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A novel non-canonical signaling pathway for μ-Opioid receptor

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Running title: OPRM1 signals as an RTK

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Nonstandard abbreviations:

AC: adenylyl cyclase; AG-1478: N- (3- chlorophenyl)- 6, 7- dimethoxy- 4-quinazolinamine; BCA: bicinchoninic acid; Co-IP: coimmunoprecipitation; DMEM: Dulbecco's Modified Eagle Medium; EGFR: epidermal growth factor receptor; FGFR: fibroblast growth factor receptor; FTS: farnesylthiosalicylic acid; GW5074: 5-Iodo-3-[(3,5-dibromo-4-hydroxyphenyl) methylene]-2-indolinone; HDBA: lavendustin C; HEKMT: HEK293 cells stably expressing HA-OPRM1; HEKMT-Y336F: HEK293 cells

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stably expressing HA-OPRM1 with Tyr^{336} mutant; IBMX: isobutylmethylxanthine; KRHB: Krebs-Ringer HEPES buffer. What does H mean? MEF: mouse embryonic fibroblasts; OPRM1: μ-opioid receptor; PAM: protein associated with myc; PD-173074: N- [2- [[4- (diethylamino)butyl]amino]- 6- (3, 5- dimethoxyphenyl)pyrido[2, 3- d]pyrimidin- 7- yl]- N'- (1, 1- dimethylethyl)- urea; PP2: 4-Amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine; RBD: Ras binding domain; RGS: regulator of G-protein signaling; RTK: receptor tyrosine kinase; SH2/3 domain: Src homology 2/3 domain; SNAPIN: SNAP-25/Snare complex.

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Abstract

μ-Opioid receptor (OPRM1) signals as a classical G protein-coupled receptor (GPCR) by activating heterotrimeric G_i/G_o proteins resulting in adenylyl cyclase (AC) inhibition. Such AC inhibition is desensitized after prolonged agonist treatment. However, after receptor desensitization, intracellular cAMP level remains regulated by OPRM1 as demonstrated by the intracellular cAMP level increase or AC superactivation upon removal of agonist or addition of antagonist. We now demonstrate that such intracellular cAMP regulation is mediated by a novel non-canonical signaling pathway resulted from OPRM1 being converted to a receptor tyrosine kinase (RTK)-like entity. This non-canonical OPRM1 signaling is initiated by the receptor recruiting and activating Src kinase within receptor complex leading to phosphorylation of the OPRM1 Tyr^{336} residue. Phospho-Tyr^{336} serves as the docking site for Grb/SOS leading to the recruitment and activation of the Ras/Raf-1, and subsequent phosphorylation and activation of AC5/6 by Raf-1. Such sequence of events was established by the absence of Ras/Raf1 recruitment and activation by the OPRM1-Y336F mutant, by the presence of Src kinase inhibitor PP2 or the absence of Src activity, by the presence of specific Raf-1 inhibitor GW5074 or the absence of Raf-1, or by the dominant negative RasN17 mutant. Src together with Ras activate Raf1 was established by the inability of the Raf1-$Tyr^{340/341}$ mutant to activate AC. Hence, the phosphorylation of OPRM1 at Tyr^{336} by Src serves as the trigger for the conversion of a classical G_i/G_o-coupled receptor into an RTK-like entity resulting in a non-canonical pathway even after the original G_i/G_o signals are blunted.

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Introduction

In the G protein-coupled receptor (GPCR) canonical signaling, agonist stimulation leads to a rapid exchange of GDP for GTP on the α subunit of the heterotrimeric G proteins and the subsequent dissociation of α and βγ subunits. Both α and βγ subunits are involved in the activation of different effectors, including adenylyl cyclase (AC), phospholipase C and ion channels (Pierce and Lefkowitz, 2001). However, this linear model does not adequately address the full range of GPCR stimulation. There are evidences to suggest that the overall cellular responses are dependent on GPCR-mediated stimulation of a large number of complex signaling cascades. Therefore, it is not surprising that cross-regulation occurs in the GPCR signaling pathways. For example, β-arrestins have been demonstrated to not only blunt the GPCR signal but also serve as signal transducers for the same GPCR. As observed with the signaling of β_2-adrenergic receptor, β-arrestins mediate agonist-induced receptor desensitization and internalization, and also mediate the recruitment of c-Src to the receptor, which facilitates the activation of MAPK/ERK cascades (Lefkowitz and Shenoy, 2005; Luttrell et al., 1999a; McDonald et al., 2000). Cross-regulation can also occur by the modulation of either upstream or downstream events of other receptors' signaling pathways upon the GPCR activation, which results in even more complicated cellular responses. An example for such cross-regulation is the growth-promoting effects of many GPCRs are mediated through transactivation of the epidermal growth factor receptor (EGFR), which elicits cellular responses mainly through its intrinsic tyrosine kinase activity, as in the case of bradykinin receptors activation in PC12 cells leading to transactivation of the EGFR and the activation of Ras/MAP kinase pathway (Zwick et al., 1997).

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In the case of opioid receptors, activation of the receptors results in the inhibition of AC activity, activation of MAP kinases and Kir3 K^+ channels, inhibition of voltage-dependent Ca^{2+} channels and modulation of other effectors such as phospholipase C (Law et al., 2000). However, prolonged agonist exposure not only blunts these cellular responses, but also results in a compensatory increase in intracellular cAMP level, which is particularly significant upon the removal of the agonist or the addition of an antagonist such as naloxone (Law et al., 1983; Pineyro and Archer-Lahlou, 2007; Sharma et al., 1977). This compensatory increase in AC activity, or AC superactivation phenomenon has been postulated to be the molecular basis of drug tolerance and dependence (Koob and Bloom, 1988). However, the classical linear GPCR signaling pathway cannot account for such change from the initial receptor-mediated AC inhibition to the eventual receptor-mediated AC activation because the G_i/G_o-mediated signaling is blunted after prolonged agonist treatment. Although the exact mechanism for such signal changes is yet to be elucidated, activation of specific protein kinases and subsequent phosphorylation of AC isoforms (Avidor-Reiss et al., 1996; Avidor-Reiss et al., 1997) and other signaling molecules, such as G protein-coupled receptor kinases (GRK) 2/3 (Chakrabarti et al., 2001) have been suggested to be the key for the observed AC activation. Among all the protein kinases studied, PKC, MAP kinases and Raf-1 have been implicated in the AC superactivation (Li and Chang, 1996; Schallmach et al., 2006; Varga et al., 2002). Alternative mechanisms, such as agonist-induced receptor internalization and the increase in the constitutive activities of the receptor or the switching from G_i/G_o-coupled to G_s-coupled, also have been suggested to play a role in AC superactivation (Szucs et al., 2004; Walwyn et al., 2007). Regardless of the mechanism, the exact molecular events

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that lead to the switching of opioid receptor from an inhibitory response to a stimulatory response remain elusive.

