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INFLUENCE OF THE ACCESSORY PROTEIN SET ON M3 MUSCARINIC RECEPTOR PHOSPHORYLATION AND G PROTEIN COUPLING

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RUNNING TITLE : SET AND M3 MUSCARINIC RECEPTOR REGULATION

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Nonstandard abbreviations: CHO - Chinese Hamster Ovary cells, CK - Casein Kinase, GPCR - G Protein-Coupled Receptor, GRK - G protein-coupled Receptor Kinase, GppNHp - 5'-guanylylimidodiphosphate, i3 loop - third intracellular loop, M3-MR – M3 Muscarinic Receptor, M3-i3 - i3 loop of M3-MR, NMS – N methylscopolamine, PP2A - Protein Phosphatase 2A.

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ABSTRACT

The proto-oncogene and inhibitor of protein phosphatase 2A (PP2A), SET, interacts with the third intracellular loop of the M3-muscarinic receptor (M3-MR) and SET knockdown with small interfering RNA (siRNA) in CHO cells augments M3-MR signaling. However, the mechanism of this action of SET on receptor signaling is not defined and we initiated studies to address this question. Knockdown of SET by siRNA in CHO cells stably expressing the M3-MR did not alter agonist-induced receptor phosphorylation or receptor internalization. Instead, it increased the extent of receptor dephosphorylation after agonist removal by ~60%. In competition binding assays, SET knockdown increased high-affinity binding of agonist in intact cells and membrane preparations. GST pull down assays and site directed mutagenesis revealed a SET binding site adjacent to and perhaps overlapping the G-protein binding site within the third intracellular loop of the receptor. Mutation of this region in the M3-MR altered receptor coupling to G-protein. These data indicate that SET decreases M3-MR dephosphorylation and regulates receptor engagement with G-protein, both of which may contribute to the inhibitory action of SET on M3-MR signaling.

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INTRODUCTION

G protein coupled receptors (GPCR) define a large family of cell-surface receptors that process signals from a great diversity of endogenous and exogenous stimuli. These receptors possess a characteristic seven transmembrane domain architecture. Agonist binding to the receptor induces conformational changes within the GPCR that are propagated to intracellular domains resulting in heterotrimeric G protein coupling to the receptor and activation of downstream signaling.

Signal transfer from the receptor to G-proteins may be regulated by intracellular proteins that bind to cytoplasmic domains of the receptor influencing receptor trafficking between plasma membrane and intracellular compartments, G protein activation and participating in the assembly of receptors into signal transduction complexes or "receptosomes" (Bockaert et al., 2004; Sato et al., 2006). Such signaling complexes may be stabilized by agonist or may be preformed and disrupted by agonist binding as part of the signal transfer process. The degree of stabilization or disruption of such signaling complexes by any given ligand is likely dependent upon the conformation of the receptor stabilized by agonist thus offering a platform for ligand-specific signaling events.

Most of the more than 80 GPCR Interacting Proteins identified to date interact with the carboxy terminal tail of GPCRs that contain interacting motifs such as the PDZ (PSD95-disc large-Zonula occludens), the Src homology 2 (SH2) and 3, the pleckstrin homology or the Ena/VASP homology domains (Bockaert et al., 2003). The third intracellular loop mediates G protein coupling and activation for most GPCRs. It is the largest intracellular portion of the receptor protein in many receptors and thus also participates in the assembly and processing of signaling complexes (Prezeau et al., 1999; Richman et al., 2001; Wang et al., 2004; Wu et al., 1998; Wu et al., 2000; Wu et al., 1997). We took advantage of recent technologies with enhanced sensitivity to detect specific interactions and identified SET protein (Template activating Factor I) as a binding partner of the third intracellular loop of M3-MR (Simon et al., 2006). Functional analysis of the interaction demonstrated that SET has an inhibitory action on M3-MR signaling through Gq and that SET likely operates at the level of M3-MR itself (Simon et al., 2006).

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In the present paper, we further characterized the regulation of M3-MR signalling by SET and addressed potential mechanisms that may account for this regulation. SET was first described as part of the SET-CAN fusion gene, a putative oncogene associated with acute undifferentiated leukaemia (von Lindern et al., 1992). SE in SET refers to the patient with leukemia containing SET translocation and the T in SET refers to translocation (Von Lindern et al., 1992).

SET is widely expressed whereas the M3-MR expression profile is more restricted. A review of mRNA expression profiles for SET and the M3-MR in human tissues indicate co-expression in several tissues including thymus, lung, prefrontal cortex and liver (SET - http://biogps.org/#goto=genereport&id=6418; http://bioinfo2.weizmann.ac.il/cgi-bin/genenote/GN_results.pl?keyword_type=2_gc_id&keyword=GC09P131445&data_type=norm2&results=yes; CHRM3 - http://biogps.org/#goto=genereport&id=1131; http://bioinfo2.weizmann.ac.il/cgi-bin/genenote/GN_results.pl?keyword_type=2_gc_id&data_type=norm2&results=yes&keyword=GC01P239549). A review of the Allen Brain Atlas indicates that SET and the M3-MR are both expressed in several specific brain tracts including the isocortex, olfactory areas, and hippocampal formation (SET - http://mouse.brain-map.org/gene/show/35371; *CHRM3* - http://mouse.brain-map.org/gene/show/12456).

Apart from its role in gene transcription regulation (Seo et al., 2001), SET is an inhibitor of the activity of PP2A (Li et al., 1996), a phosphatase involved in various signaling cascades (Lechward et al., 2001) and GPCR regulation (Krueger et al., 1997; Pitcher et al., 1995). PP2A is one of the major phosphatases involved in GPCR dephosphorylation (Pitcher et al., 1995). For many GPCRs, phosphorylation of the receptor by second messenger kinases and GRKs following agonist-mediated activation is an important aspect of receptor regulation (Budd et al., 2000). Phosphorylation of many GPCRs is associated with G protein uncoupling and receptor internalization resulting in a decreased responsiveness of the signaling system to agonist or perhaps receptor coupling to alternative signaling pathways through β-arrestin, which binds to the phosphorylated receptor (Kendall and Luttrell, 2009;

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Lefkowitz and Shenoy, 2005). Specific phosphorylation sites in the M3-MR are differentially regulated by different agonists and result in the regulation of distinct signaling pathways (Tobin et al., 2008, Butcher et al., 2011).

A phosphorylation-deficient M3-MR mutant elicits a more robust initial inositol phosphate response when compared with the wild type receptor (Budd et al., 2000). In addition, siRNA knockdown of endogenous SET in CHO cells stably expressing the M3-MR (CHO-M3 cells) augmented the increase in intracellular calcium in response to a short-term activation of the M3-MR (Simon et al., 2006). We thus hypothesized that SET regulates M3-MR signaling by influencing the phosphorylation status of the receptor and/or M3-MR - G protein coupling.

MATERIALS AND METHODS

siRNA mediated gene silencing of SET

CHO cells stably expressing the human M3-MR (CHO-M3 cells) plated in 100 mm dishes were transfected at 20-30 % confluency with SET siRNA targeting different regions of human SET mRNA (accession number #45198) using Lipofectamine 2000 as described previously (Simon et al., 2006). Three SET siRNA duplexes with 2 and 12 bp mismatch controls were used in various aspects of the study. Each SET siRNA duplex effectively reduced SET protein by 60-80%, whereas the control siRNA oligonucleotides did not alter the levels of SET protein. SET siRNA duplexes and their controls were as follows: ^{268}cagaagaggucagaauugaucgcca^{292}, 2 bp mismatch cagaagaggucGAaauugaucgcc, 12 bp mismatch agGGAGgACuUUaaCuAGGAAcca;

^{327}ccauccacaagugucugcacugcuu^{351}, 2 bp mismatch control ccauccacaagGUucugcacugcuu, 12 bp mismatch control ccaAcAcGUgAgucuUcacGCuCuu; ^{530}ccgaaaucaaauggaaaucuggaaa^{554}, 2 bp mismatch ccauccacaagGUucugcacugcuu. The siRNA duplexes 327-351 and 530-554 were generally used in combination (10). In siRNA control experiments for receptor phosphorylation and radioligand binding

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studies, cells were transfected with the corresponding predicted oligonucleotide control for the siRNA duplex (Simon et al., 2006). The transfection mixture was removed 5 h later and replaced by α-MEM medium containing 10 % fetal bovine serum (FBS, Invitrogen), 1 % penicillin-streptomycin (PS, Invitrogen, Carlsbad, USA) and 250 ug/ml geneticin (Invitrogen, Carlsbad, USA).

To assess SET knockdown, cells were homogenized in lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1 % Nonidet P-40) and incubated for 1 h on ice. Cell homogenates were then centrifuged at 10, 000 x g at 4^{o}C and 12 ug of the supernatant were separated on 10% SDS-polyacrylamide gels and transferred to a polyvinylidene difluoride (PVDF) membrane. Expression of SET was assessed by immunoblotting with a polyclonal anti-SET antibody (1:1000) kindly provided by Dr. T.D. Copeland (Adachi et al., 1994) (National Cancer Institute-Frederick, MA). Equal protein loaded was verified by reprobing blots with an anti-α-actin (Chemicon international).

