

An antibody biosensor establishes the activation of the M1 muscarinic acetylcholine receptor during learning and memory

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Establishing the *in vivo* activation status of G protein-coupled receptors (GPCRs) would not only indicate physiological roles of GPCRs but would also aid drug-discovery by establishing drug:receptor engagement. Here we develop a phospho-specific antibody-based biosensor to detect activation of the M₁ muscarinic acetylcholine receptor (M₁ mAChR) *in vitro* and *in vivo*. Mass spectrometry phosphoproteomics identified 14 sites of phosphorylation on the M₁ mAChR. Phospho-specific antibodies to four of these sites established that serine at position 228 (S²²⁸) on the M₁ mAChR showed extremely low levels of basal phosphorylation that were significantly up-regulated by orthosteric agonist stimulation. In addition, the M₁ mAChR positive allosteric modulator, BQCA, enhanced acetylcholine-mediated phosphorylation at S²²⁸. These data supported the hypothesis that phosphorylation at S²²⁸ was

an indicator of M₁ mAChR activation. This was further supported *in vivo* by the identification of phosphorylated S²²⁸ on the M₁ mAChR in the hippocampus of mice following administration of the muscarinic ligands xanomeline and BQCA. Finally, S²²⁸ phosphorylation was seen to increase in the CA1 region of the hippocampus following memory acquisition, a response that correlated closely with up-regulation of CA1 neuronal activity. Thus, determining the phosphorylation status of the M₁ mAChR at S²²⁸ not only provides a means of establishing receptor activation following drug treatment both *in vitro* and *in vivo* but also allows for the mapping of the activation status of the M₁ mAChR in the hippocampus following memory acquisition thereby establishing a link between M₁ mAChR activation and hippocampal based memory and learning.

G protein-coupled receptors (GPCRs) respond to the binding of their cognate ligands by transitioning from an inactive to an active conformation capable of engaging with intracellular signalling cascades (1-3). Whereas this process has been described in exquisite pharmacological detail (4,5), and biophysically in recent crystal structures (2,6), correlating the activation state of a GPCR subtype *in vivo* to a physiological response or drug treatment is extremely challenging and presents a considerable barrier to establishing the physiological role of GPCRs and the on-target action of GPCR ligands.

Progress in this area has been made in transfected systems where it has been possible to monitor receptor conformational changes in response to ligand occupation using fluorescent resonance energy transfer (FRET). In these studies, changes in the energy transfer between FRET-acceptor and FRET-donor moieties, engineered within the receptor sequence, provides a read-out of changes in receptor conformation on agonist binding (7-10). Alternatively, a green fluorescent protein (GFP)-biosensor based on a conformation-sensitive antibody (nanobody-80, Nb80) that preferentially recognises the active state of the β_2 -adrenoceptor has recently been employed to determine the active conformation of the β_2 -adrenoceptor at the plasma membrane and within intracellular compartments (11). These approaches, however, require transfection of either mutated receptors (7-10) or a GFP-biosensor (11), and therefore, although able to monitor receptor conformational changes quantitatively in living cells in real time, are restricted to heterologous systems.

An alternative approach considered here is to monitor the phosphorylation status of GPCRs as a read-out of receptor activation. This is based on the “classical” principle that the conformation adopted by a receptor upon agonist occupation reveals phosphorylation sites, often within the third intracellular loop and C-terminal tail, that otherwise are not accessible in the inactive receptor conformation (12). In this scenario, the phosphorylation status of a particular GPCR may serve as a read-out of the proportion of receptors that have adopted an active conformation. Therefore, phospho-specific antibodies to agonist-dependent receptor phosphorylation events could potentially be used as a probe for the activated receptor in not only recombinant

systems, but also in physiologically relevant tissues. If this were the case then it might be possible to correlate the activation status of GPCRs with physiological responses and, importantly for drug discovery, could be used to assess receptor engagement with synthetic ligands.

We test this notion here by focusing on the M_1 -muscarinic acetylcholine receptor (M_1 mAChR), which is one of five muscarinic receptor subtypes (M_1 - M_5) that respond to the natural ligand acetylcholine and is a subtype that has been implicated in a number of neurological processes (13,14) most notably learning and memory (15-17). We have shown previously that this receptor subtype is rapidly phosphorylated by agonist addition likely via members of the G protein-coupled receptor kinase family (18), although the involvement of other receptor kinases have not been ruled out. Here we used mass spectrometry based phospho-proteomics to determine the sites of receptor phosphorylation from which we developed a series of phospho-specific antibodies. This included an antibody to phosphoserine 228 (pS²²⁸) in the third intracellular loop, which we show is a phosphorylation event highly sensitive to agonist stimulation. This antibody was used here to probe the phosphorylation status of the M_1 mAChR following engagement with orthosteric and allosteric muscarinic ligands, both *in vitro* and *in vivo*. Furthermore, this antibody was used to determine that the M_1 mAChR was activated in specific regions of the hippocampus following fear conditioning in a manner that maps to the regions of the hippocampus showing increased synaptic activity during memory acquisition. In this way we not only link the activation status of the M_1 mAChR to memory acquisition but also establish the principle that phosphorylation sites can be used to probe the activation status of GPCRs during physiological responses and on drug treatment.

EXPERIMENTAL PROCEDURES

Materials

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich. Xanomeline was obtained from Eli Lilly and Co. (Lilly UK, Erl Wood Manor, Windlesham, Surrey, UK).

Generation of cell-lines

Chinese hamster ovary (CHO) cells that stably and constitutively expressed the C-terminal HA epitope-tagged mouse M_1 mAChR (CHO-M1

cells) were generated using the Flp-In™ system. CHO Flp-In cells were co-transfected with pcDNA5FRT containing M₁ mAChR and pOG44, transfected cells were selected with hygromycin B, and expression of M₁ mAChR was confirmed by immunoblotting with anti-HA antibodies and by [³H] N-methylscopolamine (NMS) radioligand binding.

[³²P]-Orthophosphate labelling and immunoprecipitation

Cells were plated in 6-well plates at 200,000 cells/well 24 h before experimentation. For phosphorylation experiments, cells were washed three times with Krebs/HEPES buffer without phosphate (118 mM NaCl, 1.3 mM CaCl₂, 4.3 mM KCl, 1.17 MgSO₄, 4.17 mM NaHCO₃, 11.7 mM glucose, 10 mM HEPES (pH 7.4)) and incubated in this buffer containing 100 µCi/ml ³²P orthophosphate for 1 h at 37 °C. Cells were stimulated for 5 min with test compounds and immediately lysed by addition of buffer containing 20 mM Tris (pH 7.4), 150 mM NaCl, 3 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate. The M₁ mAChR was immunoprecipitated from the cleared lysates using anti-HA affinity matrix (Roche Applied Science). The washed immunoprecipitates were separated by SDS-PAGE on 8% gels that were then dried, and radioactive bands were revealed using autoradiography film. The films were scanned and bands were quantified using AlphaImager software (Alpha Innotech, San Leandro, CA).

M₁ mAChR purification and mass spectrometry

Stably transfected CHO-M1 cells expressing mouse HA-tagged M₁ mAChR were grown until confluent in expanded surface rolling bottles at 0.25 rpm in a humidified CO₂ incubator. For receptor purification, cells from four rolling bottles were harvested, resuspended in 40 ml of Krebs/HEPES buffer, and stimulated with 100 µM acetylcholine for 5 min. Membranes were then prepared and solubilized by addition of 5 ml of PBS containing 1% Nonidet P-40 plus a mixture of protease and phosphatase inhibitors (Complete, Roche Applied Science). After centrifugation at 20,000 x g, the resulting supernatant was diluted 1:1 with PBS, and the receptor was then purified on anti-HA affinity matrix. After extensive washing with solubilization buffer containing 0.5% Nonidet P-40, the resin was resuspended in 2 x SDS-PAGE sample buffer. The sample was resolved by SDS-PAGE on 10% gels and stained with colloidal