In one of our previous studies, we demonstrated that AC superactivation required the recruitment and activation of Src and subsequent Src-mediated phosphorylation of OPRM1 at Tyr^{336} residue (Zhang et al., 2009). In current study, we further detailed the cellular events after Tyr^{336} phosphorylation that led to AC superactivation by demonstrating that phosphorylation of OPRM1 at Tyr^{336} serves as the docking site for Grb/SOS leading to the recruitment and the activation of the Ras/Raf-1, and subsequent phosphorylation and activation of AC5/6 by Raf-1 resulting the ultimate AC superactivation. Hence, the phosphorylation of OPRM1 at Tyr^{336} by Src kinase serves as the switch for the conversion of a classical G_i/G_o-coupled receptor into an RTK-like entity resulting in a non-canonical signal pathway even after the canonical G_i/G_o signaling has been attenuated.

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Materials and Methods

Cell culture. HA-OPRM1HEK293 (HEKMT) and HEK293 cells stably expressing HA-OPRM1 with tyrosine mutants (HEKMT-Y336F) were cultured at 37ºC in advanced MEM (Invitrogen) supplemented with 5% fetal bovine serum (Hyclone), 2 mM GlutaMAX^{TM} (Gibco), 100 units/ml penicillin, 100 ug/ml streptomycin and 0.1 mg/ml G418 (Geneticin, Gibco) in a 5% CO_2 incubator. MEF-Raf-1 wild type and cells isolated from Raf-1 knockout mice were cultured at 37ºC in advanced DMEM (Invitrogen) supplemented with 5% fetal bovine serum, 2 mM GlutaMAX^{TM}, 100 units/ml penicillin and 100 ug/ml streptomycin in a 10% CO_2 incubator. HEKMT or MEF-Raf1 wild type or MEF cells isolated from Raf-1 knockout mice were transiently transfected with various cDNA plasmids using Nucleofector transfection kits designed for HEK293 cells or for MEF cells (Lonza).

cAMP assay. Cells were seeded in 96-well plates the day before and morphine was added to the medium 4hr before assays. The culture medium was removed and cells were washed with medium at 37ºC once and then 100ul treatment buffer (0.5 mM isobutylmethylxanthine (IBMX) and 10 uM forskolin in KRHB buffer) with different concentration of naloxone was added to each well. The cells were incubated for 15 min at 37°C and then the assay was terminated by placing the plate in an 85°C water bath for 6 minutes to lyse the cells and release the intracellular cAMP. The cAMP level was measured by using AlphaScreen cAMP Detection Kit (PerkinElmer) and Biomek 2000 Laboratory Automation Workstation (Beckman Coulter). The results were analyzed and summarized with Prism4.0 software (GraphPad Software).

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Immunoprecipitation and Western blotting. Cells were treated with or without morphine and/or naloxone and then lipid raft fraction was prepared following the method as described (Zhang et al., 2009). The prepared lipid raft was incubated with mouse monoclonal anti-HA antibody (1:200, Covance) in 1 ml buffer A (100 mM NaCl, 10 mM Tris, PH 7.4) in the presence of 0.1% digitonin (Sigma-Aldrich) at 4°C overnight. Proteins in the samples were resolved by 10% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to polyvinylidene difluoride membranes (PVDF, 0.45 um, Amersham Biosciences). The presence of pRaf-1, pSrc, AC5/6 and OPRM1 in the SDS-PAGE resolved immunoprecipitates was determined with anti-pRaf1-Tyr340/341 (1:500, Millipore), anti-pSrc-Tyr416 (1:500, Millipore), anti-AC5/6 (1:500, Santa Cruz Biotechnology) or rabbit anti-HA (1:1000, Covance) antibody respectively. The band intensities were quantified and analyzed with the ImageQuant software (GE Healthcare).

Ras activation assay. The Ras activity was determined by using Ras activation assay kit (Millipore) as described by the manufacturer's protocol. Briefly, cells were lysed by adding 0.5 ml MLB buffer (Mg lysis/wash buffer: 25 mM HEPES, PH7.5, 150 mM NaCl, 1% Igepal CA-630, 10 mM $MgCl_2$ 1 mM EDTA and 10% glycerol). The solution was cleared and protein concentration was determined by BCA assay. The cell lysis solution from each treatment with same amount of protein was mixed with 5 μg Raf-1 RBD (Ras-binding domain) agarose and incubated at 4°C for 45 min with slow rotation. The agarose beads were pelleted down by brief centrifugation and washed 3 times with 0.5 ml MLB buffer each. The agarose beads were resuspended in 40 μl 2X Western sample loading buffer and boiled for 5 min. Samples were loaded on a 12% SDS-PAGE gel and Western blot was performed as described and the blot was probed with mouse

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anti-Ras antibody (1:1000, Millipore). A portion of cell lysis solution from each sample with the equal amount of protein was loaded to another gel and the result was used to determine the amount of input Ras.

Raf-1 activity assay. Raf-1 kinase activity was determined with the assay kit supplied by Millipore. Briefly, after various treatments, cells were lysed in lysis buffer containing 1% Triton X-100, 10 mM Tris, pH 7.5, 120 mM NaCl, 25 mM KCl, 100 uM Na_3VO_4 and 1 X Complete protease inhibitor (Roche). The cell lysate was cleared by centrifuging at 16,000 x g for 5 min and the supernatant was incubated with 2 ug anti-Raf-1 polyclonal antibody (Santa Cruz Biotechnology) overnight at 4°C and followed by another 3 hr incubation after the addition of 20 ul of protein G agarose beads. Afterwards, the agarose beads were washed three times with buffer A. Then, the agarose beads pellets were resuspended in 20 ul assay dilution buffer (20 mM MOPS, PH 7.2, 25 mM β-glycerol-phosphate, 5 mM EDTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol (DTT)), 10 ul Mg/ATP cocktail (75 mM $MgCl_2$, 500 uM ATP) and 1.6 ul (0.4 ug) inactive MEK1). The mixture was incubated for 30 min at 30°C with shaking. The reaction was terminated by adding 10 ul 6X Western sample loading buffer and boiled for 5 min. The samples were loaded on a 10% SDS-PAGE gel and the blot was probed with anti-pMEK1 (1:1000, Millipore) or anti-Raf-1 (1:500, Santa Cruz Biotechnology) antibody. The kinetics of Raf-1 kinase activation was determined by the use of [γ-^{32}P]ATP. The immunoprecipitation was carried out the same as above except the agarose beads pellets were suspended in 50 ul kinase buffer (50 mM Tris-HCl, pH 7.5; 10 mM $MgCl_2$, 10 mM $MnCl_2$, 25 uM ATP, 1 mM DTT and 100 uM Na_3VO_4) containing 5 uCi of [γ-^{32}P] ATP (New England Nuclear). The mixture was incubated at 30°C and 10 μl aliquots were

removed at the time points of 0, 5, 10 and 20 min. The reaction was terminated by addition of 10 μl of 40% trichloroacetic acid (TCA). The sample was then spotted onto a P81 phosphocellulose paper square (Millipore). The paper was washed extensively with 1% phosphoric acid for three times and one time with acetone. Radioactivity retained on the P81 paper was quantified by liquid scintillation counting.