SET expression

CHO-M3 cells plated in 100 mm-dishes were transfected with pcDNA3 or pcDNA3::His-SET using Lipofectamine 2000 according to the manufacturer instructions (Life Technologies). Forty eight hours later, cells were harvested for calcium measurements as described below. SET expression levels were assessed by performing electrophoresis of protein homogenates (12 ug) on denaturing polyacrylamide gels (10%) and membrane transfers were probed with anti-SET antibody.

M3 muscarinic receptor phosphorylation and dephosphorylation

CHO-M3 cells plated in 100 mm-dishes were transfected with siRNA duplexes and 48 hours later the cells (700,000 cells) were transferred to 6-well plates in complete medium. The next day, confluent cells were washed three times in phosphate-free Krebs/HEPES buffer (10 mM HEPES, 118 mM NaCl, 4.3 mM KCl, 1.17 mM $MgSO_4 \cdot 7H_2O$, 1.3 mM $CaCl_2$, 25.0 mM $NaHCO_3$, 11.7 mM glucose, pH 7.4) and incubated in phosphate-free Krebs/HEPES supplemented with [^{32}P]-orthophosphate (50 uCi/ml) for 1 h at

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37C. Cells were stimulated with the cholinergic agonist, carbachol (100 μM) for 5 minutes and reactions were terminated by rapid aspiration of the drug-containing media and application of 1 ml of ice-cold solubilization buffer (10 mM Tris, 10 mM EDTA, 500 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 500 nM microcystin, 50 mM NaF, 5nM NaPPi, 20 mM β-glycerophosphate, pH 7.4). Samples were left on ice for 20 min, passed through a needle syringe ($G26^{1/2}$) and cleared by microcentrifugation. Cell lysates were pre-cleared with protein A sepharose beads and then incubated on ice for 60 min with anti-M3-MR antibody raised against a GST-receptor fusion protein containing the third intracellular loop of the human m3-receptor (S345-L463) (Tobin et al., 1996). Immune complexes were isolated on protein A-Sepharose beads, and the beads were washed four times with solubilization buffer. Isolated immune complexes were then resolved on 8% SDS-PAGE gels. The gels were dried and phosphorylated M3-MR was visualized and quantified using a Typhoon 9400 Variable Mode Imager. The isolated immunes complexes were also visualized after electrotransfer by immunoblotting with polyclonal anti-M3MR antibody (provided by Dr. Jurgen Wess, National Institutes of Health, Bethesda) (Zeng and Wess, 1999).

For dephosphorylation experiments, [^{32}P]-orthophosphate-labeled cells were stimulated with carbachol (100 μM, 5 min), rapidly washed three times with phosphate-free Krebs/HEPES buffer and incubated in this buffer for 5 or 20 minutes at 37^{o}C. Cells were then lysed and processed as described above.

Receptor internalization

CHO-M3 cells plated in 100 mm-dishes were transfected with siRNA duplexes and 48 hours later cells were transferred to 24-well plates in complete medium. The next day, cells were washed three times in phosphate-free Krebs/HEPES buffer, serum-starved for 1 hour in phosphate-free Krebs/HEPES buffer and then stimulated with 1 mM carbachol for 2 hours. Reactions were terminated by aspiration, and the cells were washed three times with ice-cold Krebs/HEPES buffer. Cells were incubated with a saturating concentration (6 nM) of the

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hydrophilic muscarinic antagonist [^{3}H] N-methylscopolamine (NMS) for 4 hours on ice. Cells were then washed 2 times in ice-cold KREBS buffer and solubilized by the addition of 1 ml of ice-cold solubilization buffer. Non-specific binding was determined with 10 µM of the antagonist atropine. Cells were scraped, transferred to vials containing 3.5 ml of Ecoscint A (National Diagnostics) for analysis by liquid scintillation spectrometry. Under these experimental conditions, non specific [^{3}H]-NMS binding was <3 % of the total binding. Specific binding was normalized by the number of cells.

Competition binding assays

For intact cell radioligand binding assays, CHO-M3 cells plated in 100 mm-dishes were transfected with SET siRNA duplexes and 72 hours later, cells were serum-starved for 2-3 hours. Cells were detached from plates with phosphate buffered saline containing 2 mM EDTA and centrifuged at low speed. The cell pellets were washed two times and resuspended in phosphate buffered saline (4×10^6 cells/ml). Competition binding studies were conducted under predetermined equilibrium conditions. Assays were conducted in disposable borosilicate glass (WWR international) and initiated by the addition of 100,000 cells (25 ul) to 75 ul of binding buffer containing 0.5 nM of (^{3}H) NMS in the presence or the absence of increasing concentrations of carbachol (10^{-6} M to 10^{-2} M) or atropine (10^{-10} M to 10^{-5} M). The assay was incubated with shaking for 2 hours at room temperature.

For membrane radioligand binding assays, CHO-M3 cells were transfected with SET siRNA duplexes as described above. After 72 hours, cells were detached from plates with phosphate buffered saline containing 2 mM EDTA, pelleted (200 x g, 5 min) and lysed in 0.5 ml/6 well dish of hypotonic lysis buffer [(5 mM EDTA, 5 mM EGTA, 5 mM Tris-HCl, pH 7.4, with protease inhibitors (Complete Protease Inhibitor Cocktail Tablet - 1 tablet/10 ml, Roche)] using a 26.5-gauge syringe. The lysed cells were centrifuged at 15,000 x g for 10 min to obtain crude membrane and cytosol fractions. Membranes were resuspended in 0.5 ml of membrane buffer (50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA, 1

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mM EGTA with protease inhibitor). For binding assays, 25 ul membrane (50 ug of protein) samples were added to 75 ul of binding buffer containing 0.5 nM [^{3}H] NMS in the presence or absence of increasing concentrations of carbachol (10^{-6} M to 10^{-2} M) or atropine (10^{-10} M to 10^{-5} M) or GppNHp (10 uM).

For radioligand binding assays involving transient expression of receptor and receptor mutants, cells were transfected using polyethylineamine as a transfection reagent (Oner et al., 2010). Membrane and intact cell binding assays were terminated by addition of 4 ml of ice-cold binding buffer followed by filtration over GF/B glass-microfiber filters (Whatman). Filters were washed 3 times with 4 ml of ice-cold binding buffer, transferred to vials containing 6 ml of Ecoscint A (National Diagnostics) and bound radioactivity was counted the next day by scintillation spectrometry. Competition binding data were analyzed by the nonlinear curve-fitting program Prism 4.0 (GraphPad).

Measurement of cell calcium content

To evaluate receptor-effector coupling, we determined the ability of agonist to increase intracellular calcium in CHO-M3 cells using a fluorometric imaging plate reader system (Molecular Devices Corp.). Seventy two and forty eight hours following siRNA transfection and plasmid transfections, respectively, CHO-M3 cells were seeded in F12 medium containing 2 % FBS and 1 % PS (35,000 cell/100µl/well) in 96-well clear-bottomed black microplates (Corning Costar Corp.), precoated with 100 ug/ml poly-D-Lysine (Sigma-Aldrich). Four hours later, cells were dye-loaded (FLIPR calcium 3 assay kit, Molecular Devices) with 100 µl of the dye-loading buffer containing 2.5 mM of probenecid for 1 h at 37 °C in a 5 % CO_2 incubator. During a data run, cells in different wells were exposed to different concentrations of drugs, and the system recorded fluorescent signals for all 96 wells simultaneously every 5 seconds for 5 min. Increases in intracellular calcium were observed as sharp peaks above the basal fluorescent levels typically 10 seconds after drug addition. The increases in intracellular calcium levels were determined by subtracting the baseline to the peak values (heights). Data were plotted using Graphpad Prism (version 4.0). Data are representative of 3-5 independent experiments and the data point for any drug concentration is an average from 3 to 4 wells.

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Protein recombinant preparation

To generate the M3-MR third intracellular loop constructs (M3i3: T^{450}-Q^{490}, I^{474}-Q^{490}, I^{474}-I^{483} and M^{480}-Q^{490}), complementary oligonucleotides from these regions were synthesized and annealed prior to ligation into the BamHI and EcoRI restriction sites of the PGEX-4T-1 vector. All mutations were made using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). GST fusion proteins were expressed in BL21 cells and purified on glutathione-Sepharose 4B (GE Biosciences) as described previously (Wu et al., 2000; Wu et al., 1997). Immobilized fusion proteins were stored at 4^{o}C and each batch of fusion proteins used for experiments were first analyzed by SDS-PAGE and Coomassie blue staining. The full-length encoding sequence of human SET cloned into the pQE30 vector was kindly provided by Dr R.Z. Qi (Hong Kong University of Science and Technology, China). The His-tagged SET protein was expressed in M15 bacteria and purified on Ni2+-nitrilotriacetic acid beads (QIAGEN Inc.) according to the manufacturer's instruction.