Coomassie Blue. The band associated with the M₁ mAChRs was excised from the polyacrylamide and washed three times for 15 min with 100 mM triethylammonium bicarbonate (TEAB). Reduction and alkylation of cysteines were performed by addition of 10mM dithiothreitol in 50 mM TEAB at 60 °C for 30 min followed by addition of 100 mM iodoacetamide in 50mM TEAB for 30 min in the dark. Gel slices were washed three times for 5 min with 50 mM TEAB containing 50% acetonitrile, finally resuspended in TEAB containing 10% acetonitrile, and incubated overnight at 37 °C with 1 µg of sequencing grade trypsin (Promega, Southampton, UK). The resulting tryptic peptides were dried and resuspended in 1 ml of buffer containing 250 mM acetic acid and 30% acetonitrile, and phosphorylated peptides were enriched by addition of 20 µl of PHOS-Select™ iron affinity resin and incubation at room temperature for 2 h with mixing. After washing resin twice with loading buffer and once with water, tryptic phosphopeptides were eluted by addition of 200 µl of buffer containing 400 mM ammonium hydroxide and 30% acetonitrile. LC-MS/MS was carried out upon each sample using an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, Rockford, IL). Peptides resulting from in-gel digestion were loaded at high flow rate onto a reverse-phase trapping column (0.3-mm inner diameter x 1 mm) containing 5 µm C₁₈ 300-Å Acclaim PepMap medium (Dionex, UK) and eluted through a reverse phase capillary column (75-µm inner diameter x 150 mm) containing Symmetry C₁₈ 100-Å medium (Waters, Elstree, UK) that was self-packed using a high pressure packing device (Proxeon Biosystems, Odense, Denmark). The resulting spectra were searched against the UniProtKB/ Swiss-Prot database using Mascot (Matrix Science Ltd.) software with peptide tolerance set to 5 ppm and the MS/MS tolerance set to 0.6 Da. Fixed modifications were set as carbamidomethylcysteine with variable modifications of phosphoserine, phosphothreonine, phosphotyrosine, and oxidized methionine. The enzyme was set to trypsin/P, and up to two missed cleavages were allowed. Peptides with a Mascot score greater than 20 and for which the probability p that the observed match was a random event was <0.05 were included in the analysis. The spectra of peptides reported as being phosphorylated were

interrogated manually to confirm the precise sites of phosphorylation.

Generation of M_1 mAChR antiserum and phospho-specific M_1 mAChR antiserum

Phosphorylation specific antibodies, anti-phosphoserine 228, 273, 322 and 451 were raised against peptide sequences AALQGS_(p)ETPGKG, RLLQAYS_(p)WKEEE, KQPPKSS_(p)PNTVK and IPKRPGS_(p)VHRTTP corresponding to amino acid residues 223-234, 267-278, 316-327 and 445-456 of the mouse M_1 mAChR. The 87 day program which included 4 immunizations were performed by Eurogentec. The resulting antisera were purified against the immunizing peptides. To generate antibodies for immunoprecipitation and detection of M_1 AChR protein, rabbits and rats were immunized with the peptide RDRGGKGQKPRGKEQ that corresponds to amino acids 334-348 of the mouse M_1 mAChR. The resulting antiserum was purified against the immunizing peptide.

Radioligand binding assays

CHO-M1 cell membranes (50 μ g/tube) were incubated in HEPES buffer (50 mM HEPES, 110 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 1 mM MgSO₄, 25 mM glucose, 58 mM sucrose, pH 7.4) containing ~0.3 nM [³H]-NMS and increasing concentrations of acetylcholine in the presence of various concentrations of BQCA. Incubations were continued for 1 h at 37°C. Non-specific binding and filtration was carried out as above. Membrane-bound ligand was separated from free ligand by rapid filtration onto GF/B glass microfiber filters followed by three rapid washes with ice-cold 0.9% NaCl. Membrane bound radioactivity was determined by liquid scintillation (Perkin Elmer Ultima Gold) counting. Nonspecific binding was determined by the inclusion of atropine (1 μ M) during the incubation with [³H]-NMS.

M_1 mAChR immunoprecipitation from tissue samples

To establish the presence and phosphorylation status of M_1 mAChRs in preparations from the hippocampus, tissues from adult wild-type or adult M_1 mAChR knockout mice on a C57Bl6/NTAC background were dissected into ice cold HBSS buffer containing protease inhibitors and phosphatase inhibitors (Complete, Roche diagnostics). Membranes were prepared and solubilized in buffer composed of 20mM Tris [pH 7.4], 150mM NaCl, 3mM EDTA and 1%

NP-40. The receptor was immunoprecipitated with the anti M_1 mAChR polyclonal antibody. Immune complexes were washed 3 times in solubilization buffer and re-suspended in 2 x SDS PAGE sample buffer. Receptors were separated by SDS PAGE on 8% gels, transferred PVDF membranes and immunoblotted with anti-phosphoserine 228 antibodies or antibodies against the M_1 mAChR protein.

Immunocytochemistry

CHO-M1 cells expressing mouse HA-tagged M_1 AChR were seeded onto 20mm glass coverslips for 24 hours prior to experimentation. Cells were washed and incubated for 1 hour in Krebs/HEPES buffer prior to treatment. Cells were fixed in PBS containing 4% paraformaldehyde and 0.1% glutaraldehyde for 30 min at room temperature. Anti-HA antibody was used at 5ng/ml followed by Alexa FluorTM 546 goat anti rat secondary antibody at 1:1000. Anti phospho-specific serine 228 antibodies were used at 0.25 μ g/ml followed by goat anti rabbit Alexa fluorTM 488 secondary antibody at 1:1000. Data was acquired using an Axiovert 200M confocal laser scanning microscope (Zeiss).

Total [³H]-inositol phosphate accumulation assay

Cells seeded at 100 000 cells/well in 24-well plates were labelled with 2.5 μ Ci/ml myo-[³H]-inositol (PerkinElmer) for 24 hr at 37°C. Cells were washed twice in Krebs/HEPES buffer (pH7.4) and incubated with 10 mM LiCl for 20 min at 37°C. Appropriate concentrations of agonist were added for 20 min to stimulate [³H]-inositol phosphate production. Where allosteric interactions were studied, cells were pre-incubated with the allosteric modulator for 2 min prior the addition of agonist. Incubations were terminated by aspiration of buffer and rapid addition of 500 μ l ice-cold trichloroacetic acid (TCA) (0.5 M). After extraction on ice for 20-30 min samples were transferred to tubes containing 100 μ l EDTA (10 mM, pH 7.0) and 500 μ l of a 1:1 mixture of tri-n-octylamine and 1,1,2-trichlorofluoroethane added for 15 min at RT. After centrifugation at 20 000 x g for 2 min, 400 μ l of the upper aqueous phase was transferred to fresh tubes containing 60 mM NaHCO₃. [³H]-inositol mono-, bis-, and trisphosphate ([³H]InsPx) fraction was recovered by anion-exchange chromatography on Dowex AG1-X8

formate columns. Columns were regenerated with 10 ml of ammonium formate (2 M)/formic acid (0.1 M) and washed thoroughly with distilled water. Samples were applied to the columns and the columns washed with 10 ml distilled water. Columns were then washed with ammonium formate (60 mM)/sodium tetraborate (10 mM) solution. Total [^3H]InsPx was eluted in 10 ml of ammonium formate (0.75 M)/formic acid (0.1 M) and collected in large scintillation vials. A 5 ml aliquot from the eluate was mixed with 10 ml of SafeFluor scintillation cocktail and radioactivity was detected by liquid scintillation counting.

Fear conditioning training

Male C57Bl6/NTAC mice (8 to 15 weeks old) were placed in the conditioning chamber (Stoelting ANY-maze fear conditioning system) and after a 2 min adaptation period, received three tone/footshock pairings where a tone (conditioned stimulus; CS; 2.8 kHz; 85 dB; 30 s) always co-terminated with a the footshock (unconditioned stimulus; US; 2 s; 0.4 mA). The CS-US pairings were separated by 1 min intervals. After completion of training, the mice remained in the conditioning chamber for 1 min and were returned to their home cages. For the immediate footshock control, mice were put in the fear conditioning chamber, and a footshock was immediately delivered (2 s; 0.4 mA) 30 s after, they were returned to their home cages. Mice remained in their home cages for 30 min before being anaesthetized with 3% isoflurane (2 L/min O₂) and transcardially perfused with 4% PFA. Following fixation, brains were immediately removed, and further fixed overnight in 4% PFA.

Immunohistochemistry of mouse brain

Brains were processed in paraffin wax and sliced at 5 μm using a microtome. Following antigen retrieval, sections were washed in TBS containing 0.1% Triton x-100 and blocked for 2 h at RT in TBS, 0.1% Triton X-100, 10% goat serum and 5% BSA. Sections were incubated with antibodies to c-Fos (Santa Cruz), ARC (Santa Cruz) and M₁ AChR phospho-specific serine 228 at 2.5 $\mu\text{g}/\text{ml}$ in blocking buffer (overnight at 4°C). Sections were washed three times, and incubated with Alexa Fluor™ 488 fluorescent secondary antibodies for 1 h at RT in blocking buffer. Following three washes, slices were mounted in Vectashield hardset™ mounting medium with DAPI. All images were taken using

a Zeiss confocal microscope with Zen software (Zeiss).