RT-PCR. Total RNA of cultured cells was isolated using TRI Reagent (Molecular Research Center, INC) according to manufacturer's instructions. RNA samples were then treated with DNase I (Ambion). The RT-PCR reaction was performed by using One Step RT-PCR Kit (Qiagen) following the manufacturer's recommendations and 1.0 μg DNase I-treated total RNA was added to each reaction. The RT-PCR conditions were as follows: 30 min at 50°C followed by 15 min at 95°C and then 35 cycles of 1min at 94°C, 1min at 60°C (54°C for β-actin) and 1min at 72°C followed by 1 cycle of 10 min at 72°C. The PCR primers for Raf-1, forward: AAGAAAGCACGCTTAGATTGG, reverse: CAAGAGCTGTCTGATATT; for OPRM1, forward: CATCAAAGCACTGATC ACGATTCC, reverse: TAGGGCAATGGAGCAGTTTCTGC and for β-actin, forward: AGACCTCTATGCCAACACAGT, reverse: ATCTCGGTGGTTAGGTGTGTC.

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Results

Our previous study suggested that, during the chronic morphine treatment, OPRM1 recruited and activated Src that in turn phosphorylated the receptor at Tyr^{336}. This is the pivotal event that eventually leads to AC superactivation (Zhang et al., 2009). However, the function of this phosphorylated Tyr^{336} in OPRM1 signaling leading to AC superactivation has not been addressed. Classically, phosphorylated tyrosine residue often serves as a docking site for proteins with SH2 domains leading to the recruitment of the downstream signaling proteins to the receptor's proximity (Fan et al., 2001; Karoor et al., 1998; Tobin, 2008). Among them, Grb/SOS complex has been shown to bind to the phosphorylated Tyr of RTK and transduces the signal to the downstream Ras/MAP pathway (Satoh et al., 1990). If the Src-mediated recruitment of Grb/SOS to the phosphorylated OPRM1 Tyr^{336} is the key for AC superactivation, then the disruption of such complex formation should affect AC superactivation. When HEK293 cells stably expressing OPRM1 (HEKMT cells) were treated with the SOS1 SH3 domain binding peptide, which specifically blocks the interaction of SOS1 and Grb2, the chronic morphine-induced AC superactivation was significantly blunted (155.0 ± 15.6% compared to 309.1 ± 19.6% in presence of 10^{-4} M naloxone, Fig.1A). Since Grb2/SOS1 usually binds and activates Ras, we examine whether Ras is involved in the AC superactivation process. Transient expression of dominant negative RasN17 mutant reduced morphine-induced AC superactivation to 223.6 ± 20.7% as compared to the control of 336.3 ± 25.6% in presence of 10^{-4} M naloxone (Fig.1B). Similar result was obtained by pretreating the cells with Ras inhibitor farnesylthiosalicylic acid (FTS), which decreased AC superactivation to 194.4.1 ± 21.7% (Fig.1B).

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Measurements of Ras activation revealed there was a time-dependent increase in Ras activity during chronic morphine treatment (Supplemental Fig.1). Addition of antagonist naloxone after chronic morphine (4hr) further potentiated the Ras activation (193.7 ± 15.8% compared to 161 ± 14.9%, 4hr morphine w/o naloxone, Fig.2A). Furthermore, pretreatment of cells with Src kinase inhibitor PP2 or Ras inhibitor FTS totally abolished chronic morphine-induced Ras activation (106.0 ± 22.5% and 83.7 ± 25.1%, respectively, compared to that with morphine and naloxone treatment only, Fig.2A). The ability to blunt the morphine-induced Ras activation by these inhibitors correlates to the ability of these inhibitors to block the morphine-induced AC superactivation (Fig.1B and (Zhang et al., 2009)).

More importantly, Ras is observed to be within the OPRM1 signaling complex during chronic morphine treatment as revealed by co-immunoprecipitation (co-IP) studies (166.0 ± 22.1% compared to control, Fig.2B). Coincidently, addition of naloxone further increased the amount of Ras co-IP with OPRM1 by 271.0 ± 34.4% as compared to control (Fig.2B). This Ras and OPRM1 interaction can be prevented by transient expression of dominant negative RasN17 mutant (39.3 ±14.7% compared to the morphine and naloxone treatment only, Fig.2C). As discussed earlier, the phosphorylated Try^{336} could act as a docking site for downstream signal molecules to facilitate AC superactivation. Hence, it is not unexpected that in HEK293 cells expressing OPRM1 Y336F mutant (HEKMT-Y336F), when the morphine-induced, Src-mediated Tyr-phosphorylation was not observed (Zhang et al., 2009), the Ras activation could not be detected (Fig.2D) and the co-IP of Ras with OPRM1 was not observed either (Zhang, unpublished observations).

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Since Ras activation is often the consequence of RTK activation, and transactivation of RTK by opioid receptor has been reported (Belcheva et al., 2002), whether OPRM1-mediated Ras activation and subsequent AC superactivation is the consequence of RTK transactivation was examined. HEKMT cells were incubated with EGFR inhibitor, Lavendustin C (HDBA), at different concentrations as indicated (Fig.3A). Even at the highest concentration of HDBA tested, 100 nM, attenuation of AC superactivation was not observed (IC_{50}, 12 nM). These results were confirmed by using various concentrations of another EGFR inhibitor, AG-1478 (IC_{50}, 3 nM, Fig.3B). It was also reported that oipoid receptor could transactivate FGFR (Belcheva, et al., 2002), however, pretreatment the cells with various concentrations of FGFR inhibitor, PD-173074 (IC_{50}, 21.5 nM), didn't show any inhibition on chronic morphine-induced AC superactivation (Fig.3C). Based on these observations, it is unlikely that the Ras activation observed in the presence of naloxone after chronic morphine treatment is the consequence of an RTK transactivation.

Upon activation, the active Ras binds to Raf kinases with high affinity and causes Raf kinases to translocate to the cell membrane, where Raf activation takes place. The involvement of Raf-1 in chronic opioid administrations had been implicated based on the fact that pretreatment with Raf-1 specific inhibitor GW5074 significantly blunted the AC superactivation (Varga et al., 2002). By carrying out co-IP studies, we could demonstrate that activated Raf-1 (pRaf-1), activated c-Src (pSrc) and AC5/6 formed a signal complex with OPRM1 in a chronic morphine-dependent manner (264.6 ± 43.0%, 215.3 ± 22.8% and 155.6 ± 21.6% respectively, compared to the control, Fig.4A, B). Pretreatment with Raf-1 inhibitor GW5074 totally blocked the co-IP of pRaf-1 with OPRM1 but did not

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alter the morphine-induced Src activation (124.7 ± 39.1% and 187.2 ± 22.0% respectively). However, pretreatment of Src kinase inhibitor PP2 not only eliminated pSrc from the complex but also prevented the appearance of pRaf-1 in the complex (92.5 ± 23.0% and 113.0 ± 36.7%, respectively), suggesting that the presence of pRaf-1 within the OPRM1 complex requires Src kinase activation. Both PP2 and GW5074 pretreatment also reduced the presence of AC5/6 in OPRM1 complex in presence of morphine and naloxone from 155.6 ± 21.6% to 82.0 ± 13.2% and 73.3 ± 6.5%, respectively (Fig.4A, B).

The measurements of Raf-1 kinase activity revealed that the Raf-1 activity was significantly enhanced within the OPRM1 signaling complex as determined by the phosphorylation of Raf-1 substrate MEK1 (pMEK1), after chronic morphine treatment (168.0 ± 26.7%) and after chronic morphine followed by naloxone treatment (191.0 ± 36.0%) when compared to the control (Fig.4C, D). Similar result was also obtained from a Raf-1 kinase kinetics assay using [γ-^{32}P]ATP to label the substrate (Fig.4E). All these results demonstrated that the phosphorylation of OPRM1 Tyr^{336} is the key for recruiting Ras to the receptor complex leading to the translocation and activation of Raf-1 to cell membrane via binding of the protein kinase to Ras. If this is the scenario, then it should follow that without Tyr^{336} being phosphorylated, Raf-1 translocation and activation should not occur. Indeed, in cells expressing the OPRM1 Y336F mutant, the phosphorylation of Raf-1's substrate MEK1 was not observed after chronic morphine treatment (Fig.5A, B).