GST pull down assays

Human recombinant His-tagged SET protein (30 nM) was gently mixed for 1 hour at 4^{o}C with the GST fusion proteins (300 nM) bound to the glutathione sepharose 4B matrix (12.5 ul) in 750 ml of buffer A [25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 50 mM NaCl, 1 mM DTT, 0.1 % NP40 and protease inhibitors (Complete Protease Inhibitor Cocktail Tablet - 1 tablet/10 ml, Roche)]. Resins were washed 3 times with 500 ml of buffer A. The retained proteins were eluted from the resin with 25 ml of 5X loading buffer, placed in a boiling water bath for 5 min and applied to 10 % SDS-polyacrylamide gels. Separated proteins were then transferred to PVDF membranes and processed for immunoblotting with the polyclonal anti-SET antibody. Membranes were systematically re-probed with an anti-GST antibody (1:5000, GE Biosciences, Piscataway, NJ, USA) to control for equal amounts of GST fusion proteins and protein loading.

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RESULTS

In our previous study reporting the interaction of SET with the M3-MR, SET knockdown augmented carbachol-induced increases in mobilization of intracellular calcium. To further validate these observations and to reduce the chances of any off-target effects of the siRNA, we extended these studies to include siRNA oligonucleotides targeting a different region of SET mRNA and also generated additional controls with two bp mismatches. SET siRNA 268-292 effectively reduced SET protein and had the same effect on M3-MR signaling as the SET siRNA 327-351 and 530-554 oligionucleotides used in our previous study (Figure 1A) (Simon et al., 2006). Control siRNAs with two and twelve bp mismatches did not knockdown SET protein and did not affect M3-MR-mediated regulation of calcium signaling (Figure 1 A, B). P2-purinergic receptor-mediated regulation of calcium signaling in CHO-M3 cells was not altered by the new SET siRNA 268-292 (Figure 1B – right panel) as previously reported for the SET siRNA oligonucleotides 327-351 and 530-554 (10). These data indicate that any effect of SET siRNA knockdown on M3-MR signaling is not likely due to unrelated, off target effects of SET siRNA.

The SET knockdown studies were complemented with parallel studies to determine the effect of elevated SET expression on M3-MR signaling. A ~2 fold increase in SET expression level in CHO-M3 cells reduced the efficacy of carbachol to mobilize intracellular calcium relative to control cells by ~ 40% (Figure 2, left panel). Importantly, SET overexpression did not alter the ability of the P2-purinergic receptor to mobilize intracellular calcium in CHO-M3 cells (Figure 2, right panel). Collectively, these data indicate that SET exerts an inhibitory action on M3-MR calcium signaling.

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Influence of SET on M3 muscarinic receptor phosphorylation

Phosphorylation of GPCR influences receptor signaling by altering receptor – G-protein coupling. We first evaluated the effect of SET on basal and agonist-induced increases in M3-MR phosphorylation. CHO-M3-MR transfectants were labeled with [^{32}P]-orthophosphate, stimulated with the agonist carbachol (100 μM, 5 min) and the incorporation of phosphates into the receptor was measured after immunoprecipitation of the M3-MR followed by a separation on SDS-PAGE and autoradiography (Figure 3). Immunoblots indicated the immunoprecipitation of a specific receptor species from CHO-M3-MR transfectants that was absent in control CHO cells and similar amounts of receptor were immunoprecipitated in the presence and absence of carbachol (Figure 3A, left panel). The phosphorylated M3-MR appeared as a single band of ~100 kDa which was absent in CHO cells lacking the M3-MR (Figure 3A, right panel). Furthermore, stimulation of cells with the muscarinic agonist carbachol increased the intensity of this band (Figure 3A, right panel). Indeed, carbachol induced a ~2 fold increase in the level of M3-MR phosphorylation as compared with basal receptor phosphorylation. To assess the role of SET in M3-MR phosphorylation events, we successfully decreased by ~ 90% the endogenous SET expression levels by transfection of cells with SET siRNA (Figure 3C). Comparison of the extent of agonist-induced M3-MR phosphorylation in control cells and in cells transfected with SET siRNA revealed that SET does not have any significant effect on basal or agonist-induced M3-MR phosphorylation (Figure 3B, C). Indeed, quantification of the radioactivity in the immunoprecipitated receptor was similar with or without reduction of endogenous SET expression (Figure 3C, control: 2.1 ± 0.3 fold over basal, SET siRNA: 2.3 ± 0.3 fold over basal). These data suggest that SET is not dynamically involved in either basal phosphorylation of receptor or agonist-mediated increases in phosphorylation.

We then addressed the role of SET in M3-MR dephosphorylation (Figure 4). After induction of receptor phosphorylation with carbachol (100 μM, 5 min), carbachol was removed, cells were incubated in agonist free media and, at the end of the incubation, the remaining radioactivity into the receptor was

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measured. We determined that as soon as 5 min after agonist removal, receptor dephosphorylation is taking place and corresponds to a ~25% decrease of the radioactivity of the immunoprecipitated M3-MR (Figure 4A). At 20 minutes after agonist removal, the level of phosphorylated M3-MR is similar to basal levels observed in the absence of agonist. Interestingly, in CHO-M3 cells where endogenous SET expression was depleted by SET siRNA, early receptor dephosphorylation increases from ~25% to ~40% (Figure 4B, C % receptor dephosphorylation: control - 23.6 ± 8.2, SET siRNA - 37.5 ± 12.2). This corresponds to a ~60% increase of receptor dephosphorylation in CHO-M3 cells lacking SET (Figure 4C). These results demonstrate that SET inhibits M3-MR dephosphorylation after agonist removal and thus increases the period of time during which the receptor remains phosphorylated after agonist dissociates from the receptor.

Effect of SET on M3 muscarinic receptor internalization

Since GPCR phosphorylation and dephosphorylation cycles are involved in GPCR functionality by regulating receptor trafficking from the plasma membrane to intracellular compartments, we asked if SET could influence this process. We first asked if SET siRNA influenced receptor expression levels by measuring cell surface receptors with the hydrophilic muscarinic antagonist [^{3}H] NMS. SET siRNA treatment did not alter [^{3}H] NMS binding to M3-MR [control - 3078 ± 490 dpm, SET siRNA - 2873 ± 194 dpm].

For receptor internalization studies, cells were incubated with carbachol for 2 hours to induce maximal M3-MR internalization as previously described (Wu et al., 2000) leading to a 40.4 ± 5.6 % decrease in [^{3}H] NMS binding (Figure 5). When cells were transfected with SET siRNA, the extent of receptor internalization did not differ from control cells (Figure 5, 36.2 ± 3.4 %).

SET alters agonist binding to receptor and receptor signaling

We next examined M3-MR interaction with G protein by determining the ability of the agonist carbachol to stabilize a high-affinity form of the receptor as detected by computer modeling of agonist

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competition curves for [^{3}H] NMS binding. The high-affinity state of the receptor for agonist results from the formation of a ternary complex between the agonist, the receptor and the G protein.

Competitive binding assays were first conducted in intact cells following treatment with control siRNA (control) or with SET siRNA. Increasing concentrations of the agonist carbachol displaced the antagonist [^{3}H] NMS binding to M3-MRs (Figure 6A). When endogenous SET expression was reduced by SET siRNA, agonist competition curves were shifted to the left as compared to control (SET siRNA IC_{50} - 0.09 ± 0.02 mM, control IC_{50} - 0.29 ± 0.07 mM) (Figure 6A), reflecting an increase in agonist affinity. This shift to the left was quantitated as a 3.13 ± 0.08 fold decrease in the IC_{50} exhibited by carbachol. In contrast, SET siRNA knockdown did not have any effect on the displacement of [^{3}H] NMS binding by the antagonist atropine as compared with control cells (Figure 6B). Altogether, this suggests that SET decreases agonist binding to the receptor and that this may lead to decreased coupling of the M3-MR and G-protein.

To further address this question, we conducted similar experiments using membrane fractions from lysed cells. In membrane preparations from control cells, carbachol exhibited an IC_{50} value of 1.3 ± 0.7 mM (Figure 6C). Following SET knockdown, the agonist competition curve was shifted to the left and best fit to a two-site model (Figure 6C). The IC_{50} for the high- and low-affinity states of the receptor were 0.02 ± 0.01 mM and 1.1 ± 0.07 mM, respectively. SET knockdown thus induced the appearance of a high-affinity state of the receptor for agonist with an IC_{50} ~ 60 times higher than that observed in control cells (1.3 ± 0.7 mM) and the lower-affinity state of the receptor for agonist in SET siRNA transfected cells (1.1 ± 0.07 mM). In contrast, competition binding studies with the antagonist atropine were not altered by SET knockdown (Figure 6D). To confirm that the appearance of a high-affinity state receptor by SET siRNA treatment results from a better coupling of M3-MR to G protein, we evaluated the effect of the nonhydrolyzable guanine nucleotide GppNHp on agonist competition curves. GppNHp inhibits the formation of the high-affinity state of the receptor for agonist. Interestingly, pre-incubation with GppNHp of membranes reversed the effect of SET siRNA and shifted the curve rightward resulting in a

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curve that was best fit to a one site model and was indistinguishable from the curve obtained with control cells (SET siRNA + GppNHp - IC_{50} = 1.5 ± 0.7 mM).