Assay for ERK activity

Extracellular signal-regulated protein kinase 1/2 phosphorylation assay in CHO-M1 cells were seeded into transparent 96-well plates at 35,000 cells/well and grown overnight at 37°C. Cells were washed with PBS and incubated in serum-free α -MEM at 37°C for at least 4 h. Cells were incubated with varying concentrations of agonist in the presence and absence of increasing concentrations of an allosteric compound for 5 min (compounds were co-added). The reaction was terminated by the removal of compounds and addition of 50 μl of SureFire lysis buffer. The lysates were agitated for 30 min at room temperature and 4 μl of each lysate was transferred into a 384-well opaque Optiplate. SureFire detection mix (7 $\mu\text{l}/\text{well}$) consisting of detection reagent; activation reagent; donor beads and acceptor beads (660:110:11:11 v/v) were added to the plate. Plates were incubated in the dark at room temperature for 2 h with gentle agitation before fluorescence signal was measured using a PHERAStar™ plate reader.

Generation of M1 DREADD mice

Transgenic C57BL6/J mice which express a humanised, mutated form of the M₁ mAChR were generated by GenOway (Lyon, France). Briefly, humanizing mutations V5A, S254T, K320R, G337A and V413I were introduced into the mouse coding sequence to make the corresponding amino acid sequence identical to the human M₁ mAChR. In addition, two point mutations Y106A and A195G, were introduced which render the M₁ mAChR insensitive to acetylcholine and promote sensitivity to the synthetic compound clozapine-N-oxide (CNO). This receptor mutant was termed the M1 DREADD receptor. Finally, an HA-tag sequence YPYDVPDYA was appended to the C-terminus followed by a stop codon. The gene targeting construct consisted of long and short homology arms, the long arm consisted of a 5268 bp fragment containing the 3' part of intron 2 this was generated by PCR from genomic DNA template using primers 85426GAlm 5' GGGGACAACCTTTGTATAGAAAAGTTGTTG GGAGGATGCAGCCTCTCCA and 85427GAlm 5' GGGGACTGCTTTTTTTGTACAAACTTGGGC GCG

CCACGTACGCCTCTTCAAGGCTTAAGTGG
AATGAAGGGGCAGCCCC. The short arm
consisted of A 3188 bp fragment containing
3'UTR sequence of the exon 3 was amplified
from genomic DNA using primers 85424GAsm
5' ACTGTCAACCCCATGTGCTACGCAC and
85425GAsm 5'

GGGCTACAAGGGAGCATGAACAAGC. A
Diphtheria toxin negative selection cassette was
included to allow for selection of ES cells which
had undergone homologous recombination and a
positive selection neomycin gene and a STOP of
transcription cassette flanked by *loxP* sites were
inserted upstream of the *CHRM1* coding
sequence. This construct was transfected into
C57BL6 ES cells which were selected using
neomycin. Resistant colonies were screened to
verify correct integration of the 5' and 3'
homology arms by PCR using primers 85488 5'
GCAGGTCGAGGGACCTAATAACTTCG and
85489sa

5'AAGCTCTCAAGGTCCAACAGTTTCTGG
for short arm integration and primers 85487la 5'
AGTCTTACCAGGGAAGAAAGCCTGATCC
and 0070-Neo-16219sa 5'
CCTGCTCTTTACTGAAGGCTCTTTACTATT
GC for long are integration.

Positive clones were expanded and injected into
blastocysts derived from C57BL6/J female mice
to generate chimeric animals which were then
bred with C57BL6 wildtype C57BL6 mice to
generate heterozygous F1 animals. The
neomycin/STOP cassette was excised by
breeding heterozygous animals with Cre deleter
mice to generate M1 DREADD constitutive
knock-in mice. Genotyping was performed by
PCR on genomic DNA obtained from tail
biopsies using primers mM1 451 F 5' TTG GTT
TCC TTC GTT CTC TGG GC and mM1 1227 R

5' GAC GTA GCA AAG CCA GTA
GCC CAG C. The resulting PCR products were
subjected to restriction digestion with *SacI*, the
products of which discriminated between wt
C57BL6 and heterozygous and homozygous M1
DREADD animals.

RESULTS

Determination of the phosphorylation sites on the M₁ mAChR

Our initial evaluation of the phosphorylation status of the M₁ mAChR was conducted on Chinese hamster ovary cells expressing the mouse M₁ mAChR (CHO-M1 cells) stimulated with the natural ligand acetylcholine or the M₁/M₄-preferring agonist xanomeline (19,20) (**Figure 1A**). These two ligands stimulated inositol phosphate production in a concentration-dependent manner with acetylcholine and xanomeline both acting as full agonists (**Figure 1A**).

In phosphorylation studies, where total receptor phosphorylation was determined using metabolically labelled CHO-M1 cells, both muscarinic agonists increased the phosphorylation status of M₁ mAChRs (**Figure 1B**). To establish the precise sites of phosphorylation, a mass spectrometry-based phospho-proteomic study was conducted which identified nine serine and three threonine phosphorylation sites in the third intracellular loop of the receptor, as well as two serine phosphorylation sites in the C-terminal tail (**Figure 1C-F**). Four of these sites at serine residues 228, 273, 322 and 451 (S²²⁸, S²⁷³, S³²², S⁴⁵¹) were selected for the generation of phospho-specific antibodies based on the antigenicity of the sites.

The phospho-specific nature of the antibodies generated were tested by immunoprecipitation of M₁ mAChRs from CHO-M1 cells (using antibodies that recognised the HA-epitope engineered at the C-terminus of the receptor) followed by treatment of the immunoprecipitate with calf intestinal alkaline phosphatase (CIAP) to remove the phosphate groups (**Figure 2A**). Control immunoprecipitates were treated with vehicle. The immunoprecipitates were then probed in Western blots with the phospho-specific antibodies. In these experiments, treatment with CIAP removed the immunoreactivity for the M₁ mAChR of all four phospho-specific antibodies (**Figure 2B**) confirming that the antibodies were all phospho-specific.

Agonist-dependent M₁ mAChR phosphorylation

We next used the phospho-specific antibodies to determine if phosphorylation on S²²⁸, S²⁷³, S³²² and S⁴⁵¹ were regulated by agonist stimulation.

Probing lysates derived from CHO-M1 cells stimulated with vehicle, acetylcholine or xanomeline in Western blots using the phospho-specific antibodies revealed that there were low levels of basal phosphorylation on residues S²²⁸ and S²⁷³ but that agonist treatment significantly increased phosphorylation at these residues (**Figure 3**). This is in contrast to phosphorylation at S⁴⁵¹ that showed notable basal phosphorylation that was only increased to a small extent in response to agonist treatment (**Figure 3**). This compared to phosphorylation at S³²² that demonstrated high levels of basal phosphorylation and showed no substantive change in response to agonist (**Figure 3**). In this respect our panel of phospho-specific antibodies revealed that different phosphorylation sites on the M₁ mAChR had different sensitivities to agonist stimulation.

Since, the antibody used to detect phosphorylation at S²²⁸ gave a strong signal and phosphorylation at this site was exquisitely agonist-sensitive this antibody was considered an excellent candidate for a biosensor that would identify the activated M₁ mAChR receptor and as such was characterized further.

Determination of changes in M₁ mAChR phosphorylation in response to an allosteric ligand

The above data demonstrated that the activated state of the M₁ mAChR in response to two orthosteric agonists (acetylcholine and xanomeline) could be established by monitoring the phosphorylation status of the receptor at S²²⁸. It is now well understood that the active conformation of GPCRs can also be promoted by ligands that bind at allosteric sites (21,22) (**Figure 4A**). Previous studies had established that the compound, 1-(4-methoxybenzyl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (benzyl quinolone carboxylic acid; BQCA), is a selective positive allosteric modulator (PAM) to the M₁ mAChR (23-25). We confirm these studies by establishing in our system that BQCA can promote acetylcholine binding at the M₁ mAChR with a co-operativity factor of >100 (α factor = 128; **Figure 4B**). The enhanced binding of acetylcholine observed in the presence of BQCA was correlated with an augmentation of acetylcholine signalling as demonstrated by a leftward shift in the inositol phosphate (InsPx) concentration-response curve to acetylcholine when conducted in the presence of BQCA (co-

operativity $\alpha\beta$ factor = 85; **Figure 4C**). Hence, BQCA was seen to promote the active conformation of the M_1 mAChR, which was reflected in the ability of BQCA to augment agonist-binding and agonist-mediated signalling. Based on these observations, and given that phosphorylation at S^{228} likely reflects the proportion of M_1 mAChRs in an agonist-occupied (R^*) conformation, we reasoned that BQCA would promote acetylcholine-mediated phosphorylation at S^{228} . This was indeed the case, with BQCA mediating a ~ 100 fold shift in the potency of acetylcholine-mediated phosphorylation at S^{228} (pEC_{50} (-Log M) for acetylcholine = 7.5 ± 0.1 and pEC_{50} for acetylcholine in the presence of BQCA ($3 \mu M$) = 8.9 ± 0.3 ; **Figure 4E**). Thus, phosphorylation at S^{228} not only reflected the action of orthosteric ligands at the M_1 mAChR (**Figure 3**), but also reported the action of an allosteric modulator at this receptor.