To further confirm the involvement of Raf-1 in chronic morphine-induced AC superactivation, MEF cells from both wild type and Raf-1 knockout mice were used to transiently express the OPRM1 and/or Raf-1. Figure 6A shows the RT-PCR results of

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Raf-1 levels that were similar in various transfection combinations as indicated. The cAMP assays using these transfected cells suggested that only when cells expressing both OPRM1 and Raf-1, the AC superactivation was significantly potentiated (Raf1WT+OPRM1, 194.8 ± 15.6% and Raf1KO+OPRM1+Raf1, 278.5 ± 19.7%). Other combinations with the absence of either OPRM1 or Raf-1 did not exhibit AC superactivation (Fig.6B, C). These MEF cells studies support the Raf-1 inhibitor GW5074 studies and clearly indicate that Raf-1 activation is critical for the observed AC superactivation.

At present, the exact mechanism for Raf-1 kinase activation remains elusive. However, the Src kinase phosphorylation at 340 and/or 341 tyrosine residues is one of the keys for the Raf-1 activation (Marais et al., 1995). Since activated Raf-1 and activated Src can be observed within the OPRM1 signaling complex in co-IP experiments (Fig.4A), it is likely that Src within the receptor complex could phosphorylate and thus activate Raf-1 leading to AC superactivation. To test this hypothesis in AC superactivation, the Tyr^{340} or Tyr^{341} was mutated to Ala singly or in combination to generate $Raf1^{Y340A}$ or $Raf1^{Y341A}$ single mutant, or $Raf1^{Y340/341AA}$ double mutant. In order to mimic the phosphorylated state at these Tyr residues, they were mutated to Asp to generate constitutively active $Raf1^{Y340/341DD}$ mutant. These mutants or wild type Raf-1 were transiently expressed in MEF-Raf1 knockout cells and their expression levels were similar as determined by RT-PCR (Fig.7A). To test their effect on chronic morphine induced AC superactivation, the wild type or mutant Raf-1 was co-transfected with OPRM1 to the MEF-Raf-1 knockout cells. Both single mutant $Raf1^{Y340A}$ and $Raf1^{Y341A}$ and double mutant $Raf1^{Y340/341AA}$ significantly blunted the AC superactivation to 155.2 ±

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14.0%, 177.5 ± 16.0% and 131.4 ±12.1% respectively, as compared to the cells expressing wild type Raf-1 where AC superactivation was at 286.3 ± 21.9% above the control level. In contrast, expression of constitutively active mutant $Raf1^{Y340/341DD}$ significantly potentiated the AC superactivation to 436.9 ± 29.7% above the control level (Fig.7B, C). These studies suggest that the phosphorylation of Raf-1 at these Tyr residues is another key for the observed AC superactivation.

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Discussion

In past decades, the classical view of GPCRs as simple heterotrimeric G protein activators is gradually evolved into intracellular signaling pathways with complex modulations. Emerging evidence suggests that the efficiency, specificity and fidelity of GPCR signaling can be achieved by a more confined and organized system and it may involve crosstalk, fine-tuning and specific regulation at multiple levels, even within the same GPCR signal pathway. For example, β-arrestins have been shown to recruit different downstream signal molecules to control the outcome of the signal transduction in addition to their roles as signal terminators (Cao et al., 2000; Lefkowitz and Shenoy, 2005). Crosstalk can also happen between different GPCRs' pathways leading to distinct signal pathways that are different with the original ones. For example, activation of PLC-coupled $P2Y_2$ receptors specifically inhibited β_2-adrennergic receptor-mediated cAMP production via G_i proteins in pGT-β cells (Suh et al., 2001). The cross-regulations may also happen between GPCRs and other signal pathways, among them, the transactivation between GPCR and RTK has been intensively studied (Daub et al., 1996). It has been reported that stimulation of various kinds of GPCRs, including the receptors for bradykinin, angiotensin, thrombin, LPA, bombesin, endothelin and muscarinic acetylcholine, can transactivate EGFR system (Gschwind et al., 2001; Kalmes et al., 2000; Pierce et al., 2001; Prenzel et al., 1999).

For opioid receptors, agonists-induced acute inhibition of AC activity is a classical GPCR signaling event (Zhang et al., 2006). However, following the prolonged treatment, the inhibition effect is blunted and it is gradually converted to a compensatory increase in intracellular cAMP level and is particularly significant upon the removal of the agonist or

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the addition of an antagonist (Law et al., 1982; Pineyro and Archer-Lahlou, 2007; Sharma et al., 1977). This observed change from receptor-mediated AC inhibition to receptor-mediated AC activation cannot be explained by classical GPCR signal paradigm but reflects possible intracellular signal switching. Our previous studies suggested that Src kinase activation and subsequent Src-mediated phosphorylation of OPRM1 at Tyr^{336} were the keys for such signal switching. Similar GPCR phosphorylation-induced signal switching had been intensively studied in β-arrestin-mediated signaling. Lefkowitz and co-workers are the first to report that β-arrestins bound to GRK-phosphorylated receptor is not only a signal termination event but also β-arrestins are able to serve as adaptors to recruit Src to the receptor complex leading to a Src-dependent, receptor-stimulated activation of downstream effectors such as the MAP kinases (Luttrell et al., 1999b). Since β-arrestins can recruit a broad spectrum of signaling molecules, GRK/β-arrestin system has been demonstrated to function not solely in the receptor desensitization process, but also in signaling pathways participate in biased agonism (Lefkowitz and Shenoy, 2005).

In current study, by identifying various molecular events after chronic morphine administration, a novel signal pathway for opioid receptor has emerged. Distinct from the canonical G_i/G_o-mediated receptor signaling, the phosphorylation of OPRM1 Tyr^{336} serves as the docking site for Grb/SOS (Supplemental Fig.2) leading to the recruitment and the activation of the Ras/Raf-1, and subsequent phosphorylation and activation of AC5/6 by Raf-1. A number of GPCRs have been shown to activate or inhibit the MAPK cascade by either a Ras dependent or independent pathway requiring the GPCRs transactivation of RTKs, such as EGFR (Daub et al., 1996). For example, expression of

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polypeptides that sequester free Gβγ subunits blocks Ras-dependent MAPK/ERK activation via the LPA receptor (Herrlich et al., 1998), α_{2A}-adrenergic receptor (van Biesen et al., 1995), M_2-muscarinic receptor (Koch et al., 1994) and D_2-dopamine receptor (Faure et al., 1994). Our data suggest that Ras activation occurs during chronic morphine treatment and is even more pronounced upon the addition of antagonist-naloxone, which mimic the drug withdrawal state (Fig.2A). This Ras activation does not require the transactivation of RTKs (Fig.3); instead, it occurs within the OPRM1 signal complex in a Src-dependent manner (Fig.2A). Furthermore, in cells expressing Tyr^{336} mutant receptor, the Ras activation was not observed and Ras could not be detected in the OPRM1 signal complex either (Fig.2*D and* Zhang, unpublished observations). Together with the observation that disruption of SOS1 and Grb2 interaction significantly blunted the AC activation (Fig.1), we conclude that the phospho-Tyr^{336} is a docking site for the recruitment of Grab2/SOS1/Ras to the OPRM1 thus forming a signal complex in which Ras is activated.