Based upon our experience, high affinity agonist binding and associated effects of GppNHp in competitive radioligand binding studies for systems coupled to Gq (e.g. M3-MR) may be quite subtle as compared to systems involving coupling to Gαi/o. Nevertheless, these data are also consistent with the interpretation that SET knockdown results in facilitated interaction of the M3-MR with G-protein.

Localization of the SET binding site within M3 muscarinic receptor

SET interaction with the M3-MR involves the carboxyl terminal part of the third intracellular loop of the M3-MR (M3i3; amino acids 450 to 490, T^{450}-Q^{490}) and more specifically the last 17 amino acids (Figure 7A) (10). Interestingly, among those 17 amino acids are key residues for Gq protein coupling and activation (Blin et al., 1995; Schmidt et al., 2003; Zeng et al., 1999). We thus addressed the role of specific regions in this 17 amino acid segment in the interaction with SET. SET interaction with the carboxyl terminal end of M3i3 was disrupted by incubation with high salt buffer (0.5 M NaCl) whereas buffer containing detergent (1% NP40) did not alter SET binding (Figure 7A). These data indicate that charged rather than hydrophobic amino acids likely mediate SET binding to the M3i3 segment. There are two clusters of positively charged amino acids within the last 17 amino acids of the i3 loop (Figure 7B, fragment A, underlined amino acids KRKR and KEKK). To identify the amino acids required for SET binding, we generated mutants in which the first charged amino acid cluster was replaced by alanine or glutamic acid residues either neutralizing or reversing the positive charge (Figure 7B, fragments B and C). Mutation of the first cluster KRKR into AAAA or EAEA abolished SET binding (Figure 7B). This indicates that only the first cluster of charged amino acids is required for SET binding and this is further confirmed by the fact that SET binds to a fragment containing only this cluster (Figure 7B, fragment D) and not to a fragment containing only the second cluster (Figure 7B, fragment E). SET thus binds to a site (amino acids 476-479) in close vicinity to amino acids involved in G protein coupling

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and activation (amino acids 484-490). This suggests that SET may complete with G protein for receptor binding and thus reduce M3-MR calcium signaling.

We then asked if disruption of the putative SET binding site (amino acids 476-479) in the context of the full length receptor influenced receptor coupling in a manner similar to that observed following siRNA-mediated SET knockdown. We generated M3-MR constructs with mutations (^{476}KRKR/AAAA M3-MR and ^{476}KRKR/EAEA M3-MR) identical to those that disrupted SET binding in the M3i3 fragment I^{474}-Q^{490}. Both constructs were expressed at levels comparable to wild type receptor as determined by whole cell and membrane radioligand binding with ^{3}H-scopolamine (Unpublished observations). However, both receptor mutants exhibited altered agonist affinity as determined by both competitive radioligand binding studies and calcium measurements. The receptor mutants both exhibited a reduction in the amount and/or affinity of the receptor population exhibiting high affinity for agonist indicating that that these mutations influence receptor interaction with G-protein (Supplemental Figure 1). Agonist-induced increases in intracellular calcium were also examined following expression of wild type receptor and the two mutant receptors in CHO cells. Both receptor mutants exhibited maximal responses to carbachol that were comparable to wild type. However, the carbachol concentration response curves were shifted to the right for the mutant receptors as compared to wild type receptor (Supplemental Figure 1). Thus, the mutations apparently influence receptor interaction with G-protein complicating interpretation of the data in regards to SET engagement.

DISCUSSION

In the present study, we analyzed the role of SET in the regulation of M3-MR function. We first examined the impact of SET on the phosphorylation status of M3-MR. SET knockdown has no effect on

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M3-MR phosphorylation following receptor activation, but M3-MR dephosphorylation was accelerated by ~60% after knockdown of SET.siRNA. Considering the well-established role of SET in the inhibition of the activity of PP2A, a phosphatase involved in GPCR dephosphorylation, one hypothesis is that the action of SET to inhibit receptor dephosphorylation is due to such an inhibitory action on PP2A. A growing number of GPCRs serve as substrates for PP2A. Among them, the chemokine receptor CXCR2 (Fan et al., 2001), the metabotropic glutamate receptor 5 (Mao et al., 2005), the β2-adrenergic receptor (Pullar et al., 2003) and more recently the G protein coupled receptor GPR54 (Evans et al., 2008).

Although there is no direct functional evidence that PP2A dephosphorylates the M3-MR, per se, a recent study indicates the binding of both SET and PP2A to the third intracellular loop of selected muscarinic receptor subtypes (Borroto-Escuela et al., 2011). Although not reported for all subtypes, the interaction of SET with the M1-MR was dependent upon agonist activation, whereas the interaction with PP2A was independent of agonist binding. Although the M3-MR and SET co-immunoprecipitated from CHO M3-MR cell lysates (Simon et al., 2006), we could not detect any changes in the amount of SET in membrane preparations or at the cell cortex as determined by immunohistochemistry following agonist exposure (Unpublished observations). The amount of SET coimmunoprecipitating with the M3-MR from CHO M3-MR cell lysates was also not apparently altered by agonist exposure (Supplemental Figure 2). Initial studies to coimmunoprecipitate the M3-MR and SET from mouse brain tissue have been challenging due to the relatively low expression of the M3-MR (Unpublished observations, Butcher and Tobin).

GPCR dephosphorylation is classically thought to occur in the late endosomal compartment after agonist-induced internalization of plasma membrane receptors in order to regenerate a functional receptor. Internalization of plasma membrane M3-MRs in intracellular compartments is initiated in CHO-M3 cells 30 minutes after incubation with the agonist carbachol (unpublished observations) (Wu et al., 2000). In our experiments, M3-MR dephosphorylation appears to occur at a faster rate than that observed for receptor internalization. The different time courses of M3-MR internalization and dephosphorylation in CHO-M3 cells suggest that receptor dephosphorylation events are taking place at the plasma membrane

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rather than after receptor internalization. Several studies demonstrated that other GPCRs such as the D1 dopamine receptor (Gardner et al., 2001), the TRH receptor (Jones and Hinkle, 2005) and the β2-adrenergic receptor (Tran et al., 2007) are dephosphorylated at the plasma membrane. Dephosphorylation of GPCRs at the plasma membrane rather than in endosomes may provide a more rapid way of regeneration of a functional receptor without the need to enter the internalization and recycling pathways. This would allow a cell that was exposed to a brief agonist stimulation to respond again rapidly.

Receptor phosphorylation initiates receptor internalization and desensitization. Surprisingly, the decreased phosphorylation level of M3-MR observed after depletion of endogenous SET did not result in any significant change in receptor internalization. Despite the fact that M3-MR internalization is a phosphorylation-dependent process, recent data suggest that M3-MR internalization depends on the nature of the kinase phosphorylating M3-MR (Torrecilla et al., 2007). Indeed, Torrecilla et al. recently demonstrated that inhibition of CK2-mediated M3-MR phosphorylation did not significantly affect M3-MR internalization, whereas it is well established that M3-MRs are internalized in a GRK dependent manner (Torrecilla et al., 2007; Tsuga et al., 1998). One could hypothesize that, in the absence of SET, receptor dephosphorylation occurs preferentially on sites specifically phosphorylated by CK2 and not by GRKs.

Since receptor phosphorylation impacts receptor-G protein coupling, we examined G protein coupling to M3-MR by pharmacological approaches. Our data obtained from both intact cells and membrane fractions demonstrate that SET reduces G protein coupling to M3-MR. One possible hypothesis is that phosphatase inhibition by SET increases the life span of the phosphorylated receptor following dissociation of agonist increasing β-arrestin binding that promotes G protein uncoupling. However, SET knockdown had no effect on receptor internalization, a process initiated by β-arrestin binding to the receptor. SET could also inhibit dephosphorylation of other signaling components involved in M3-MR signaling such as the $G\alpha_{q/11}$ protein itself. Indeed, phosphorylation of $G\alpha_{q/11}$ on serine 154 decreased $G_{q/11}$ protein coupling to 5-HT_{2A} receptors and induced receptor desensitization (Shi et al.,

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2007). This hypothesis remains to be explored.

Our interaction studies in GST pull down assays suggest that SET could inhibit G protein coupling by directly competing with G protein binding to the receptor. Indeed, the SET binding site (^{476}KRKR^{479}) sits next to the Gq protein binding site (^{484}KEKKAAQ^{490}) within the i3 loop of M3-MR (Blin et al., 1995; Schmidt et al., 2003; Zeng et al., 1999). Given the size of these two proteins, it is possible that binding of one will affect binding of the other.

Based upon the increased maximal response to carbachol for intracellular calcium measurements observed following reduction of SET protein by SET siRNA and the reduction in maximal response to carbachol observed with increased SET expression, one might predict that the elimination of putative docking sites for SET in the third intracellular loop would also increase the maximal response to carbachol. However, this was not the case (Supplemental Figure 1) as the disruption of these putative docking sites in the context of the intact receptor compromised receptor engagement with G-protein.

