (Felder et al. 2001; Langmead et al. 2008).

Role of muscarinic receptors in Alzheimer‘s disease

The most important role of mAChRs-mediated cholinergic neurotransmission in CNS relates to cognitive function. Disruptions of the cholinergic system in rodents revealed that its function is important for short and long-term memory processing (Brito et al., 1983; Meck et al., 1987). Clinical studies with muscarinic receptor agonists demonstrated the potential for this class of compounds to reverse cognitive deficits associated with disrupted cholinergic neurotransmission. For instance, in AD clinical studies, the inhibitor of acetylcholinesterase physostigmine and the muscarinic receptor agonist arecoline (Fig. 1, compound **3**) have been shown to improve cognition (Christie et al., 1981). Several lines of evidence indicate that the most prominent adverse effects of acetylcholinesterase inhibitors and non-selective muscarinic agonists are mediated by activation of peripheral M_2 and M_3 receptors. They include bradycardia, gastrointestinal distress, excessive salivation, and sweating (Bymaster et al., 2003; Wess et al., 2007). In contrast, distribution of M_1 receptors in the forebrain and the deleterious effects of M_1 antagonists on memory and learning indicate a primary role of this subtype of mAChRs in cognition, attention mechanisms, and sensory processing (Langmead et al., 2008). Therefore, enormous effort has been devoted to develop selective M_1 agonists. In addition to the potential symptomatic cognitive benefits provided by muscarinic receptor agonists, recent findings suggest that these drugs impinge the underlying key pathological process in AD (Growdon, 1997; Hock et al. 2003; Nitsch 1996) because M_1 muscarinic receptor agonists promote non-amyloidogenic processing of the amyloid precursor protein (APP) (cleavege by a-secretase in the middle of the Aβ sequence) that prevents formation of noxious amounts of Aβ fragments (Caccamo et al., 2006; Jones et al., 2008). This shift in APP processing is mediated by ERK1/2 and PKC activation (Caccamo et al., 2006). Involvement of muscarinic receptors in accelerating progression of the disease is supported by demonstration of accelerated amyloid pathology in Parkinson´s disease patients treated with muscarinic receptor antagonists (Perry et al., 2003). Similarly, the decline in strength of muscarinic signal transduction in cerebral cortex that develops along with soluble β-amyloid accumulation and markedly precedes behavioral impairments and amyloid pathology has been demonstrated in a transgenic mouse model of Alzheimer´s disease (Machova et al. 2008 and 2010).

Classical agonists



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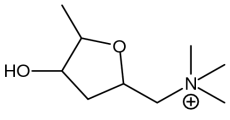

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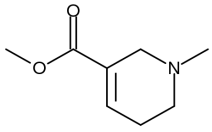

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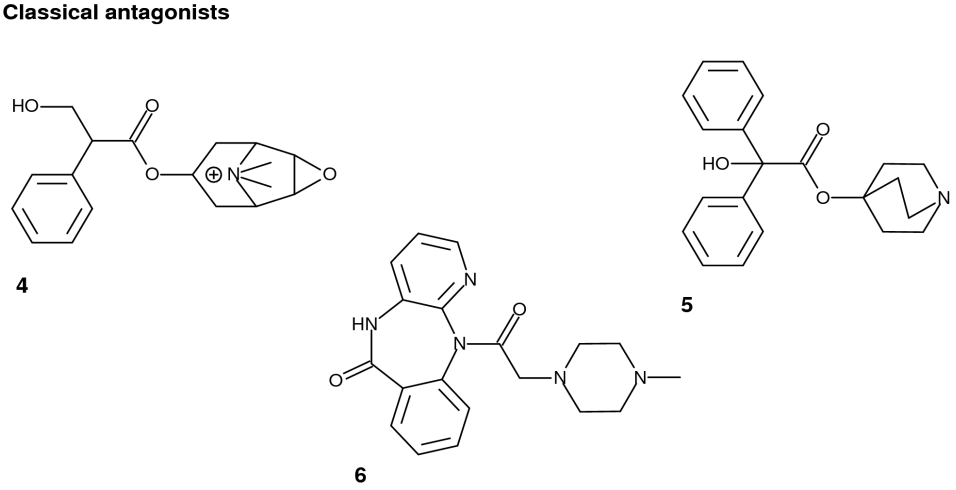