In the classic view, the activated Ras will bind to Raf-1 and recruit the protein kinase to the cell membrane, where the Raf-1 is activated (Baccarini, 2005). Based on the observation that Raf-1 inhibitor GW5074 attenuates the AC activation, Raf-1 has been implicated in the opioid receptor mediated chronic morphine-induced AC superactivation (Varga et al., 2002). Our current study is the first to suggest that Raf-1 is recruited to the OPRM1 signal complex and activated during chronic morphine/naloxone treatment, and such Raf-1 activation is Src kinase dependent (Fig.4A, B). Based on our previous studies indicating that the OPRM1 could recruit and activate Src during chronic morphine treatment, together with the fact that in cells expressing Tyr^{336} mutant OPRM1, the Raf-1

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activation is not observed (Fig.5), we conclude that the phospho-Tyr^{336} recruited the Raf-1 to the OPRM1 signal complex via activated Ras and where Raf-1 was activated by Src. By using MEF-Raf-1 wild type and knockout cells, Raf-1 activation is demonstrated as a pre-requisite for AC superactivation and this activation is mediated by OPRM1 directly and not by other receptor signal pathways (Fig.6). The current accepted model for Raf-1 activation involves the recruitment to the plasma membrane by the activated Ras via the Ras binding domain (RBD) in Raf-1, the dephosphorylation of the inhibitory PKA/PKB site Ser^{259} at Raf-1 by phosphatase PP2A, and the eventual phosphorylation at Tyr^{340} and/or Tyr^{341} probably by Src kinase (Baccarini, 2005; Kolch, 2002; Marais et al., 1995). Our data have shown that Src is required for Raf-1 activation (Fig.4A, B), the mutation at Tyr^{340} or Tyr^{341} significantly attenuates the AC activation and the double mutation at these sites almost totally abolishes the AC activation, suggesting that these two sites are equally important for the Raf-1 activation. The observations with constitutively active mutant Tyr$^{340/341DD}$ significantly enhanced AC activation further substantiate such conclusion (Fig.7B, C). One may expect that the constitutively active Raf-1-Tyr$^{340/341DD}$ mutant itself is enough to cause the AC superactivation, however, without chronic morphine/naloxone treatment, Src activation and subsequent phosphorylation at Tyr336 of OPRM1 were not observed. Furthermore, without recruitment of Ras to and activated of Ras by OPRM1, Raf-1 was not recruited to OPRM1 signal complex. Hence, the constitutively active Raf-1 itself could not lead to AC superactivation. Except phosphorylation and activation by Src, other protein kinases, such as PKC (Kolch et al., 1993), Akt (Zimmermann and Moelling, 1999), PAK3 (King et al., 1998) and PKA (Dhillon et al., 2002), have been suggested to involve in Raf-1 regulation either positively

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or negatively. Whether these protein kinases are involved in the Raf-1 activation during chronic morphine administration remains to be examined.

In RTK/Ras/MAPK cascade, the activated Raf-1 will directly phosphorylate MEK1/2 that will leads to the activation of MAPK/ERK, which in turn can activate AC. However, during the chronic morphine treatment, the MAPK activation is not observed (Zhang, unpublished observation). Instead, several studies suggest that the Raf-1 can phosphorylate several AC isoenzymes directly, such as AC2, 5 and 6 (Ding et al., 2004). Avidor-Reiss *et al*. reported that AC activation can be reproduced in COS-7 cells with co-transfection of AC5 and OPRM1 (Avidor-Reiss et al., 1997). It is also shown that Raf-1-mediated phosphorylation of AC6 leads to the sensitization of this isoenzyme to stimulators (Tan et al., 2001), such as forskolin (i.e. AC activation). Furthermore, mutagenesis studies from the same group suggest that Ser^{750} on AC6 is phosphorylated by Raf-1 and mutation of Ser^{750} to Ala significantly impaired AC6 activity (Ding et al., 2004). Our current data clearly indicate that AC5/6 was recruited to OPRM1 signal complex during chronic morphine treatment in a Src- and Raf-1-dependent manner (Fig.4A, B). Because other protein kinases, such PKA and PKC, and also some regulatory proteins, such as RGS2 (Roy et al., 2006), SNAPIN (SNAP-25/Snare complex) (Chou et al., 2004) and PAM (protein associated with myc) (Gao and Patel, 2005) have been suggested to be capable of modulating the AC5/6 activity, chronic morphine-induced AC activation could involve multiple protein kinases in addition to Src and Raf-1 and the regulatory proteins as discussed above.

In conclusion, our current study provided a new model for the OPRM1-mediated chronic morphine-induced AC superactivation. The phosphorylation at Tyr^{336} by Src

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kinase serves as a docking site to recruit Grb/SOS/Ras and Raf-1. This chronic agonist-induced, site-specific phosphorylation results in a distinct signaling outcome. As a result, the OPRM1 is converted from a classical G_i/G_o-coupled receptor into an RTK-like entity resulting in a non-canonical signaling pathway even when the canonical G_i/G_o signaling has been attenuated (summarized in Fig.8). It is noteworthy that a similar model has been suggested in RTK signaling that RTKs exert some of their effects by engagement of GPCR signaling molecules, including heterotrimeric G proteins and β-arrestins, suggesting that RTKs can also act as GPCR-like receptors (Cao et al., 2009; Dalle et al., 2001; Liang and Garrison, 1991; Luttrell et al., 1990; Povsic et al., 2003). Since chronic agonist-induced AC superactivation has been implicated in drug addiction processes, if the Src-mediated Tyr^{336} phosphorylation can be prevented while still maintaining the overall cellular Src activity, then a specific treatment paradigm for opioid drug addiction might be attainable.

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Authorship Contributions:

Participated in research design: Zhang and Law.

Conducted experiments: Zhang.

Performed data analysis: Zhang, Loh and Law.

Wrote the manuscript: Zhang and Law.

References

Avidor-Reiss T, Nevo I, Levy R, Pfeuffer T and Vogel Z (1996) Chronic opioid treatment induces adenylyl cyclase V superactivation. Involvement of Gbetagamma. *J Biol Chem* **271**:21309-21315.

Avidor-Reiss T, Nevo I, Saya D, Bayewitch M and Vogel Z (1997) Opiate-induced adenylyl cyclase superactivation is isozyme-specific. *J Biol Chem* **272**:5040-5047.

Baccarini M (2005) Second nature: biological functions of the Raf-1 "kinase". *FEBS Lett* **579**(15):3271-3277.

Belcheva MM, Haas PD, Tan Y, Heaton VM and Coscia CJ (2002) The fibroblast growth factor receptor is at the site of convergence between mu-opioid receptor and growth factor signaling pathways in rat C6 glioma cells. *J Pharmacol Exp Ther* **303**(3):909-918.