Altogether, our work highlights for the new functions for the accessory protein SET in the regulation of M3-MR. We report that SET decreases M3-MR dephosphorylation and regulates M3-MR engagement with G protein. These actions of SET on M3-MR coupling probably contribute to the inhibitory effect of SET on M3-MR calcium signaling. The functional characterization of SET as a component of a muscarinic receptor signaling complex and an inhibitor of M3-MR signaling provides not only an expanded functionality for SET, but also presents a totally unappreciated mechanism for regulation of GPCR signaling capacity.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Simon, Oner, Tobin, Lanier

Conducted experiments: Simon, Oner

Contributed new reagents or analytical tools: Cohen-Tannoudji, Tobin

Performed data analysis: Simon, Oner, Lanier,

Wrote or contributed to the writing of the manuscript: Simon, Lanier with contributions from all author

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. **SET siRNA knockdown in CHO-M3 cells and effect of SET knockdown on calcium mobilization by the Gq-coupled M3 muscarinic and P2 purinergic receptors.** *(A)* 12 ug of protein homogenates from CHO-M3 cells transfected with SET siRNA oligonucleotides 268-291or SET siRNA oligonucleotides 327-351 and 530-554 and their controls (C and C*, control siRNA with 12 or 2 mismatches from the siRNA SET, respectively) were electrophoresed on denaturing polyacrylamide gels (10 %) and probed with anti-SET or anti-actin antibodies. *(B)* CHO-M3 cells transfected with SET siRNA (327/530 or 268) or their respective controls were plated in 96-well black plates pre-coated with poly D-Lysine and loaded with fluorescent dye. Cells were stimulated with increasing concentrations (10^{-9} M to 10^{-4} M) of the muscarinic agonist, carbachol (left panel), or the P2 purinergic agonist, UTP, (right panel) and intracellular calcium mobilization was measured. Excitation fluorescence was 485 nm and emission was detected at 520 nm using a 515 nm emission cutoff filter and fluorescence emissions were measured with the FlexStation III (Molecular Devices). The increases in intracellular calcium were determined by subtracting the baseline to peak values (heights). Results were expressed as the percentage of the maximal response in control cells (control). The data are presented as the mean ± SEM of 3 independent experiments in triplicate.

Figure 2. **Effect of increased SET protein on calcium mobilization by the Gq-coupled M3 muscarinic and P2 purinergic receptors in CHO-M3 cells.** *Upper panel* - 12 ug of protein homogenates from CHO-M3 cells transfected with pcDNA3 or pcDNA3::His-SET were electrophoresed on denaturing polyacrylamide gels (10 %) and probed with anti-SET antibody. *Lower panel* – Transfected CHO-M3 cells were plated in 96-well black plates pre-coated with poly D-Lysine and loaded with fluorescent dye. Cells were stimulated with increasing concentrations (10^{-9} M to 10^{-5} M) of the muscarinic agonist, carbachol (left panel), or the P2

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purinergic agonist, UTP, (right panel) and intracellular calcium mobilization was measured. Excitation fluorescence was 485 nm and emission was detected at 520 nm using a 515 nm emission cutoff filter and fluorescence emissions were measured with the FlexStation III (Molecular Devices). The increases in intracellular calcium were determined by subtracting the baseline to peak values (heights). Results were expressed as the percentage of the maximal response in cells transfected with pcDNA3 (control). The data are presented as the mean ± SEM of 2-4 independent experiments in triplicate.

Figure 3. **M3 muscarinic receptor phosphorylation.** A) CHO or CHO-M3 cells were plated in 6-well plates and labeled with [^{32}P]-orthophosphate (50 μCi/ml, see "Materials and Methods"). Cells were stimulated (+) or not (-) with the cholinergic agonist, carbachol (100 μM) for 5 minutes. After agonist removal, cells were lysed and processed as indicated in "Materials and Methods". Immunoprecipitated M3-MRs were resolved by 8% SDS-PAGE and the gel either dried and subjected to autoradiography or electrotransferred and probed with a polyclonal anti M3-MR antibody. Phosphorylated M3-MRs were visualized using a Typhoon 9400 Variable Mode Imager. The autoradiogram shown is representative of 4 independent experiments. B) M3-MR phosphorylation was measured in CHO-M3 cells transfected with control siRNA or SET siRNA oligonucleotides 327-351 and 530-554 as described in (A). The autoradiogram shown is representative of 4 independent experiments. C) Quantitative analysis of the agonist dependent receptor phosphorylation. Data are presented as the mean ± S.E.M of 4 independent experiments. Basal receptor phosphorylation values were not significantly different among control cells and cells transfected with SET siRNA (SET siRNA - 90.4 ± 32.2 % of control). Individual pairwise comparisons were performed using Student's t test, **$p<0.01$ compared with unstimulated. Upper panel – Visualization of SET knockdown efficiency by immunoblotting - 12 ug of protein homogenates from CHO-M3 cells transfected with control siRNA (control) or SET siRNA were

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electrophoresed on denaturing polyacrylamide gels (10 %) and probed with anti-SET or anti-actin antibodies.

Figure 4. **M3 muscarinic receptor dephosphorylation.** A) Receptor dephosphorylation time course. Receptor phosphorylation in CHO-M3 cells was induced by incubating cells with carbachol (100 uM, 5 min). At the end of the incubation, carbachol was removed by extensive washes and receptor dephosphorylation was initiated (post-wash: 5 or 20 min) or not (post-wash: 0 min) by incubating cells in agonist-free medium for 5 or 20 minutes at 37C. Cells were then processed as described in Figure 1. The autoradiogram shown is representative of 2 to 3 independent experiments. *B)* Receptor dephosphorylation in CHO-M3 cells transfected with control siRNA or SET siRNA oligonucleotides 327-351 and 530-554 was measured as described in *(A)*. The autoradiogram shown is representative of 3 independent experiments. *C)*, Quantitative analysis of receptor dephosphorylation in control cells and cells transfected with SET siRNA. Data are presented as the mean ± S.E.M of 3 independent experiments. Individual pairwise comparisons were performed using Student's t test, *$p<0.05$ compared with control cells.

Figure 5. **M3 muscarinic receptor internalization**. CHO-M3 cells transfected with control or SET siRNA oligonucleotides were stimulated (+) or not (-) with 1 mM carbachol for 2 hours. After removal of the agonist, cells were incubated for 4 hours on ice with a saturating concentration of the hydrophilic muscarinic antagonist [^{3}H] NMS (6 nM). At the end of the incubation, unbound [^{3}H] NMS was removed by extensive washes, cells solubilized and radioactivity was measured by scintillation spectrometry. The data are expressed as the mean ± S.E.M of 4 independent experiments performed in triplicate. Results are indicated as specific radioligand binding for 100,000 cells. Individual pairwise comparisons were performed using Student's t test, **$p<0.01$ compared with receptor binding in absence of carbachol.

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Figure 6. **Competition binding studies with muscarinic ligands**. *(A, B)* Cells (100,000 cells) were incubated with 0.5 nM of [^{3}H] NMS in presence or absence of increasing concentrations of *(A)* carbachol (10^{-6} M to 10^{-2} M) or *(B)* atropine (10^{-10} M to 10^{-6} M). *(C, D)* Membrane proteins (35 ug) prepared from cells transfected with control siRNA or siRNA SET oligonucleotides 327-351 and 530-554 were incubated with 0.5 nM of [^{3}H] NMS in presence or absence of GppNHp (10 uM) and [^{3}H] NMS binding was measured after addition of increasing concentrations of *(C)* carbachol (10^{-6} M to 10^{-2} M) or *(D)* atropine (10^{-10} M to 10^{-6} M). Competition curves were plotted as the percentage of maximal [^{3}H] NMS binding in the absence of carbachol or atropine. The results shown are representative of 3 to 5 independent experiments each performed in triplicate.

Figure 7**. Localization of SET binding site within the M3-i3 loop.** A) The carboxy terminal end of the third intracellular loop of the M3-MR (T^{450}-Q^{490}) was expressed in BL21 cells as a GST fusion protein and purified on glutathione Sepharose-4B. Purified GST fusion proteins were incubated with recombinant His-SET as described in "Materials and Methods". Protein complexes were washed with buffer A (-) or buffer A containing 0.5 M NaCl or 1% NP40 and bound proteins were analyzed by SDS-PAGE immunoblotting with SET and GST antibodies. The immunoblot shown is representative of 3 independent experiments. B) The carboxy terminal part or the last 17 amino acids of the M3i3 loop (T^{450}-Q^{490} and fragment A, respectively) were expressed as a GST fusion protein in BL21 cells and purified on glutathione Sepharose-4B. Mutants and truncated fragments of the last 17 amino acids were also generated (fragments B to E). Each purified construct was electrophoresed on denaturing polyacrylamide gels (10 %) and visualized by Coomassie blue staining. Purified GST fusion proteins were incubated with recombinant His-SET and processed as described in "Materials and Methods". The input

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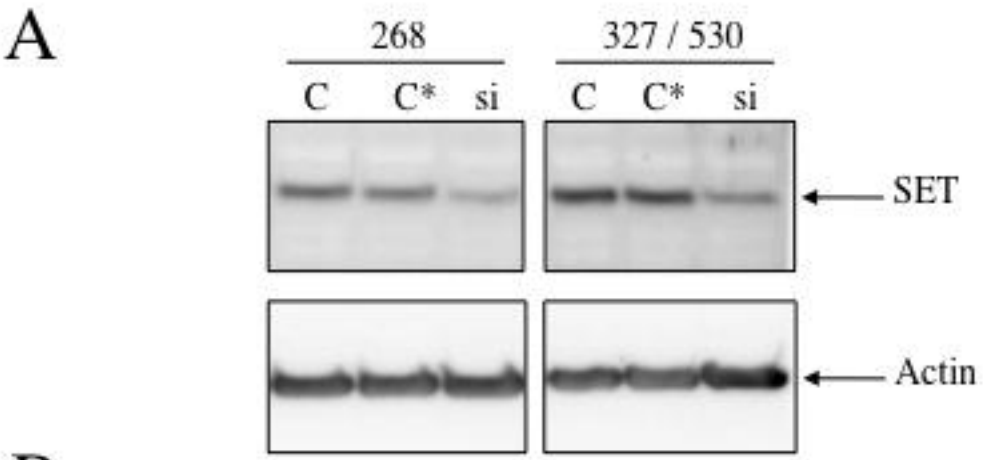
represents the total amount of His-SET used for the interaction assay. The immunoblot shown is representative of 3 independent experiments.