Fig. 1. Structures of classical agonists and antagonists

1	carbachol	2-carbamoyloxyethyl(trimethyl)azanium
2	muscarine	[(2S,4R,5S)-4-hydroxy-5-methyloxolan-2-yl]methyl-trimethylazanium
3	arecoline	methyl 1-methyl-3,6-dihydro-2H-pyridine-5-carboxylate
4	N-methylscopolamine	(1*R*,2*R*,4*S*,7*S*)-7-{[(2*S*)-3-hydroxy-2-phenylpropanol]oxy}-9,9-dimethyl-3-oxa-9-azoniatricyclo[3.3.1.$0^{2,4}$]nonane
5	quinuclidinyl benzilate	1-azabicyclo[2.2.2]octan-8-yl 2-hydroxy-2,2-diphenylacetate
6	pirenzepine	1-[2-(4-methylpiperazin-1-yl)acetyl]-5H-pyrido[2,3-b][1,4]benzodiazepin-6-one

Role of muscarinic receptors in schizophrenia

Schizophrenia is a diagnosis that covers a set of disorders of different etiologies with the same symptoms. This disorder can be divided based on character of negative symptoms to deficit and non-deficit ones or according to DSM-IV (The Diagnostic and Statistical Manual of Mental Disorders) to paranoid, disorganized, catatonic, undifferentiated, and residual types. The common etiological aspect of schizophrenia is believed to be based upon dysregulation of dopamine and glutamate neurotransmission in the mesolimbic and mesocortical brain regions, excessive dopaminergic transmission in the forebrain – so-called "dopamine hypothesis" or "dopamine hyperfunction hypothesis". An alternative hypothesis for the etiology of schizophrenia, based on involvement of mAChRs, surfaced from clinical observations that anticholinergic agents, such as the antagonist scopolamine, were shown to induce a psychotic state similar to schizophrenia and exacerbate symptoms in schizophrenic patients. Clinical trials provided evidence that muscarinic agonists were moderately effective as neuroleptic agents (Biel et al., 1962; Mego et al., 1988). In the neuropathological studies it has been shown that levels of both M_1 and M_4 receptors are reduced in the prefrontal cortex, hippocampus, caudate and putamen in postmortem samples from schizophrenic patients (Dean et al., 1999, 2002; Crook et al., 1999, 2000, 2001). Knock-out studies have been employed to further link mAChR to the pathology of schizophrenia (Bymaster et al., 2003; Raedler et al., 2007). From studies in knock out mice, the M_1 receptor subtype has

been viewed as the most likely candidate for mediating the effects on cognition, attention mechanisms, and sensory processing. The M_4 receptor is localized in dopamine rich brain regions (the mesolimbic dopaminergic pathway), and regulates dopamine levels in this region (Tzavara et al., 2004). Thus the "dopamine hyperfunction hypothesis" and the "cholinergic hypothesis" of schizophrenia are compatible. Another study found that the attenuation of amphetamine-induced activity by the muscarinic agonist xanomeline (Fig. 2, compound **12**) was absent in M_4 knock out mice and attenuated in M_1 knock out mice (Wool et al., 2009). The authors conclude that the efficacy of xanomeline in amphetamine-induced hyperlocomotion is predominantly driven by M_4 receptors on dopaminergic neurons involved in motor control, cognition and psychosis. The importance of the cholinergic system in schizophrenia has been further validated clinically by the use of clozapine (Fig. 2 **7**), one of the most clinically useful atypical antipsychotics (Kane et al., 1988; Hagger et al., 1993; Goldberg et al., 1994). Numerous studies suggest that the unique efficacy of clozapine is due to its major circulating metabolite, N-desmethylclozapine (NDMC) (Fig. 2, compound **8**) acting as an M_1 allosteric agonist (Weiner et al., 2004; Davies et al., 2005; Burstein et al., 2005) in combination with its inhibition of D_2 receptors.