Cao C, Huang X, Han Y, Wan Y, Birnbaumer L, Feng GS, Marshall J, Jiang M and Chu WM (2009) Galpha(i1) and Galpha(i3) are required for epidermal growth factor-mediated activation of the Akt-mTORC1 pathway. *Sci Signal* **2**(68):ra17.

Cao W, Luttrell LM, Medvedev AV, Pierce KL, Daniel KW, Dixon TM, Lefkowitz RJ and Collins S (2000) Direct binding of activated c-Src to the beta 3-adrenergic receptor is required for MAP kinase activation. *J Biol Chem* **275**(49):38131-38134.

Chakrabarti S, Oppermann M and Gintzler AR (2001) Chronic morphine induces the concomitant phosphorylation and altered association of multiple signaling proteins: a novel mechanism for modulating cell signaling. *Proc Natl Acad Sci U S A* **98**(7):4209-4214.

Chou JL, Huang CL, Lai HL, Hung AC, Chien CL, Kao YY and Chern Y (2004) Regulation of type VI adenylyl cyclase by Snapin, a SNAP25-binding protein. *J Biol Chem* **279**(44):46271-46279.

Dalle S, Ricketts W, Imamura T, Vollenweider P and Olefsky JM (2001) Insulin and insulin-like growth factor I receptors utilize different G protein signaling components. *J Biol Chem* **276**(19):15688-15695.

Daub H, Weiss FU, Wallasch C and Ullrich A (1996) Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors. *Nature* **379**(6565):557-560.

Dhillon AS, Pollock C, Steen H, Shaw PE, Mischak H and Kolch W (2002) Cyclic AMP-dependent kinase regulates Raf-1 kinase mainly by phosphorylation of serine 259. *Mol Cell Biol* **22**(10):3237-3246.

Ding Q, Gros R, Gray ID, Taussig R, Ferguson SS and Feldman RD (2004) Raf kinase activation of adenylyl cyclases: isoform-selective regulation. *Mol Pharmacol* **66**(4):921-928.

Fan G, Shumay E, Malbon CC and Wang H (2001) c-Src tyrosine kinase binds the beta 2-adrenergic receptor via phospho-Tyr-350, phosphorylates G-protein-linked receptor kinase 2, and mediates agonist-induced receptor desensitization. *J Biol Chem* **276**(16):13240-13247.

Faure M, Voyno-Yasenetskaya TA and Bourne HR (1994) cAMP and beta gamma subunits of heterotrimeric G proteins stimulate the mitogen-activated protein kinase pathway in COS-7 cells. *J Biol Chem* **269**(11):7851-7854.

Gao X and Patel TB (2005) Histidine residues 912 and 913 in protein associated with Myc are necessary for the inhibition of adenylyl cyclase activity. *Mol Pharmacol* **67**(1):42-49.

Gschwind A, Zwick E, Prenzel N, Leserer M and Ullrich A (2001) Cell communication networks: epidermal growth factor receptor transactivation as the paradigm for interreceptor signal transmission. *Oncogene* **20**(13):1594-1600.

Herrlich A, Daub H, Knebel A, Herrlich P, Ullrich A, Schultz G and Gudermann T (1998) Ligand-independent activation of platelet-derived growth factor receptor is a necessary intermediate in lysophosphatidic, acid-stimulated mitogenic activity in L cells. *Proc Natl Acad Sci U S A* **95**(15):8985-8990.

Kalmes A, Vesti BR, Daum G, Abraham JA and Clowes AW (2000) Heparin blockade of thrombin-induced smooth muscle cell migration involves inhibition of epidermal growth factor (EGF) receptor transactivation by heparin-binding EGF-like growth factor. *Circ Res* **87**(2):92-98.

Karoor V, Wang L, Wang HY and Malbon CC (1998) Insulin stimulates sequestration of beta-adrenergic receptors and enhanced association of beta-adrenergic receptors with Grb2 via tyrosine 350. *J Biol Chem* **273**(49):33035-33041.

King AJ, Sun H, Diaz B, Barnard D, Miao W, Bagrodia S and Marshall MS (1998) The protein kinase Pak3 positively regulates Raf-1 activity through phosphorylation of serine 338. *Nature* **396**(6707):180-183.

Koch WJ, Hawes BE, Allen LF and Lefkowitz RJ (1994) Direct evidence that Gi-coupled receptor stimulation of mitogen-activated protein kinase is mediated by G beta gamma activation of p21ras. *Proc Natl Acad Sci U S A* **91**(26):12706-12710.

Kolch W (2002) Ras/Raf signalling and emerging pharmacotherapeutic targets. *Expert Opin Pharmacother* **3**(6):709-718.

Kolch W, Heidecker G, Kochs G, Hummel R, Vahidi H, Mischak H, Finkenzeller G, Marme D and Rapp UR (1993) Protein kinase C alpha activates RAF-1 by direct phosphorylation. *Nature* **364**(6434):249-252.

Koob GF and Bloom FE (1988) Cellular and molecular mechanism of drug dependence. *Science* **242**:715-723.

Law PY, Hom DS and Loh HH (1982) Loss of opiate receptor activity in neuroblastoma X glioma NG108-15 hybrid cells after chronic opiate treatment. A multiple-step process. *Mol Pharmacol* **22**(1):1-4.

Law PY, Hom DS and Loh HH (1983) Opiate receptor down-regulation and desensitization in neuroblastoma X glioma NG108-15 hybrid cells are two separate cellular adaptation processes. *Mol Pharmacol* **24**(3):413-424.

Law PY, Wong YH and Loh HH (2000) Molecular mechanisms and regulation of opioid receptor signaling. *Annu Rev Pharmacol Toxicol* **40**:389-430.

Lefkowitz RJ and Shenoy SK (2005) Transduction of receptor signals by beta-arrestins. *Science* **308**(5721):512-517.

Li LY and Chang KJ (1996) The stimulatory effect of opioids on mitogen-activated protein kinase in Chinese hamster ovary cells transfected to express mu-opioid receptors. *Mol Pharmacol* **50**:599-602.

Liang MN and Garrison JC (1991) The epidermal growth factor receptor is coupled to a pertussis toxin-sensitive guanine nucleotide regulatory protein in rat hepatocytes. *J Biol Chem* **266**(20):13342-13349.

Luttrell L, Kilgour E, Larner J and Romero G (1990) A pertussis toxin-sensitive G-protein mediates some aspects of insulin action in BC3H-1 murine myocytes. *J Biol Chem* **265**(28):16873-16879.

Luttrell LM, Daaka Y and Lefkowitz RJ (1999a) Regulation of tyrosine kinase cascades by G-protein-coupled receptors. *Curr Opin Cell Biol* **11**(2):177-183.

Luttrell LM, Ferguson SS, Daaka Y, Miller WE, Maudsley S, Della Rocca GJ, Lin F, Kawakatsu H, Owada K, Luttrell DK, Caron MG and Lefkowitz RJ (1999b) Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes. *Science* **283**(5402):655-661.

Marais R, Light Y, Paterson HF and Marshall CJ (1995) Ras recruits Raf-1 to the plasma membrane for activation by tyrosine phosphorylation. *Embo J* **14**(13):3136-3145.

McDonald PH, Chow CW, Miller WE, Laporte SA, Field ME, Lin FT, Davis RJ and Lefkowitz RJ (2000) Beta-arrestin 2: a receptor-regulated MAPK scaffold for the activation of JNK3. *Science* **290**(5496):1574-1577.