These phosphorylation experiments were conducted with BQCA at a concentration that in itself gave no response (i.e. $3 \mu M$), but rather acted to enhance acetylcholine responses (i.e. a concentration where BQCA acted as a PAM). However, at higher concentrations, BQCA could be seen to act as an agonist in its own right (23,25). The agonist properties of BQCA can be readily determined in pERK1/2 assays which, due to the high coupling efficiency between the M_1 mAChR and pERK1/2 signalling in this system, was sufficiently sensitive to detect the agonist action of BQCA (**Figure 4D**). Thus, high concentrations of BQCA in a pERK1/2 assay showed a direct agonist action (**Figure 4D**). This agonist activity was reflected in complex concentration-response curves, where increasing concentrations of BQCA in the presence of very low concentrations of acetylcholine revealed the agonist activity of BQCA (green arrow, **Figure 4D**), whereas higher concentrations of acetylcholine revealed mixed agonist and PAM activities of BQCA (black arrow, **Figure 4D**). Importantly, when examined in phosphorylation studies it was seen that at a high concentration ($100 \mu M$) BQCA mediated phosphorylation at S^{228} to a similar extent to that seen with a maximal concentration of acetylcholine ($500 \mu M$) (**Figure 4F**). In contrast, a low concentration of BQCA ($3 \mu M$) alone did not stimulate any change in receptor phosphorylation (**Figure 4F**).

Phosphorylation at S^{228} was further characterised in immunocytochemistry studies. In these studies the S^{228} phospho-specific antibody detected very little immunoreactivity in non-transfected CHO cells and in CHO-M1 cells under basal conditions (**Figure 4G**). However, following agonist treatment (acetylcholine, 5 min) or treatment with a high concentration of BQCA ($100 \mu M$) punctate cytoplasmic staining was detected (**Figure 4G**). Interestingly, the punctate localization of the phosphorylated M_1 mAChR represented only a small proportion of the total receptor population (identified using the antibodies against the HA-epitope tag) that, after 5 min stimulation, appeared concentrated at or very close to the plasma membrane (**Figure 4G**).

Drawing together all these data it can be concluded that muscarinic ligands that promote the transition of the receptor from an inactive R -state to an active R^* -state, promote phosphorylation of the M_1 mAChR on S^{228} . This includes orthosteric ligands, such as acetylcholine and xanomeline, as well as allosteric ligands (i.e. BQCA). In this sense S^{228} phosphorylation is an ideal sensor of receptor activation – since this phosphorylation event reflects activation of the receptor regardless of the mechanism of activation.

Determination of the active conformation of the M_1 mAChR during memory acquisition and drug treatment

The data presented above support the notion that phosphorylation of S^{228} on the M_1 mAChR can act as a biosensor for receptor activation in response to either orthosteric or allosteric ligands. To test if this could be used to monitor the activation status of the M_1 mAChR *in vivo*, we first employed a novel mouse model where the wild type M_1 mAChR gene was humanized so that the translated amino acid sequence was identical to the human M_1 mAChR and where a HA-epitope tag was inserted at the C-terminus. A further two point mutations were introduced into the orthosteric binding site (Y106A and A195G) (**Figure 5A**) which resulted in a HA-tagged humanized M_1 mAChR mutant which was unable to be activated by the natural ligand acetylcholine but rather could be activated by the synthetic drug clozapine-N-oxide (CNO) (26,27). This receptor mutant has been previously described and characterized *in vitro* and termed as a designer receptor exclusively activated by designer drugs (M1 DREADD) (26). The design

of this mouse means that expression of the M1 DREADD will be under the control of the native M_1 mAChR promoter ensuring that the M1 DREADD would be expressed in the same cell types and at the same expression levels as the native M_1 mAChR.

Analysis of the ability of acetylcholine and CNO to induce phosphorylation of the M1 DREADD was tested in transfected cells. It was found that acetylcholine had no significant effect on M1 DREADD phosphorylation at S^{228} (**Figure 5B**). In contrast, the M1 DREADD was phosphorylated by CNO in a concentration-dependent manner (**Figure 5B**). Immunohistochemical labelling of the HA-epitope tag identified expression of the M1 DREADD mutant in the molecular layer of the CA1 region of the hippocampus (**Figure 5C**). Using the S^{228} phospho-specific antibody in M1 DREADD identified light immunohistochemical staining in the CA1 region of the hippocampus that was up-regulated within 30 mins of i.p. administration of CNO (**Figure 5D**).

Co-staining with anti-HA, to reveal the expression of the M1-DREADD-HA tagged receptor, and the S^{228} phospho-specific antibody revealed neurons where the M1-DREADD receptor existed in a phosphorylated state (indicated by arrows in **Figure 5E**). Interestingly, not all of the M1-DREADD expressing neurons stained with the phospho-specific S^{228} antibody suggesting that in some neurons the M1-receptor remained non-phosphorylated (at least at this residue)(**Figure 5E**). It should be noted that M1-KO controls also revealed some weak non-specific nuclear staining with the S^{228} phospho-specific antibody (**Figure 5D,E**).

The data in the M1 DREADD mice supported the notion that the S^{228} phospho-specific antibody can be used to monitor M_1 mAChR activation following stimulation by a synthetic ligand. We therefore tested this further by the administration of xanomeline (5 mg/kg i.p.) into wild-type mice followed by immunohistochemical analysis of phosphorylation at S^{228} . Pharmacokinetic analysis determined that xanomeline levels in the brain peaked 30 min after injection. At this time the animal was sacrificed by perfusion fixation and the brain removed and sectioned for immunohistochemical staining. This procedure revealed phosphorylation of the M_1 mAChR at S^{228} in the neuronal cell bodies in the pyramidal layer of the CA1 region of the hippocampus (**Figure 6A**). In contrast, low levels of staining

were observed in vehicle-treated mice (**Figure 6A**).

To test if phosphorylation at S^{228} could also act as a biosensor for M_1 mAChR activation in response to the administration of a PAM, BQCA was administered at 15 mg/kg, which resulted in a free concentration of BQCA in the hippocampus of 51.8 ± 3.6 nM; a concentration where BQCA would show PAM activity at the M_1 mAChR. In these experiments Western blots of hippocampal lysates prepared from BQCA-treated animals showed an increase in the phosphorylation status of the M_1 mAChR at S^{228} in response to BQCA (**Figure 6B, C**).

M1 mAChR is activated during memory acquisition

Having demonstrated that the action of an orthosteric agonist and a PAM at the M_1 mAChR in the hippocampus could be monitored using the phosphorylation status of the M_1 mAChR at S^{228} we next asked whether the phosphorylation of S^{228} could also be used as a biosensor for M_1 mAChR activation during a physiological process. Previous studies had implicated a role for the M_1 mAChR in learning and memory (15-17) and since the M_1 mAChR is highly expressed in the hippocampus (28), an area associated with learning and memory, we asked if changes in the phosphorylation status of S^{228} on M_1 mAChRs could be detected in the hippocampus following fear conditioning training, a classical protocol used to induce a learning response (29). The mice were therefore subjected to fear conditioning training and 30 min later sacrificed, tissues fixed by transcardial perfusion and brains sectioned and probed with phospho-specific antibodies to S^{228} phosphorylation. In these experiments an increase in the phosphorylation status of S^{228} was observed in the stratum pyramidale of the CA1 region of the hippocampus following fear conditioning training when compared to an immediate unpaired foot shock control (**Figure 6D**). Importantly, this increase in S^{228} phosphorylation correlated with an increase in c-Fos immunoreactivity, a marker of neuronal activity (**Figure 6D**).