Together these studies clearly demonstrate the potential benefit of muscarinic receptor agonists in the treatment of cognitive deficits in diseases such as schizophrenia and AD. However, results from clinical studies with mAChR agonists have also highlighted the need to identify which muscarinic receptor subtype(s) underlies the pro-cognitive actions of muscarinic agonists and to develop appropriate subtype selective ligands.

Getting selective musacrinic ligands

Identification and cloning of individual muscarinic receptors (Bonner et al., 1987) and subsequent availability of heterologous expression systems catalyzed search of selective muscarinic ligands. However, due to high sequence conservation within the orthosteric binding site for all five mAChR subtypes it has proven to be difficult to develop mAChR subtype selective agonists that bind to the orthosteric (acetylcholine binding) site (Heinrich et al., 2009). Search for selective muscarinic antagonists has been far more successful than search for selective agonists. Up todate more or less selective antagonists for all muscarinic subtypes (except M_5) have been identified (Böhme et al., 2002) while only few agonists with limited selectivity are known so far (see below). The reason why it is harder to get selective agonists than antagonists is that in general agonists are smaller molecules than antagonists (Fig. 1) and bind exclusively to the conserved amino acids in the orthosteric binding site, whereas relatively bigger antagonists additionally interact with less conserved amino acids in the close vicinity of the orthosteric binding site. In principle there are two possible approaches to design selective agonists. The first is through combination of two chemical moieties; an agonist structure connected by a flexible linker to a second moiety that binds to the less conserved extracellular domain of the receptor. The latter confers selectivity among subtypes and thus serves "addressing" function (Disingrini et al., 2006; Anthony et al., 2009). Another approach to attain selectivity of muscarinic agonists is to develop relatively bulky agonists like AF102B (Fig. 2 compound 10) (Fisher et al., 1989) or WAL2014 (Fig. 2, compound 11) (Ensinger et al., 1993). These compounds, however, do not posses long „arms" that could

reach far out of the orthosteric binding site to interact with distal less conserved parts of the receptor (like the O-hexyl moiety of xanomeline, Fig. 2, compound 12). The M_1-selective agonist AF102B differs from the non-slective AF102A only in the stereochemistry of the 2-methyl group (cis for alanog B and trans for analog A). The same applies to other AF102 isomeric analogues (Fisher et al., 1991). Although the exact molecular mechanism of M_1 selectivity of the cis compomuds is not fully understood, it is very likely that it is due to interaction of their small alkyl moieties in the cis orientation with amino acids in the tight vicinity of acetylcholine binding site that are unique to the M_1 receptor subtype.