Pierce KL and Lefkowitz RJ (2001) Classical and new roles of beta-arrestins in the regulation of G-protein-coupled receptors. *Nat Rev Neurosci* **2**(10):727-733.

Pierce KL, Tohgo A, Ahn S, Field ME, Luttrell LM and Lefkowitz RJ (2001) Epidermal growth factor (EGF) receptor-dependent ERK activation by G protein-coupled receptors: a co-culture system for identifying intermediates upstream and downstream of heparin-binding EGF shedding. *J Biol Chem* **276**(25):23155-23160.

Pineyro G and Archer-Lahlou E (2007) Ligand-specific receptor states: implications for opiate receptor signalling and regulation. *Cell Signal* **19**(1):8-19.

Povsic TJ, Kohout TA and Lefkowitz RJ (2003) Beta-arrestin1 mediates insulin-like growth factor 1 (IGF-1) activation of phosphatidylinositol 3-kinase (PI3K) and anti-apoptosis. *J Biol Chem* **278**(51):51334-51339.

Prenzel N, Zwick E, Daub H, Leserer M, Abraham R, Wallasch C and Ullrich A (1999) EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature* **402**(6764):884-888.

Roy AA, Baragli A, Bernstein LS, Hepler JR, Hebert TE and Chidiac P (2006) RGS2 interacts with Gs and adenylyl cyclase in living cells. *Cell Signal* **18**(3):336-348.

Satoh T, Endo M, Nakafuku M, Akiyama T, Yamamoto T and Kaziro Y (1990) Accumulation of p21ras.GTP in response to stimulation with epidermal growth factor and oncogene products with tyrosine kinase activity. *Proc Natl Acad Sci U S A* **87**(20):7926-7929.

Schallmach E, Steiner D and Vogel Z (2006) Inhibition of AC-II activity following chronic agonist exposure is modulated by phosphorylation. *J Mol Neurosci* **29**(2):115-122.

Sharma KS, Klee WA and Nirenberg M (1977) Opiate-dependent modulation of adenylate cyclase. *Proc Natl Acad Sci U S A* **74**:3365-3369.

Suh BC, Kim JS, Namgung U, Han S and Kim KT (2001) Selective inhibition of beta(2)-adrenergic receptor-mediated cAMP generation by activation of the P2Y(2) receptor in mouse pineal gland tumor cells. *J Neurochem* **77**(6):1475-1485.

Szucs M, Boda K and Gintzler AR (2004) Dual effects of DAMGO [D-Ala2,N-Me-Phe4,Gly5-ol]-enkephalin and CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-

NH2) on adenylyl cyclase activity: implications for mu-opioid receptor Gs coupling. *J Pharmacol Exp Ther* **310**(1):256-262.

Tan CM, Kelvin DJ, Litchfield DW, Ferguson SS and Feldman RD (2001) Tyrosine kinase-mediated serine phosphorylation of adenylyl cyclase. *Biochemistry* **40**(6):1702-1709.

Tobin AB (2008) G-protein-coupled receptor phosphorylation: where, when and by whom. *Br J Pharmacol* **153 Suppl 1**:S167-176.

van Biesen T, Hawes BE, Luttrell DK, Krueger KM, Touhara K, Porfiri E, Sakaue M, Luttrell LM and Lefkowitz RJ (1995) Receptor-tyrosine-kinase- and G beta gamma-mediated MAP kinase activation by a common signalling pathway. *Nature* **376**(6543):781-784.

Varga EV, Rubenzik M, Grife V, Sugiyama M, Stropova D, Roeske WR and Yamamura HI (2002) Involvement of Raf-1 in chronic delta-opioid receptor agonist-mediated adenylyl cyclase superactivation. *European Journal of Pharmacology* **451**(1):101-102.

Walwyn W, Evans CJ and Hales TG (2007) Beta-arrestin2 and c-Src regulate the constitutive activity and recycling of mu opioid receptors in dorsal root ganglion neurons. *J Neurosci* **27**(19):5092-5104.

Zhang L, Tetrault J, Wang W, Loh HH and Law PY (2006) Short- and long-term regulation of adenylyl cyclase activity by delta-opioid receptor are mediated by Galphai2 in neuroblastoma N2A cells. *Mol Pharmacol* **69**(6):1810-1819.

Zhang L, Zhao H, Qiu Y, Loh HH and Law PY (2009) Src phosphorylation of micro-receptor is responsible for the receptor switching from an inhibitory to a stimulatory signal. *J Biol Chem* **284**(4):1990-2000.

Zimmermann S and Moelling K (1999) Phosphorylation and regulation of Raf by Akt (protein kinase B). *Science* **286**(5445):1741-1744.

Zwick E, Daub H, Aoki N, Yamaguchi-Aoki Y, Tinhofer I, Maly K and Ullrich A (1997) Critical role of calcium- dependent epidermal growth factor receptor transactivation in PC12 cell membrane depolarization and bradykinin signaling. *J Biol Chem* **272**(40):24767-24770.

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Footnotes

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Figure Legend

Fig. 1. **Grb/SOS/Ras is involved in chronic morphine-induced AC superactivation.** A. HEKMT cells were treated with 1 μM morphine for 4 hr (■ HEKMT) or were added 10 μM Grb2 and SOS1 interaction inhibitor, SOS1 SH3 peptide, for 3 hr during the 4hr-morphine treatment (● +SH3 peptide). The cAMP assays were carried out as described in the Materials and Methods. **, $p<0.01$ and ***, $p<0.001$ (n=6) when compared with the 4r-morphine treatment (■) using unpaired *t* test. B. HEKMT cells were either transiently transfected with 0.5 μg mock cDNA (■ HEKMT) or dominant negative RasN17 cDNA (◇ +RasN17). After 48 hr, cells were treated with 1 μM morphine for 4 hr and then performed the cAMP assays. Ras inhibitor, farnesylthiosalicylic acid (FTS, 50 μM) was added for 3hr during the morphine treatment (● +FTS). ***, $p<0.001$ (n=6) when compared with the 4hr-morphine treatment (■) using one-way ANOVA. Forskolin-induced cAMP in cells without drug treatment is used as 100% control.

Fig. 2. **Ras is activated and colocalized with OPRM1 during chronic morphine treatment.** A. *The effect of naloxone or Src and Ras inhibitors on Ras activation.* HEKMT cells were treated with 1 μM morphine for 0, 10 min or 4hr as indicated followed by the addition of 0 or 10 μM of naloxone (Nal) for 15 min. Src inhibitor (PP2, 2 μM) or Ras inhibitor (FTS, 50 μM) was added for 1 or 3hr during the 4hr-morphine treatment. The assays were performed as described in Materials and Methods. **, $p<0.01$ when compared with control (the ratio of the density of active Ras to the density of input Ras from untreated cells was used as 100% control) using unpaired *t* test. $, $p<0.05$ when compared with 4hr-morphine treatment using unpaired *t* test. ##, $p<0.01$ when

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compared with 4hr morphine and naloxone treatment using unpaired *t* test. B. *Co-IP of Ras with OPRM1*. HEKMT cells were treated with or without 1 μM morphine for 4 hr with or without 10 μM naloxone for 15 min as indicated. The co-IP was carried out as described in Materials and Methods. The ratio of the density of Ras to the density of OPRM1 from untreated cells was used as 100% control. **, $p<0.01$ and ***, $p<0.001$ when compared with control using unpaired *t* test. The no IP Ab control was carried out the same as above with the absence of IP Ab, (anti-HA). C. *RasN17 inhibited Ras and OPRM1 interaction*. HEKMT cells were either transiently transfected with 0.5 μg mock cDNA (Ctrl) or dominant negative RasN17 cDNA (RasN17). After 48 hr, cells were treated with morphine (1 μM, 4 hr) and naloxone (10 μM, 15 min) and then the co-IP was carried out as described in Materials and Methods. The ratio of the density of Ras to the density of OPRM1 from mock cDNA transfected cells was used as 100% control. **, $p<0.01$ when compared with control using unpaired *t* test. D. *Ras was not activated in cells expressing Y336F mutant OPRM1*. Cells were treated with or without 1 μM morphine for 4hr followed by with or without 10 μM naloxone for 15 min and assays were performed as described in Materials and Methods. Each experiment was repeated three times.