DISCUSSION

Here we show that phosphorylation at serine 228 in the third intracellular loop of the M_1 mAChR can be used as a read-out for receptor activation. Using this antibody as a biosensor for the activated receptor we show that the M_1 mAChR

is activated in the hippocampus following fear conditioning training. This correlates with an increase in neuronal activity in the hippocampus thereby strongly supporting a link between hippocampal M_1 mAChR activity and hippocampal based learning and memory (15-17). Our study also illustrates how analysis of GPCR phosphorylation status can be used to assess the activation of receptors following drug treatment. Establishing target-drug engagement is highly valued in drug discovery but up to now no method has been described to monitor this for a GPCR target.

In order to establish the phospho-specific antibody to S^{228} as a sensor for M_1 mAChR activation we first conducted a mass spectrometry based phospho-proteomic analysis of the M_1 mAChR expressed in a recombinant system. This revealed that the M_1 mAChR, like many other GPCRs (30,31), is multiply phosphorylated at sites within the third intracellular loop and C-terminal tail. The multi-site nature of M_1 mAChR phosphorylation is consistent with the suggestion made by us, and others, that the complex pattern of receptor phosphorylation is cell type specific. This idea lead to the notion that a receptor expressed in different cell types might show a different phosphorylation pattern and this pattern would contribute to cell type-specific receptor signalling and regulation (30,32,33). This notion, has been coined the receptor phosphorylation barcode (30,32) and has been further extended to suggest that different agonists might drive different patterns of phosphorylation in a manner that encodes for different signalling outcomes (30,32,33). In this way the phosphorylation barcode is thought to contribute to stimulus bias where a ligand can direct signalling down one pathway in preference to another (34,35), possibly by mediating a specific pattern of receptor phosphorylation.

Given the notion that phosphorylation of GPCRs might be a dynamic process regulated, at least in part, by the pharmacological properties of the agonist used to stimulate the receptor it was important in this study to carefully characterize the agonist-dependency of the phosphorylation events revealed by the mass spectrometry studies. Hence, we generated a panel of phospho-specific antibodies to the M_1 mAChRs that could be used to further probe at least four of the 14 sites of phosphorylation. Firstly, all four of the antibodies generated were phospho-specific recognising

phosphorylation at S^{228} , S^{273} , S^{322} and S^{451} . These antibodies established that there were high levels of constitutive phosphorylation at S^{322} and S^{451} . In contrast, phosphorylation at S^{228} and S^{273} was seen to be highly sensitive to agonist stimulation and showed little phosphorylation in the basal state. Thus, phosphorylation at S^{228} and S^{273} appeared to fit the traditionally held view that only the agonist-occupied receptor undergoes phosphorylation (36,37) and thus these sites were ideal candidates as sensors of the activated receptor.

Due to the high signal-to-noise ratio obtained with the phospho-specific antibody to phosphorylated S^{228} we used this in further studies and established that orthosteric agonists, acetylcholine and xanomeline, mediated phosphorylation of S^{228} . Furthermore, the positive allosteric modulator, BQCA, was also able to drive S^{228} phosphorylation. Hence, it would appear that at least in the case of S^{228} phosphorylation, ligands with different pharmacological properties, but that all promoted the transition of the receptor from an inactive R-conformation to an active R*-conformation, resulted in phosphorylation of the receptor on S^{228} . In this sense an antibody that specifically recognised S^{228} phosphorylation could be considered as a biosensor of the active R*-conformation of M_1 mAChR.

It is important to point out that not all phosphorylation sites on GPCRs will show the characteristics of S^{228} phosphorylation described here. Careful evaluation of the agonist-sensitivity of the phosphorylation event with a range of pharmacological ligands would need to be conducted since other sites of phosphorylation might be less sensitive to agonist stimulation (such as S^{322} and S^{451}) or might show different levels of phosphorylation in response to ligands. Such phosphorylation sites would not be suitable as indicators of receptor activation.

To determine if the phospho-specific S^{228} antibody could be used to detect phosphorylation (and thereby activation) of the M_1 mAChR *in vivo* we employed a mutant mouse line that expressed a M1 DREADD receptor in place of the wild-type M1 mAChR. This receptor could be selectively activated by CNO *in vivo* (26). Importantly, our *in vitro* studies had established that CNO-stimulated phosphorylation at S^{228} on the M1 DREADD. We show here that the M1

DREADD expressed in the CA1 region of the hippocampus was phosphorylated on S²²⁸ following administration of CNO. These data confirmed the hypothesis that the phospho-specific S²²⁸ antibody could be used to identify activated receptors in the hippocampus. This was further supported by receptor stimulation using xanomeline and BQCA in wild-type animals, where receptor activation could similarly be identified by an up-regulation of phosphorylation at S²²⁸.

Given that phosphorylation at S²²⁸ fitted the criteria for use as a biosensor for receptor activation we investigated if the M₁ mAChR was activated in the hippocampus during memory acquisition. Previous studies had established a role for muscarinic receptor signalling in learning and memory (14,38,39). The involvement of the M₁ mAChR in this process is suspected not only because this receptor subtype is highly expressed in the hippocampus (28) but also because gene knockout and pharmacological disruption of M₁ mAChRs resulted in defective learning and memory (14). Thus, there is a great deal of interest in targeting the M₁ mAChR as a mechanism to treat cognitive deficits in neurodegenerative disease, such as Alzheimer's disease (14,40,41). Despite this it is still unclear if M₁ mAChRs acting directly at the level of the hippocampus mediate memory processing or

whether these receptors are more subtly involved by promoting interactions between the prefrontal cortex and hippocampus (16,42-44). We were therefore interested to test the possibility that M₁ mAChRs were activated in the hippocampus during memory acquisition. This was found to be the case with a significant up-regulation of M₁ mAChR phosphorylation at S²²⁸ following fear conditioning training in the CA1-region of the hippocampus. Importantly, this region was also seen to show high levels of neuronal activity, as indicated by an increase in c-FOS expression, following fear conditioning training. These data support the hypothesis that hippocampal M₁ mAChR are activated during memory acquisition and that this may contribute to the process of learning and memory.

In conclusion, by determining the phosphorylation sites within the M₁ mAChR and generating and characterizing phospho-specific antibodies we have established that at least one of the phosphorylation events (S²²⁸) is exquisitely sensitive to agonist stimulation. Using this phosphorylation event as a biosensor for receptor activation we have been able to establish the activation of the M₁ mAChR *in vivo* in the CA1 region of the hippocampus following drug treatment and memory acquisition.

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Conflicts of interest

Authors declare that there are no conflicts of interest.

Author Contributions

AJB, conducted primary experiments, assisted in writing paper. SJB, RP, SMB, AM, JMB, TMH, JME, ARB, contributed to the experimental data. RAJC, LMB, CCF, contributed to experimental design and data analysis. ABT, conceived and lead study, and wrote the paper.

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Abbreviations

M₁ mAChR, M1 muscarinic acetylcholine receptor; M₃ mAChR, M3 muscarinic acetylcholine receptor ; BQCA, 1-(4-methoxybenzyl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid; CHO, Chinese hamster ovary; FRET, fluorescent resonance energy transfer; GPCR, G protein-coupled receptor; GRK, G protein coupled receptor kinase; NMS, N-methylscopolamine; TEAB, triethylammonium bicarbonate; CNO, clozapine-N-oxide.

Figure legends

Figure 1. Agonist-mediated phosphorylation of the M₁ mAChR

A. CHO cells expressing a C-terminally tagged mouse M₁ mAChR (CHO-M1 cells) were treated with various concentrations of the muscarinic receptor agonists; acetylcholine (ACh), xanomeline (Xan). Cells were then lysed and the inositol phosphate levels (IPx) determined. Shown is the mean data of 3 experiments $\pm$ SEM. **B.** Phosphorylation of the M₁ mAChR was monitored in CHO-M1 cells metabolically labelled with [³²P]-orthophosphate and treated with vehicle, acetylcholine (ACh, 100 μ M) or xanomeline (Xan, 10 μ M) followed by immunoprecipitation, gel electrophoresis and autoradiography. Also shown is the mean data of 5 experiments $\pm$ SEM. The total M₁ mAChR was also established using an anti-HA in a Western blot (Loading Control). **C.** Summary of the mass spectrometric determination of the phosphorylation sites on the M₁ mAChR. The phospho-sites indicated in red were the sites to which phospho-specific antibodies were raised. **D.** Representative ms/ms spectrum and fragmentation table of M₁ mAChR peptide phosphorylated on S²²⁸. **E.** Summary of all the M₁ mAChR phospho-peptides obtained from five experiments. Phosphorylated amino acids are highlighted in red. **F.** Primary amino acid sequence of the human M₁ mAChR receptor highlighting the phospho-acceptor sites in red.