Selective muscarinic agonists

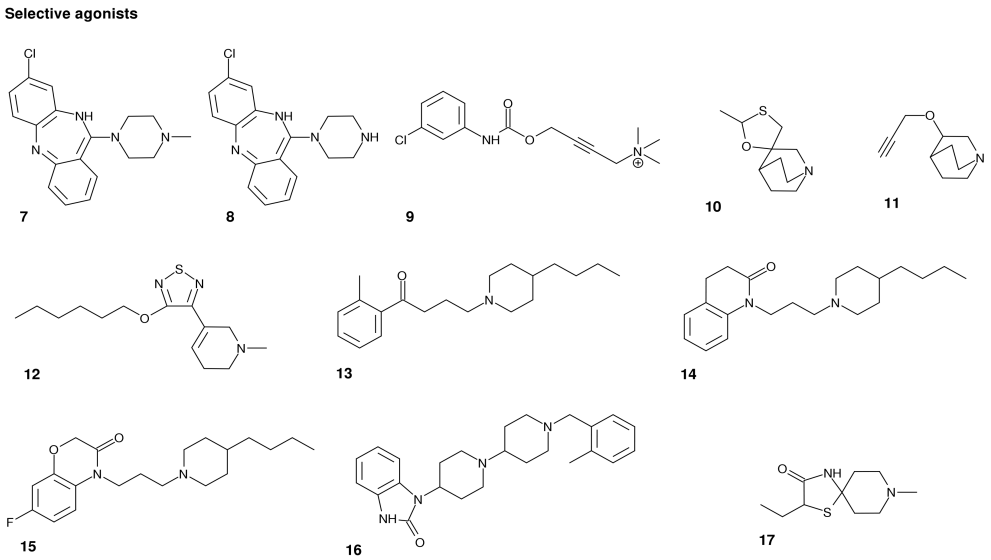

Fig. 2. Structures of selective agonists

7	clozapine	3-chloro-6-(4-methylpiperazin-1-yl)-5H-benzo[c][1,5]benzodiazepine
8	N-desmethylclozapine	3-chloro-6-piperazin-1-yl-5H-benzo[c][1,5]benzodiazepine
9	McN-A-343 (lopac)	4-[(3-chlorophenyl)carbamoyloxy]but-2-ynyl-trimethylazanium
10	AF102B (cevimeline)	cis-2-methyl-spiro(1,3-oxathiolane-5,3')quinuclidine
11	WAL 2014 (talsaclidine)	(8R)-8-prop-2-ynoxy-1-azabicyclo[2.2.2]octane
12	xanomeline	3-hexoxy-4-(1-methyl-3,6-dihydro-2H-pyridin-5-yl)-1,2,5-thiadiazole
13	AC-42	4-n-Butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl]-piperidine
14	77-LH-28-1	(1-[3-(4-butyl-1-piperidinyl)propyl]-3,4-dihydro-2(1H)-quinolinone)
15	AC-260584	(4-[3-(4-butylpiperidin-1-yl)-propyl]-7-fluoro-4*H*-benzo[1,4]oxazin-3-one)
16	TBPB	([1-(1 - 2-methylbenzyl)-1,4_-bipiperidin-4-yl)-1*H*-benzo[*d*]imidazol-2(3*H*)-one])
17	AF267B	(2S)-2-ethyl-8-methyl-1-thia-4,8-diazaspiro[4,5]decan-3-one

Based on the need to treat AD, M_1 selective agonists have been intensively searched to avoid severe side effects of inhibitors of acetylcholinesterase or non-selective agonists. Compound McN-A-343 (lopac) (Fig. 2, compound **9**) was synthesized and reported to be M_1 selective in primary tissues before the cloning of individual muscarinic receptor subtypes and its putative selectivity was based solely on its interaction with musacrinic antagonists (e.g. M_1 selective antagonist pirenzepine, Fig. 1, compound **6**). Its selectivity was later questioned. It is now known that McN-A-343 is not selective and, in addition, acts as a nicotinic agonist. The first agonists with some selectivity towards M_1 receptors were AF102B (cevimeline) (Fig. 2, compound **10**) that reversed cognitive impairments in a step-through passive avoidance task and in an 8-arm radial maze (Fisher et al., 1989) and WAL 2014 (talsaclidine) (Fig. 2, compound **11**) that exhibited full agonist properties in the rabbit vas deferens (putative M_1 receptor) and behaved like a partial agonist at M_2 receptors in the atrium and M_3 receptor in the ileum (Ensinger et al., 1993). Other putative M_1 selective agonists are discussed below.

Xanomeline

Xanomeline (Fig. 2, compound **12**) was initially reported to be M_1 and later M_1 and M_4 selective agonist based on its functional effects *in vivo*, *ex vivo* and *in vitro*. In Phase II and Phase III clinical trials xanomeline has been shown to improve cognitive performance in AD patients but the trials were discontinued due to intolerable gastrointestinal and cardiovascular side effects. Interestingly, in addition to improving cognitive performance, xanomeline had robust effects on the psychotic symptoms and behavioral disturbances associated with AD, including hallucinations, delusions and vocal outbursts (Bodick et al., 1997). Based on the ability of xanomeline to reduce the psychotic behavior in AD patients an effort to evaluate this agonist as an antipsychotic agent was initiated (Shannon et al., 2000; Stanhope et al., 2001). In a clinical study xanomeline showed improvements in Brief Psychiatric Rating Scale and Positive and Negative Syndrome Scale scores as well as in verbal learning and short-term memory in schizophrenic patients (Shekar et al., 2008).