Figure 1



C: control siRNA (12 mismatches)
C*: control siRNA (2 mismatches)
Si: siRNA SET

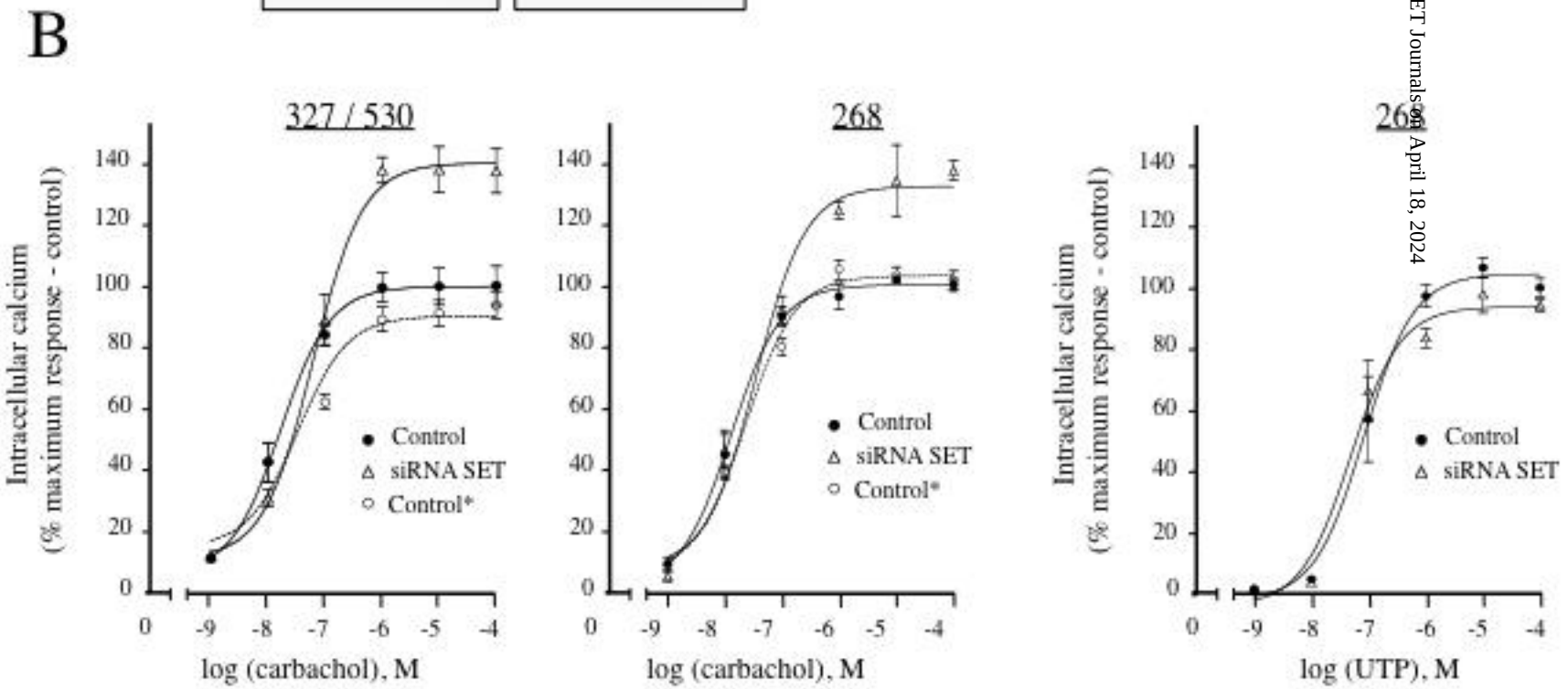


Figure 2

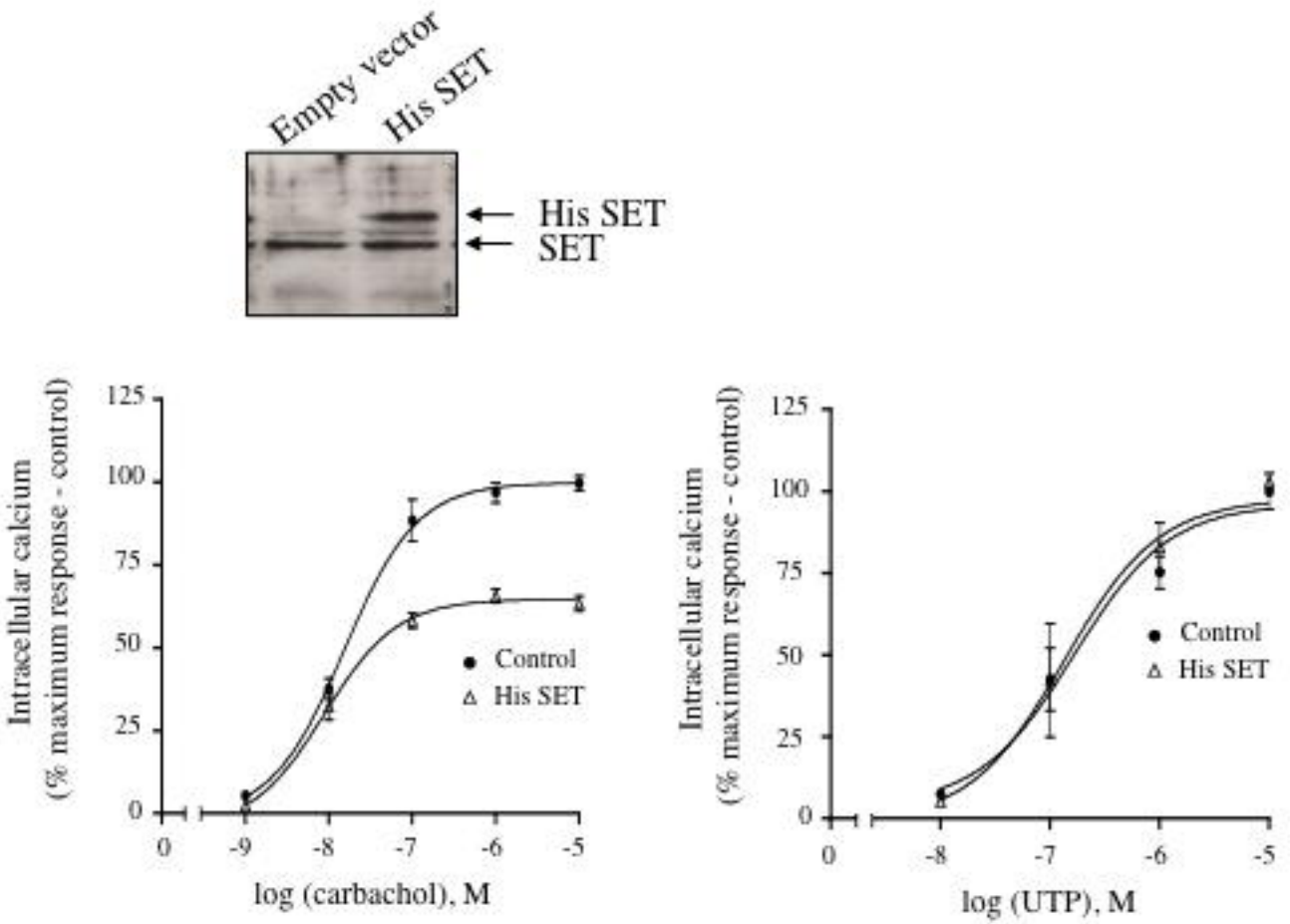
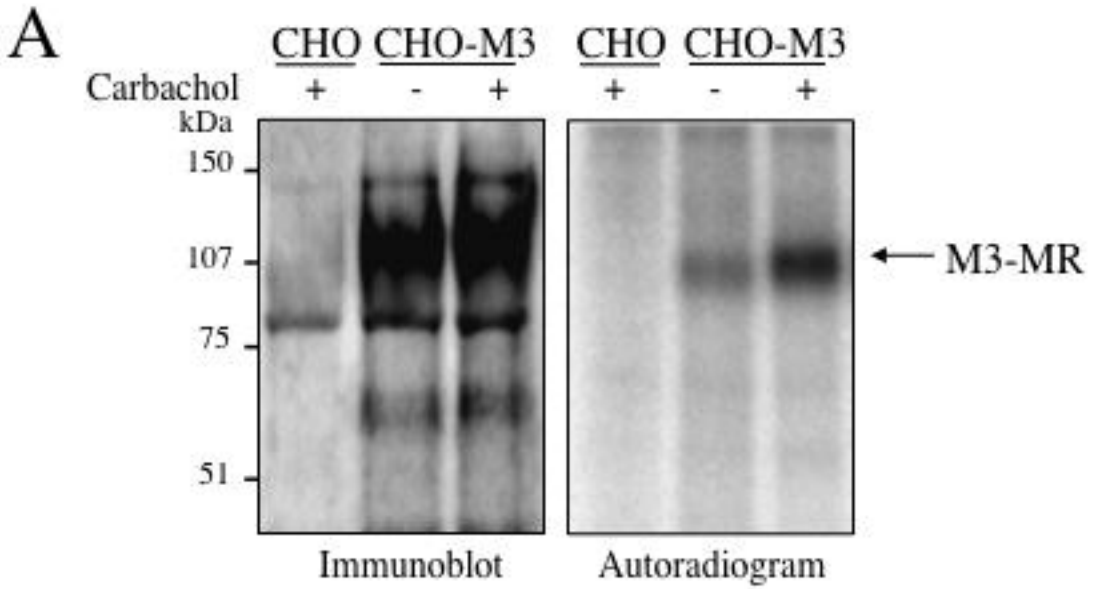
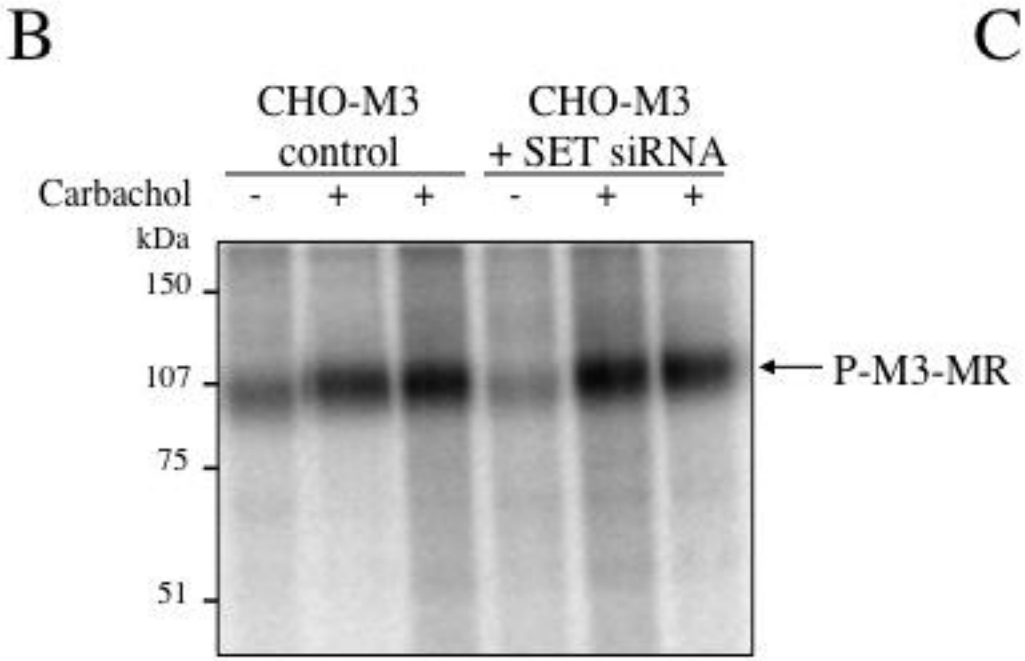