Figure 2. Characterisation of M₁ mAChR phosphorylation-specific antibodies

A. Schematic representation of the experimental procedure used to characterise M₁ mAChR phosphorylation specific antibodies where calf intestinal alkaline phosphatase (CIAP) was used to dephosphorylate immunoprecipitated M₁ mAChR receptor derived from cells that had been treated with acetylcholine (ACh) before being probed in Western blots with the phospho-specific antibodies. **B.** Results of Western blots from the experiments that are illustrated in A. Blots were probed with phospho-specific antibodies directed towards pS²²⁸, pS²⁷³, pS³²² and pS⁴⁵¹. Also shown is the loading control from this particular experiment using an anti-HA antibody to detect the epitope tagged M₁ mAChR. The * indicates slight cross reactivity of the phospho-specific antibodies with CIAP.

Figure 3. Agonist-dependent phosphorylation revealed by phospho-specific antibodies to the M₁ mAChR

M₁ mAChRs immunoprecipitated from non-transfected CHO cells (NT) or CHO-M1 cells that were treated with vehicle, acetylcholine (ACh, 100 μ M), or xanomeline (Xan, 10 μ M) for 5 minutes and resolved by SDS PAGE and probed in Western blots with phospho-specific antibodies raised against pS²²⁸, pS²⁷³, pS³²² and pS⁴⁵¹. Total M₁ mAChR was determined in the loading control using anti-HA antibodies. The experiment shown is typical of at least five independent experiments.

Figure 4. A positive allosteric modulator (BQCA) promotes M₁ mAChR phosphorylation at S²²⁸

A. Schematic representation of the M₁ mAChR illustrating distinct binding modes for orthosteric ligands, acetylcholine (ACh) and xanomeline (Xan), from that of allosteric ligands, such as BQCA. The chemical structure of BQCA is also shown. **B.** CHO-M1 cell membranes were used in competition radioligand binding experiments where acetylcholine (ACh) displacement of [³H]-NMS was tested in the presence of a range of concentrations of BQCA. The data presented are means ± S.E.M of three independent experiments conducted in triplicate. **C.** The positive allosteric modulator (PAM) activity of BQCA was revealed in concentration-response curves for acetylcholine (ACh)-mediated inositol phosphate (IP_x) production in CHO-M1 cells in the presence of various concentrations of BQCA. **D.** Both the mixed agonist (green arrow) and PAM (black arrow) activity of BQCA was revealed in concentration-response curves for acetylcholine (ACh) pERK1/2 response in CHO-M1 cells in the presence of various concentrations of BQCA. **E.** Representative Western blots of CHO-M1 cell lysates stimulated with increasing concentrations of acetylcholine (ACh) in the presence or absence of BQCA (3 μM) and probed with the phospho-specific serine 228 antibody or anti-HA antibodies as a loading control for total M₁ mAChR. Also shown is the mean data of 3 independent experiments ± SEM. **F.** CHO-M1 cells were stimulated with a high concentration of acetylcholine (ACh, 500 μM) or with BQCA at two concentrations; a low concentration (3 μM) where BQCA might be expected to show no intrinsic agonist activity and a high concentration (100 μM) where BQCA would show agonist activity. Cell lysates were prepared, resolved by SDS PAGE and Western blots probed with the phospho-specific serine 228 antibody. The IgG band was used as a loading control in these experiments. Also shown is the mean data ± S.E.M of Western blots from 3 independent experiments. **G.** CHO-M1 cells stimulated with acetylcholine (ACh) or BQCA were fixed and processed for immunocytochemistry using an anti-HA antibody to show total M₁ mAChR or with the phospho-specific serine 228. Non-transfected CHO cells were similarly treated. All the images and gels shown were typical of at least 3 independent experiments. All the graphical data represents the mean ± SEM of at least three independent experiments. Statistical analysis uses student's paired t-test

Figure 5. Phosphorylation of S²²⁸ on the M1 DREADD receptor could be detected in the hippocampus following receptor activation with a selective agonist

A. Illustration of the two point mutations (Y106C and A195G) used to generate the HA-epitope tagged M1 DREADD receptor mutant in which activation by acetylcholine (ACh) is abolished but instead the receptor could be activated by clozapine-N-oxide (CNO). **B.** CHO FlpIn cells expressing HA-tagged M1 DREADD receptor were stimulated with increasing concentrations of ACh or CNO. Western blots were probed with either anti-HA (as a loading control) or phospho-specific serine 228 antibodies. **C-D.** M1 DREADD knock-in mice or M₁ mAChR-knockout

mice (M1-KO) were injected (i.p.) with CNO (0.3mg/kg). After 30 minutes tissue was fixed by transcardial perfusion and sections stained with **C.** anti-HA antibodies or **D.** phospho-specific serine 228 antibodies. **E.** Fixed sections from M₁ mAChR-knockout mice (M1-KO) or M1 DREADD knock-in mice treated with vehicle or CNO (0.3mg/kg) were co-stained with anti-HA (green) and anti phospho-specific serine 228 (red) antibodies. Two neurons where the staining for the receptor and the phosphorylated receptor occur in the same neuron are indicated by the arrows. The areas marked by the white box are magnified in the lower panels.

Figure 6. M₁ mAChR is activated in the hippocampus following drug treatment and memory acquisition

A. C57/BL6/NTAC mice were injected (i.p) with vehicle or xanomeline (5 mg/kg). After 30 min tissues were fixed by transcardial perfusion, sections obtained and stained with the phospho-specific serine 228 antibody and with DAPI stain to reveal the nuclei. Shown are representative sections through the CA1 region of the hippocampus **B.** C57/BL6/NTAC mice (Wild-Type) or M₁ mAChR-knockout mice (M1-KO) were injected (i.p.) with BQCA (15 mg/kg) or vehicle and after 30 min hippocampal membranes were prepared from which the M₁ mAChR was immunoprecipitated. The sample was then processed in Western blots, which were probed with phospho-specific serine 228 antibody or an M₁ mAChR-specific antibody to detect total M₁ mAChR. **C.** The quantification of Western blots from (B). The data are presented as means $\pm$ S.E.M (n=3). Statistical analysis uses students paired t-Test. **D.** C57/BL6/NTAC mice were subjected to a fear conditioning training protocol or to an un-paired immediate foot shock as a control; 30 mins later tissue was fixed by transcardial perfusion and sections obtained and stained with phosphorylated S²²⁸ specific antibody (upper panel) or anti-c-FOS antibody (lower panel). All the data shown are typical of at least three independent experiments.