AC-42 and its analogues

Similar to xanomeline, Acadia‘s compound AC-42 (Fig. 2, compound **13**) fully activates M_1 receptors and displays allosteric properties (Spalding et al., 2002). AC-42 is highly selective for M_1 relative to other muscarinic receptor subtypes. Mutations that render the M_1 receptor insensitive to activation by acetylcholine do not alter the activity of AC-42. Selectivity of AC-42 is thus likely accomplished by targeting a site distinct from the acetylcholine binding site. On the other hand the activity of AC-42 can be eliminated by mutations in transmembrane domains one and seven that do not alter receptor activation by acetylcholine (Spalding et al., 2006; Lebon et al., 2009). From a series of AC-42 analogues a compound 77-LH-28-1 (Fig. 2, compound **14**) was identified as a systematically active M_1 allosteric agonist (with partial agonism at M_3) (Langmead et al., 2008). Another Acadia‘s AC-42-based agent AC-260584 (Fig. 2, compound **15**) displayed high

potency and full efficacy (compared to carbachol), exerted pro-cognitive action and was found to be orally bioavailable in rodents (Bradley et al., 2009).

N-desmethylclozapine

Clozapine (Fig. 2, compound **7**) was developed as a D_2 receptor antagonist to treat schizophrenia according to the "dopamine hyperfunction hypthesis" of psychosis and was the first of atypical antipsychotics introduced to clinical practice in 1971. Clozapine was withdrawn by the manufacturer in 1975 after it was shown to cause agranulocytosis that led to death in some patients. In 1989, when studies demonstrated that it was more effective than any other antipsychotic for treating schizophrenia, clozapine was approved for clinical use in treatment-resistant schizophrenia. Clinical studies show clozapine's ability to improve negative symptoms of schizophrenia (Breier et al., 1994; Brar et al., 1997; Rosenheck et al., 1999; Gaertner et al., 2002; Zoccali et al., 2004). So far clozapine remains the only antipsychotic in clinical practice that alleviates negative symptoms. Interestingly, it is clozapine's major metabolite N-desmethylclozapine (NDMC) (Fig. 2, compound **8**) that acts as M_1 agonist (Weiner et al., 2004; Davies et al., 2005; Burstein et al., 2005). So alleviation of negative symptoms can be attributed to NDMC. The binding of NDMC to muscarinic receptors is not surprising as it is structurally close to another benzodiazepine, pirenzepine (Fig. 2, compound **6),** an M_1 selective antagonist, but considering its large size it is surprising that NDMC acts as an agonist.

The second generation of M_1 selective agonists

Based on robust structure-activity relationship (SAR) of known M_1 selective agonists the second generation of M_1 allosteric agonists exerting antipsychotic and procognitive effects was generated. TBPB (Fig. 2, compound **16**) is a potent, highly selective and centrally penetrating M_1 allosteric agonist (Jones et al., 2008). In addition, at low doses TBPB showed efficacy in reversing scopolamine-induced memory deficits and regulation of non-amyloidogenic APP processing in vitro, suggesting ability to provide both symptomatic and disease-modifying effects in AD patients. Subsequent studies (Bridges et al., 2008, Miller et al., 2008) showed that TBPB analogues are full or partial M_1 allosteric agonists and that halogenated ones inhibit D_2 receptors like NDMC.

SAR of the spirone-based prototype agonist AF102B (Fig. 2, compound **10**) yielded a promising analogue coded AF267B (Fig. 2, compound **17**) that when administered peripherally attenuated major hallmarks of AD (rescued the cognitive deficits in spatial tasks but not contextual fear conditioning and reduced production of both the A□ and phosphorylated tau protein in the hippocampus and cortex, but not in the amygdala) (Caccamo et al., 2006; Fisher, 2007).