Figure 3





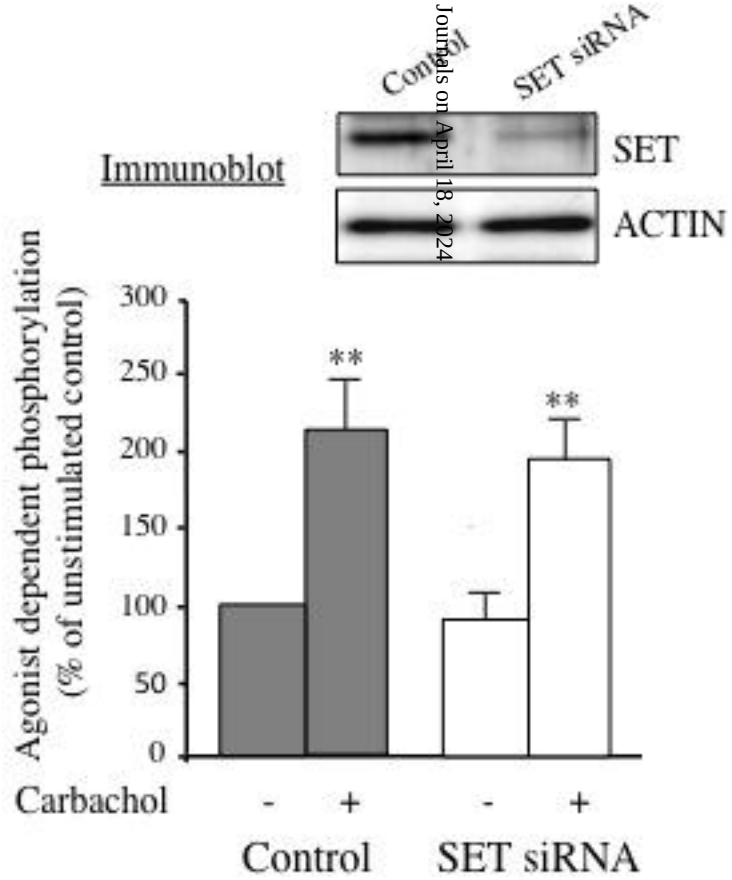


Figure 4

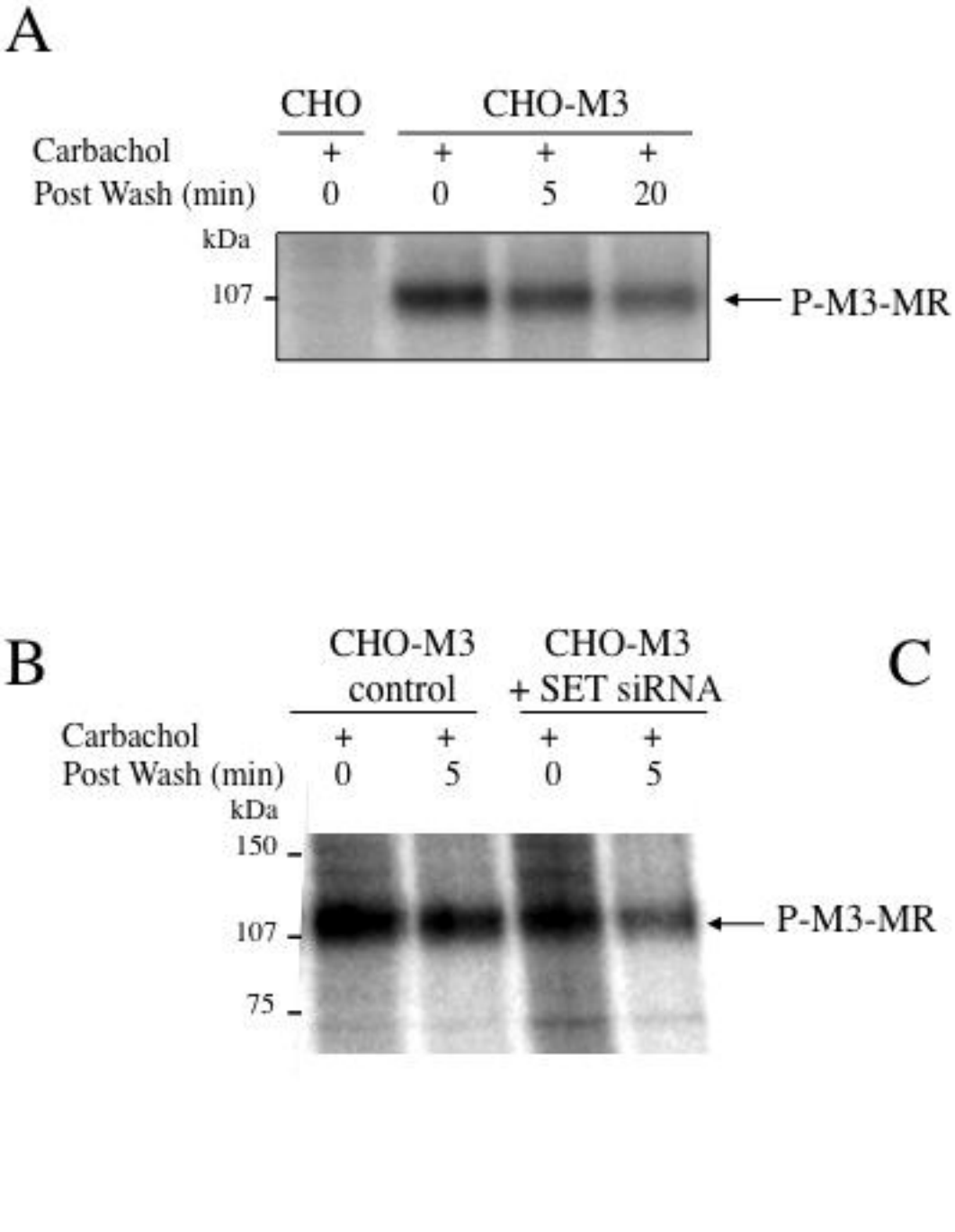


Figure 5

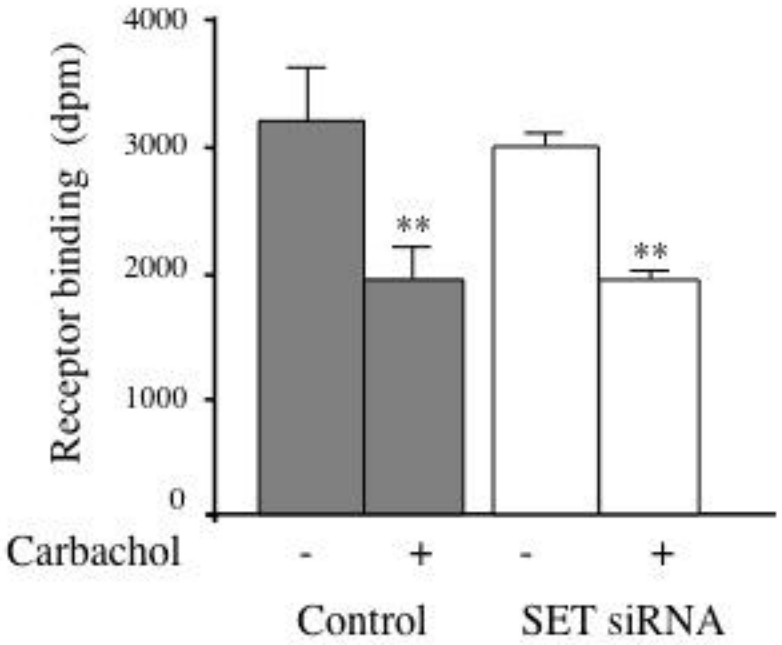


Figure 6

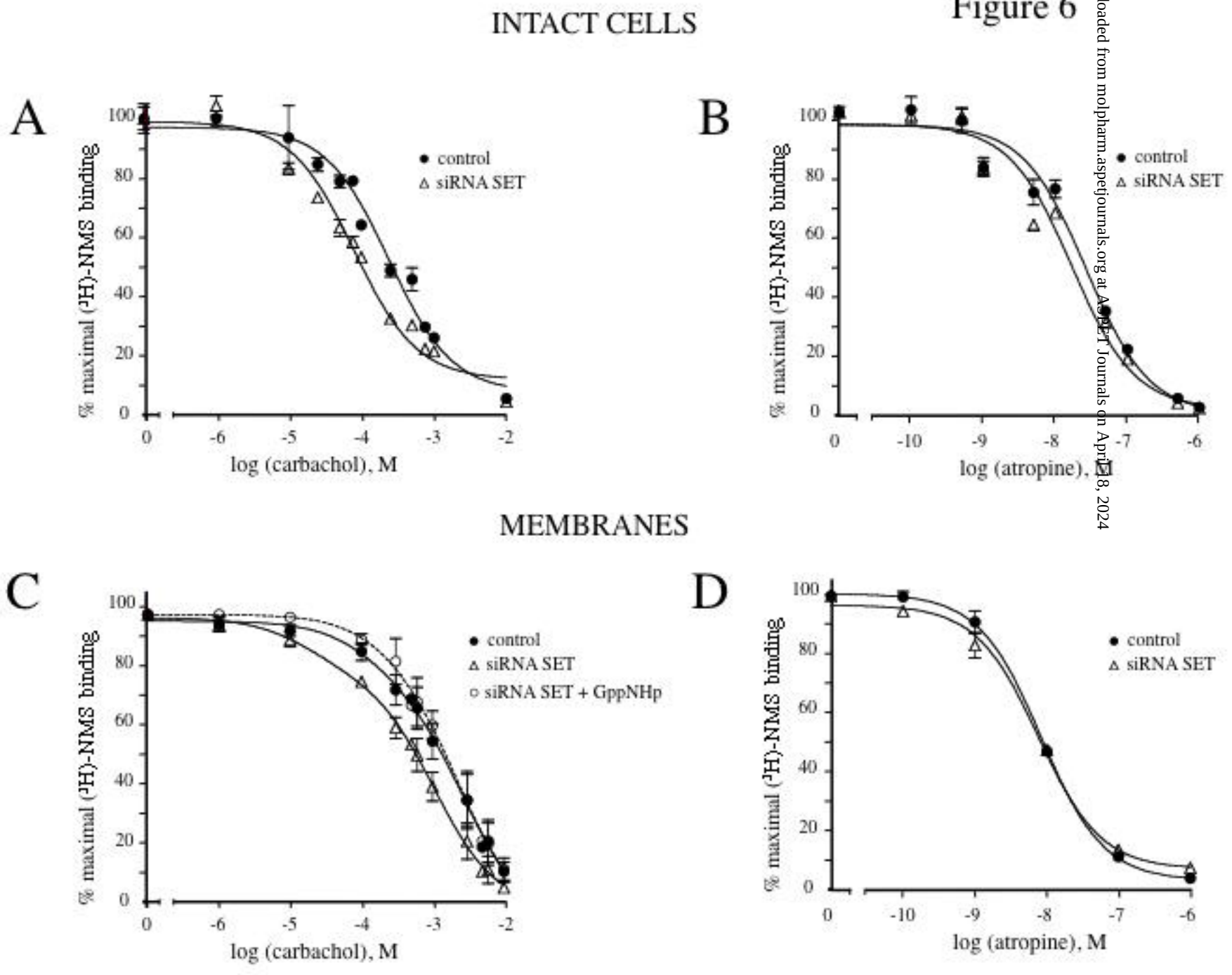
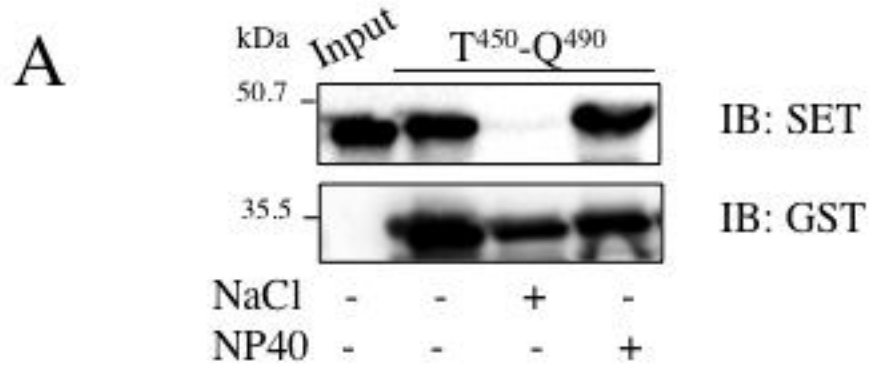
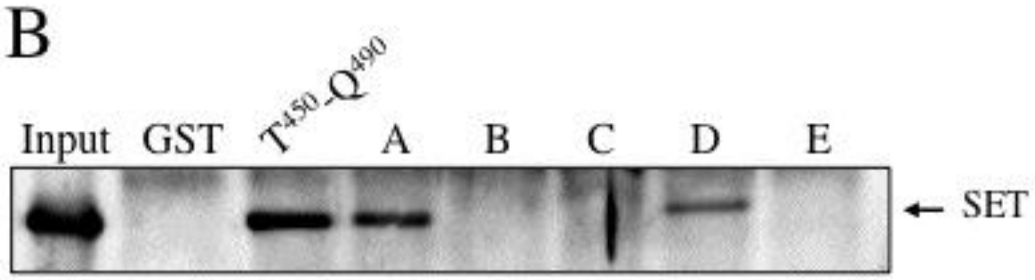
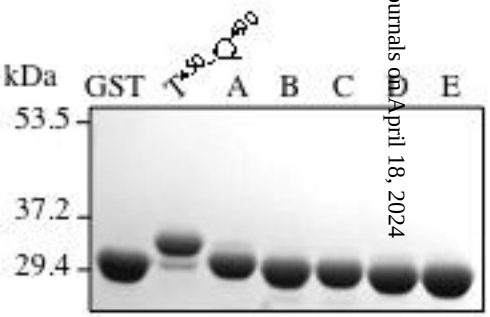


Figure 7







Coomassie blue
(fusion proteins)

	Binding
A- ^{474} ITKRKRMSLIKEKKAAQ ^{490}	+++
B- ^{474} ITAAAAMSLIKEKKAAQ ^{490}	-
C- ^{474} ITEAEAMSLIKEKKAAQ ^{490}	-
D- ^{474} ITKRKRMSLI ^{483}	++
E- ^{480} MSLIKEKKAAQ ^{490}	-