Figure 1

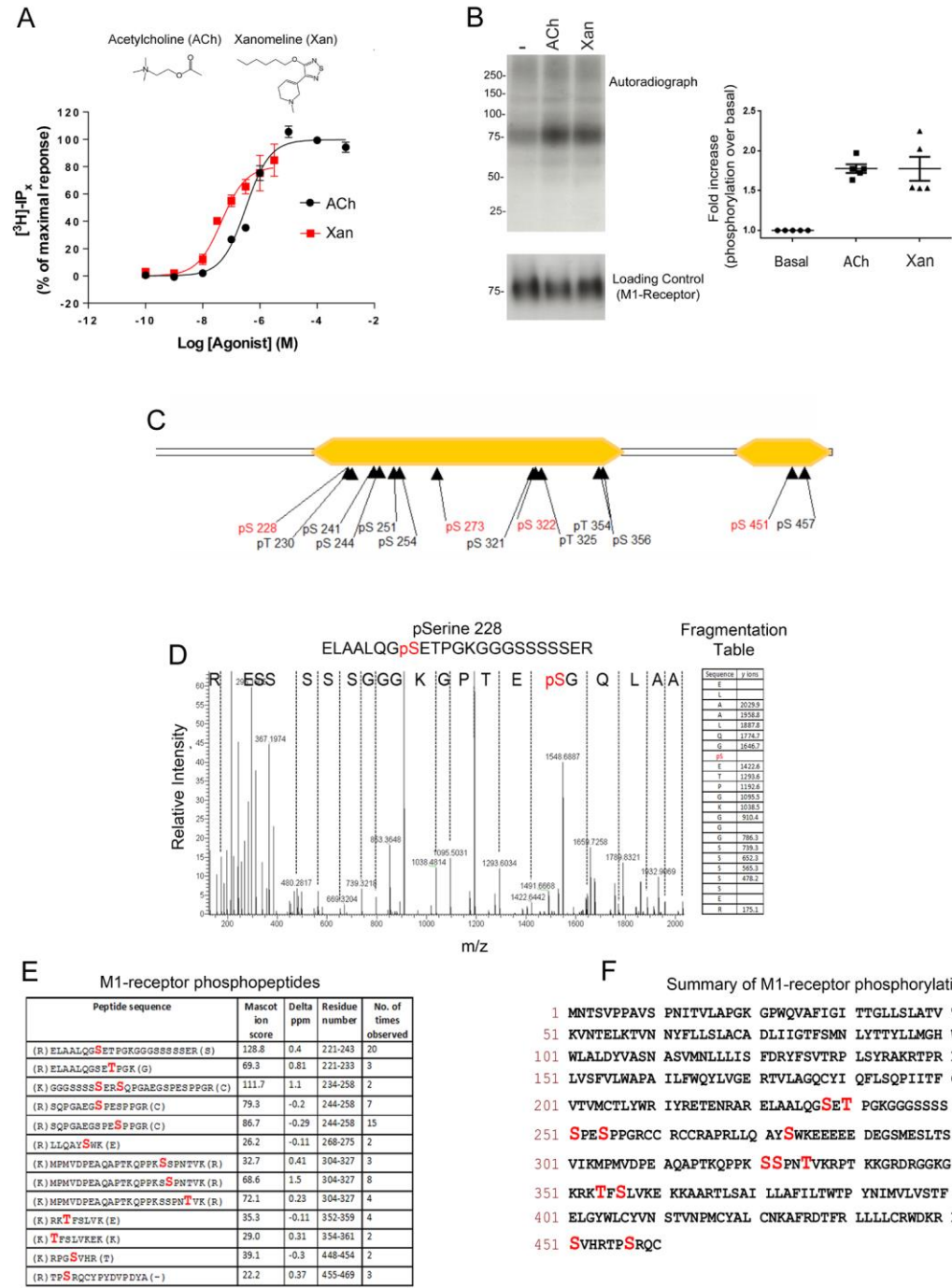


Figure 2

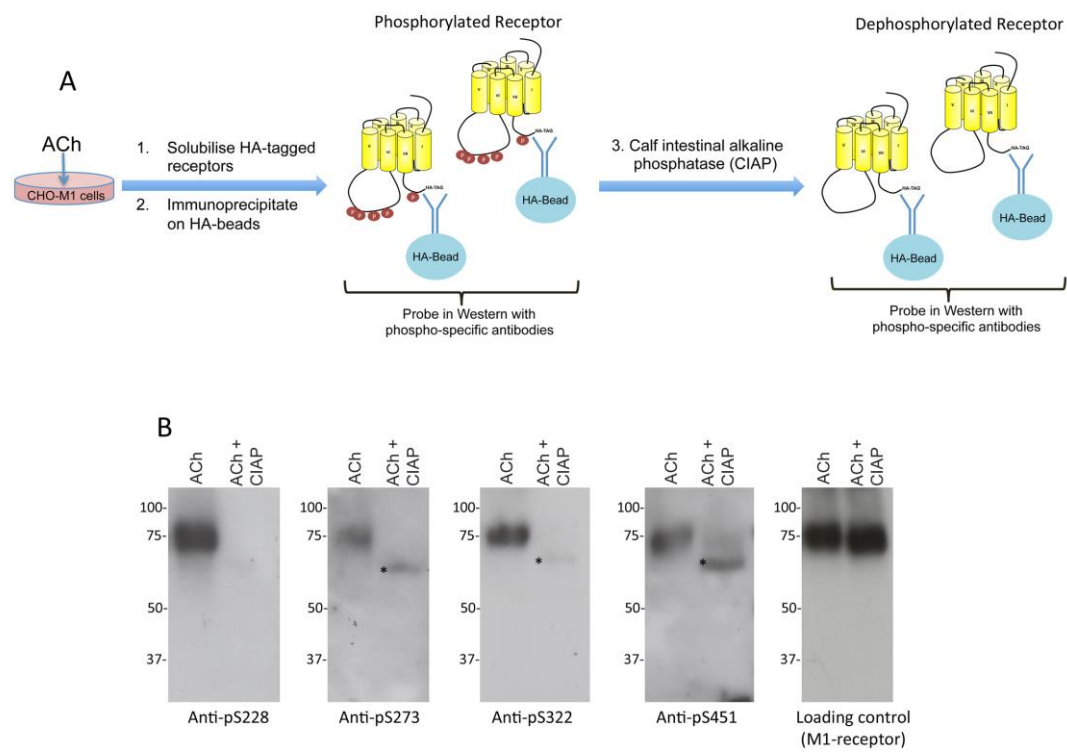


Figure 3

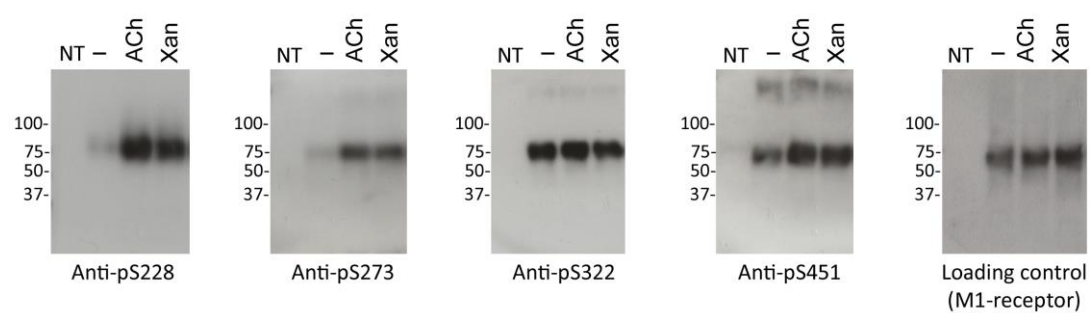


Figure 4

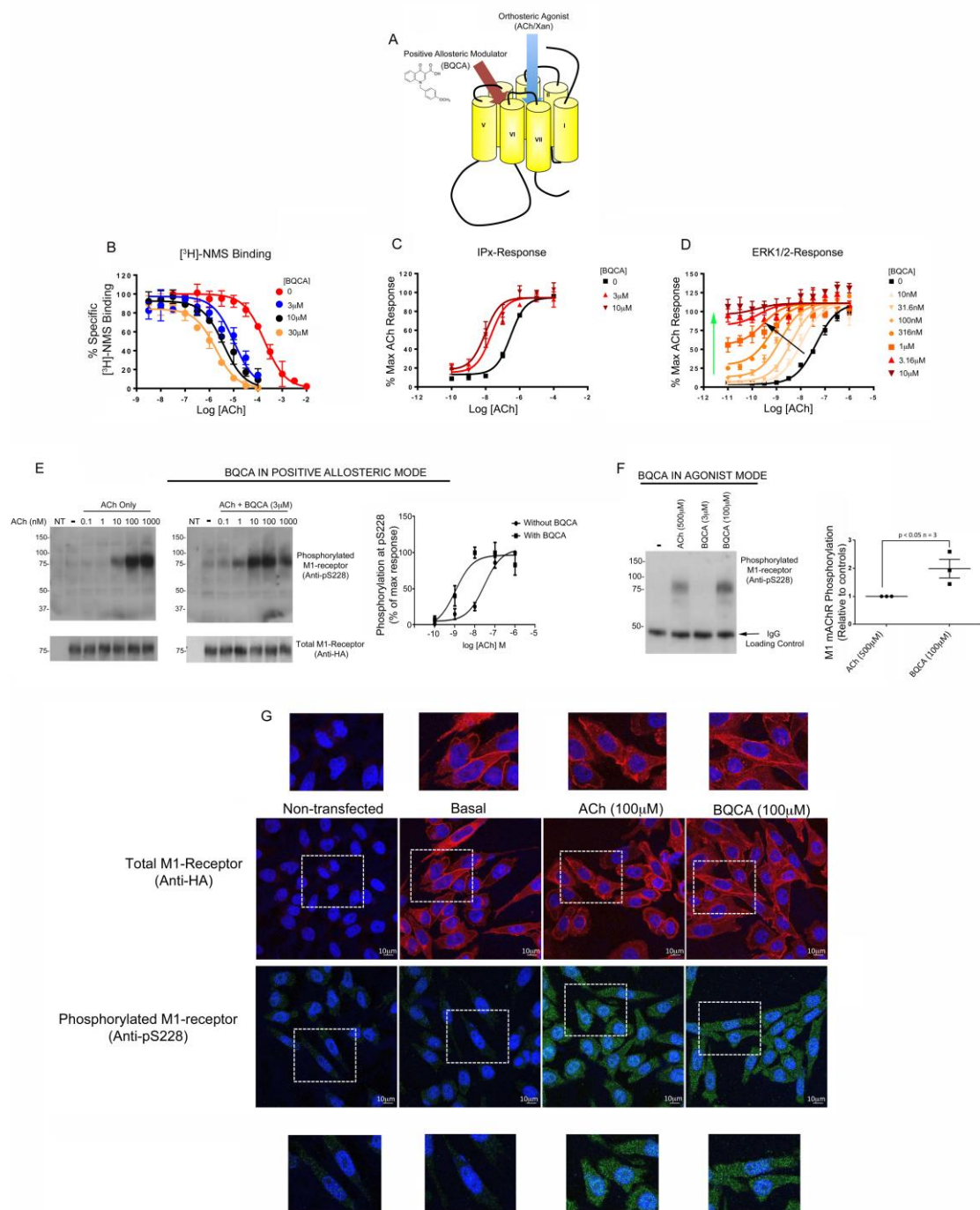


Figure 5

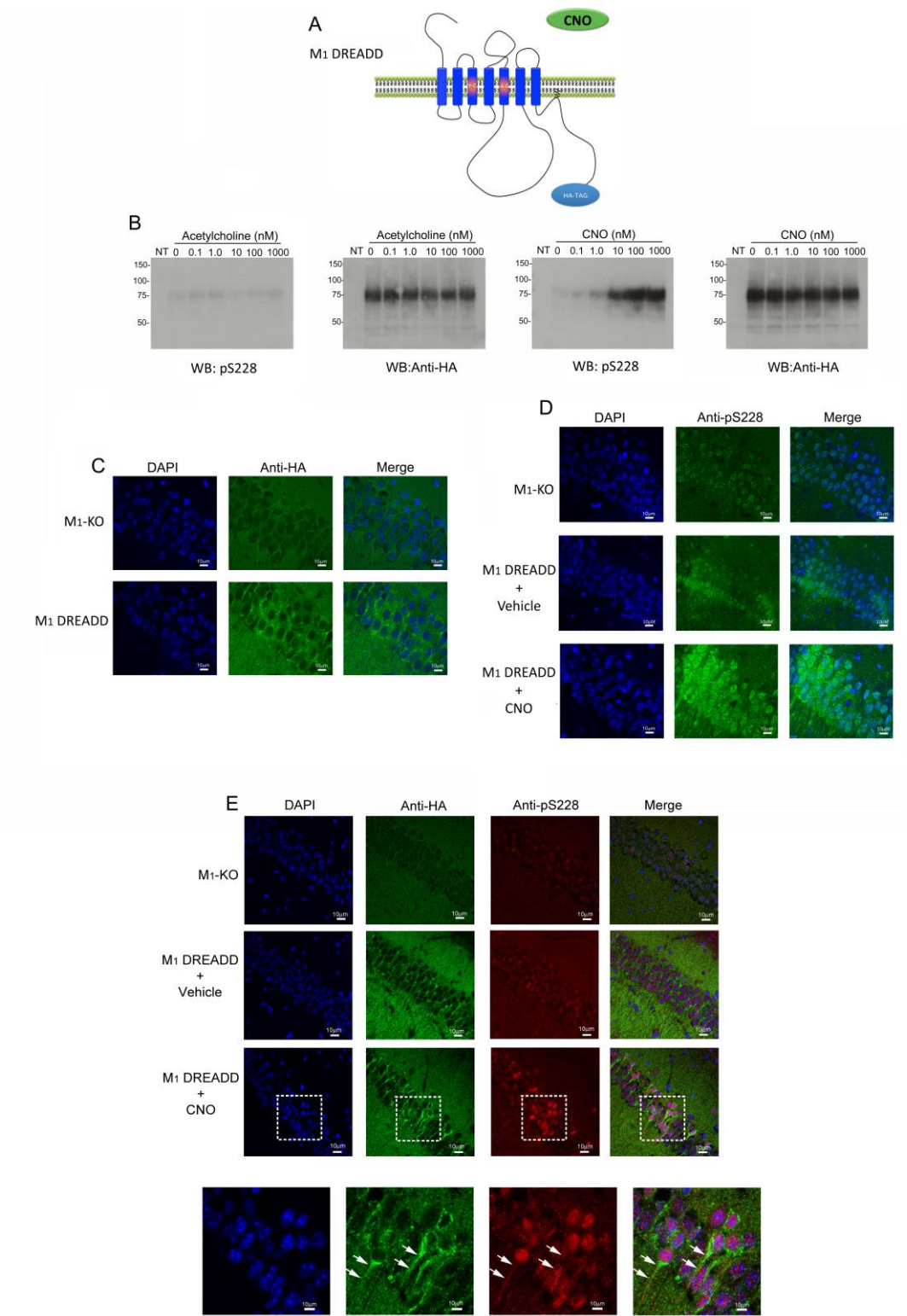