Unique properties of xanomeline

Besides its high affinity reversible binding, xanomeline (Fig. 2, compound **12**) also binds to muscarinic receptors in a manner resistant to washing (Christopoulos et al., 1997) with a half-life over 30 hours (Jakubik et al., 2002). Formation of xanomeline wash-resistant (WR) binding cannot be prevented by the orthosteric antagonists atropine

or N-methylscopolamine (Jakubik et al., 2002). The necessity of the presence of lipid environment for formation of xanomeline WR binding and SAR of xanomeline analogs differing in the length of the O-hexyl chain suggest that xanomeline WR binding involves interhelical penetration of the M_1 muscarinic receptor by the compound's O-alkyl chain and interaction with membrane lipids surrounding the receptor (Jakubik et al., 2004). The selectivity of xanomeline is based on its functional effects in vivo, ex vivo, and in vitro. However, in binding experiments xanomeline displays the same affinity both for high affinity reversible and low affinity WR binding at all subtypes of mAChRs (Jakubik et al. 2006). Several lines of evidence suggest that the differential kinetics of WR xanomeline binding may constitute the basis for xanomeline functional selectivity (Jakubik et al., 2006; Machova et al., 2007). Interestingly, although acute xanomeline action at M_5 receptors is (partially) agonistic, WR xanomeline acts as antagonist at this receptor subtype (Grant et al., 2005). Current results from our laboratory show that differential kinetics of xanomeline WR binding and action is reflected in differences in both short-term (seconds) and long-term (minutes and hours) changes in levels of intracellular calcium of CHO cells expressing individual subtypes of mAChRs (Šantrůčková et al., manuscript in preparation) and thus may constitute the basis for xanomeline functional selectivity.

Allosteric properties of selective muscarinic agonists

The common feature of emerging selective muscarinic agonists is their binding to the amino acids out of the acetylcholine orthosteric binding site towards extracellular domain. Therefore they are also named ectopic (on top of) agonists or allosteric (Greek for "other site") agonists or bi-topic (bind to two domains) agonists. The nuance between ectopic and allosteric agonist is that while an ectopic agonist binds to amino acids away from the orthosteric binding site it activates it through interaction with the orthosteric site, an allosteric agonist is totally capable of receptor activation at a site distinct from the orthosteric domain. Bi-topic ligands (e.g., xanomeline) are capable of activating the receptor both from the orthosteric and allosteric sites. Allosteric and bi-topic agonists exert typical allosteric properties, i.e. change in the binding kinetics of orthosteric ligands (Jakubik et al., 2002). Incidentally, the first published allosteric agonists that weakly stimulated production of inositol phosphates in CHO cells expressing the M_1 or the M_3 receptors and inhibited synthesis of cAMP in CHO cells expressing the M_2 or the M_4 receptors (Jakubik et al., 1996) were prototypic allosteric modulators of muscarinic receptors, e.g., alcuronium, gallamine, and strychnine.

From allosteric agonists to allosteric modulators

An allosteric modulator is a ligand that binds to an allosteric site on the receptor and changes receptor conformation to produce increase (positive cooperativity) or decrease (negative cooperativity) in the binding or action of an orthosteric agonist (e.g., acetylcholine). As opposed to classical agonists, positive allosteric modulators of natural neurotransmitters have the following advantages: 1/ They mimic neurotransmission under pysiological conditions – preserve time and space pattern of the signal. 2/ Greater subtype selectivity can be obtained as neutral cooperativity (no change in binding or action of classical agonist

upon binding of the allosteric modulator) equals to no binding to any particular receptor subtype. 3/ The magnitude of the effects of an allosteric modulator on action of a natural neurotransmitter is limited by the magnitude of allosteric interaction (Kenakin, 2007). The effects of an allosteric modulator reach a maximum that is not excedded by increasing the dose. 4/ Positive modulation at one subtype may be combined with negative modulation at the other. For example, common cholinergic synapses in the forebrain contain M_1 postsynaptic receptors and M_2 presynaptic receptors that mediate feedback inhibition of acetylcholine release. Positive allosteric modulation of postsynaptic M_1 receptors and negative modulation of presynaptic M_2 receptors by a given allosteric modulator would have desired synergistic effects.

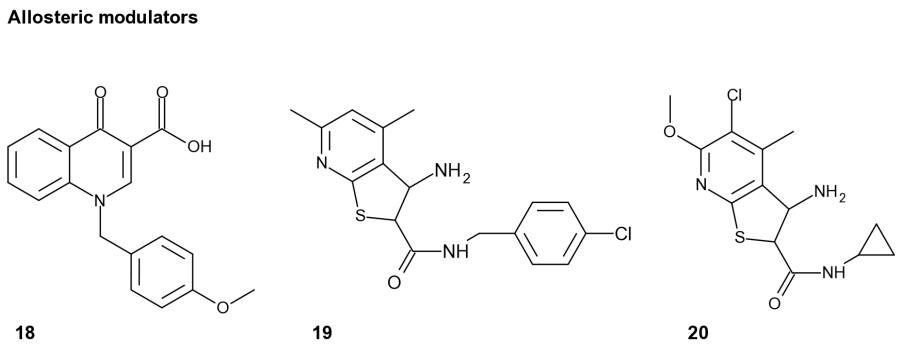

Fig. 3 Structures of positive allosteric modulators of acetylcholine binding

18	BQCA	1-(4-methoxy)-4-oxo-1,4-dihydroquioline-3-carboxylic acid
19	VU0010010	3-amino-*N*-(4-chlorobenzyl)-4,6-dimethylthieno[2,3-b]pyridine-2-carboxamide
20	LY2033298	3-amino-5-chloro-6-methoxy-4-methyl-thieno[2,3-b]pyridine-2-carboxylic acid cyclopropylamide

Early proof-of concept studies by several laboratories were successful in identifying positive allosteric modulators of acetylcholine binding at M_1, M_3 and M_4 receptors (Jakubik et al., 1997; Lazareno et al., 1998, 2002, 2004; Lanzafame and Christopoulos, 2004). However, these compounds lacked efficacy and physiochemical properties in *in vivo* studies. A major breakthrough in the reseach for muscarinic allosteric modulators was the discovery of BQCA (Fig. 3, compound **18**), the positive allosteric modulator of acetylcholine binding and action at M_1 receptors that positively regulates non-amyloidogenic APP processing *in vitro* (Ma et al., 2009, Shirley et al., 2009). Besides the expected procognitive effects BQCA also increased blood flow to cerebral cortex that is beneficial in neurodegenerative diseases like AD. Also two very effective positive allosteric modulators with high M_4 selectivivity showed promising results in rats *in vivo* were recently discovered: VU0010010 (Fig. 3, compound **19**) (Barady et al., 2008) and LY2033298 (Fig. 3, compound **20**) (Chan et al., 2008). However, allosteric modulation of neurotransmitter action is not always in concert with modulation of its binding. Our study shows that although the allosteric modulator rapacuronium strongly decreases affinity of mAChRs for acetylcholine it accelerates its

binding at M_3 receptors that leads to facilitation of its action *in vitro* (Jakubik et al., 2009) that explains severe brochospasm observed *in vivo* (Goudsouzian, 2001).

Conclusions

Both historical clinical data and current preclinical and clinical data on new compounds acting as selective activators or potentiators of M_1 and/or M_4 receptors suggest that selective mAChR activation has high potential in the treatment of schizophrenia and AD. Muscarinic ectopic agonists, allosteric agonists and allosteric modulators target structural diversity of the domains of muscarinic receptors and therefore have a different mode of action in comparison to orthosteric agonists. Targeting these unique domains in synthesis of highly selective activators or potentiators of mAChRs has proven to be useful in development of novel antipsychotic agents. Provided that cholinergic synapses are preserved, positive allosteric modulators of acetylcholine action are theoretically superior to subtype selective muscarinic agonists in modulating cholinergic neurotransmission. Current *in vivo* and clinical studies demonstrate usability of muscarinic allosteric agonists in the treatment of AD and schizophrenia.

Aknowledgement

This work was supported by Project AV0Z 50110509, the grants of the Grant Agency of the Czech Republic 305/09/0681, the Grant Agency of the Czech Academy of Sciences IAA500110703, and the Ministry of Education, Youth and Sports, Czech Republic LC554, and NIH grant NS25743.

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