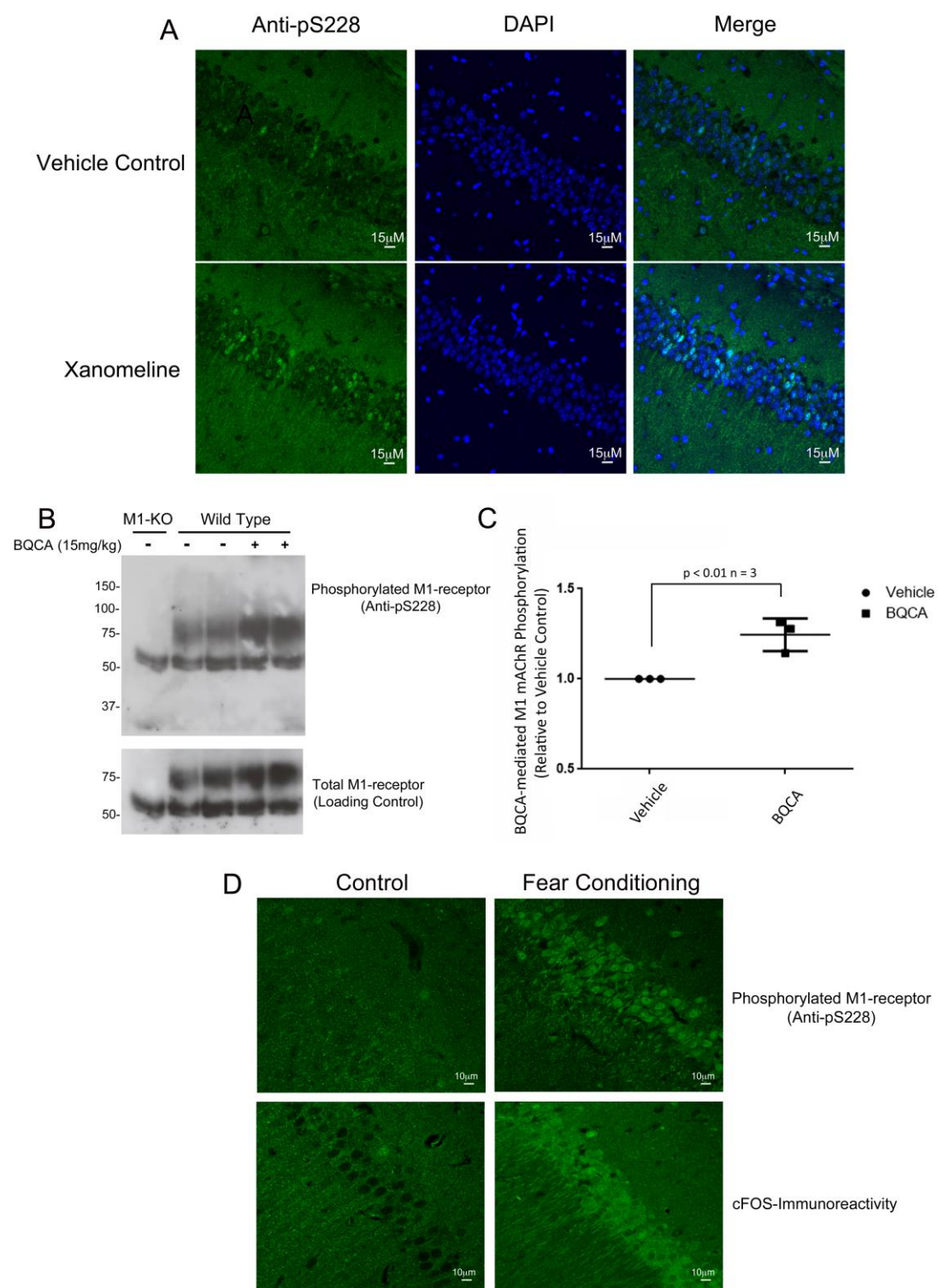


Figure 6



**An antibody biosensor establishes the activation of the M1 muscarinic
acetylcholine receptor during learning and memory**

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