Fig. 3. **AC superactivation is EGFR or FGFR independent.** HEKMT cells were treated with 1 μM morphine for 4 hr (■) or pretreated with EGFR inhibitor, HDBA (A) or AG-1478 (B), at a concentration of 10nM (▼), 50nM (◆) or 100 nM (●); or pretreated with FGF inhibitor PD-173074 (C) at a concentration of 20nM (▼), 50nM (◆) or 100 nM (●) for 2 hr during the 4hr morphine treatment before cAMP assays were

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performed (n=6). Forskolin-induced cAMP in cells without drug treatment is used as 100% control.

Fig. 4. **Raf-1 is activated during chronic morphine treatment.** A. *Raf-1 is activated within OPRM1 signal complex in a chronic morphine- and Src kinase-dependent manner*. Cells were treated with or without morphine (Mor, 1 μM, 4hr) and naloxone (Nal, 10 μM, 15 min) as indicated. Src inhibitor (PP2, 2 μM) or Raf-1 inhibitor, GW5074 (GW, 10 μM) was added 1 or 2hr during the 4hr-morphine treatment, respectively. Co-IP was carried out as described in Materials and Methods. B. *Bar graph summary of western blots as shown in A*. The ratio of the density of pRaf1, pSrc or AC5/6 to the density of OPRM1 from untreated cells was used as 100% control. *, $p<0.05$, **, $p<0.01$ and ***, $p<0.001$ when compared with control; $^{\#}$, $p<0.05$ and $^{\#\#}$, $p<0.01$ when compared with morphine and naloxone treatment using unpaired *t*-test. The bars represent averages from 3 separate western blots. C. *Raf-1 activation requires chronic morphine treatment*. Cells were treated with or without morphine (1 μM, 4hr) and naloxone (10 μM, 15 min) as indicated. Raf-1 activity assays were performed as described in Materials and Methods. The blot was probed with anti-pMEK1 and anti-Raf-1. D. *Bar graph summary of western blots as shown in C*. The ratio of the density of pMEK1 to the density of Raf-1 from untreated cells was used as 100% control. **, $p<0.01$ when compared with control using unpaired *t*-test. The bars represent averages from 3 separate western blots. E. *Kinetics of Raf-1 kinase activation*. Cells were treated with or without morphine (1 μM, 4hr) and naloxone (10 μM, 15 min) as indicated. The assays were carried out following the procedure in Materials and Methods. *, $p<0.05$, **, $p<0.01$ and ***, $p<0.001$ when

compared with control; #, $p<0.05$ when morphine and naloxone treatment (▼ +Mor+Nal) compared with morphine alone treatment (▲ +Mor) using unpaired *t* test. Each experiment was repeated three times.

Fig. 5. **Raf-1 activation is not observed in cells expressing Tyr mutant OPRM1.** A. *Raf-1 activation*. HEKMT or HEKMT-Y336F cells were treated with or without 1 μM morphine for 4hr followed by 0 or 10 μM of naloxone for 15 min as indicated. Raf-1 activity assays were performed as described in Materials and Methods. The blots were probed with anti-pMEK1, anti-Raf-1 and anti-Gβ. B. *Bar graph summary of western blots as shown in A*. The ratio of the density of pMEK1 to the density of Raf-1 from untreated cells was used as 100% control. *, $p<0.05$ and **, $p<0.01$ when compared with control using unpaired *t* test. The bars represent averages from 3 separate western blots.

Fig. 6. **Raf-1 is required for chronic morphine-induced AC superactivation.** A. *RT-PCR analysis of Raf-1 wild type (Raf1WT) or Raf-1 Knockout (Raf1KO) MEF cells transiently expressing OPRM1 and/or Raf-1*. Cells were transfected with cDNAs as indicated. After 48 hr, cells were lysed and total RNAs were purified. RT-PCR was carried out as described in the Materials and Methods. β-actin was used as control. B. *Both OPRM1 and Raf-1 are required for AC superactivation*. MEF-Raf-1 WT cells were transfected with OPRM1 (△ Raf1WT+OPRM1) or Raf-1 (◇ Raf1WT+Raf1) cDNAs. Raf-1 KO cells were transfected with OPRM1 (■ Raf1KO+OPRM1), Raf-1 (● Raf1KO+Raf1) or both (▼ Raf1KO+OPRM1+Raf1) cDNAs. After 48 hr, cells were treated with morphine (1 μM, 4hr) and cAMP assays were performed in the presence of

various concentrations of naloxone. Forskolin-induced cAMP in cells without drug treatment is used as 100% control. C. *Bar graph representation of the results from B when 10^{-4} M naloxone was added in the assay*. **, $p<0.01$ and ***, $p<0.001$ when compared with control using unpaired *t* test (n=6).

Fig. 7. **Tyr 340 and 341 on Raf-1 are critical for chronic morphine-induced AC superactivation.** A. *RT-PCR analysis of MEF-Raf-1 KO cells transiently expressing OPRM1 and wild type or Tyr mutant Raf-1*. Cells were transfected with cDNA as indicated. After 48 hr, cells were lysed and total RNAs were isolated. RT-PCR was carried out as described in the Materials and Methods. β-actin was used as control. B. *AC activity in the presence of naloxone after 4 hr of morphine treatment*. MEF-Raf-1 KO cells were transfected with OPRM1 and wild type Raf-1 (■ OPRM1+Raf1), or Raf-1 Y340A mutant (● OPRM1+Y340A), or Raf-1 Y341A mutant (□ OPRM1+Y341A), or double Tyr mutant Y340/341AA (▼ OPRM1+Y340/341AA), or constitutive active mutant Y340/341DD (◇ OPRM1+Y340/341DD) cDNAs. After 48 hr, cells were treated with 1 μM morphine for 4hr and then cAMP assays were performed in the presence of the indicated naloxone concentrations. Forskolin-induced cAMP in cells without drug treatment is used as 100% control**.** C. *Bar graph representation of the results from B in the presence of 10^{-4} M naloxone.* **, $p<0.01$ when compared with the cells expressing OPRM1 and wild type Raf-1 using unpaired *t*-test (n=6).

Fig. 8. **Schematic summary of OPRM1 differential signal pathways upon acute and chronic morphine administration.** During acute morphine treatment, OPRM1 interacts

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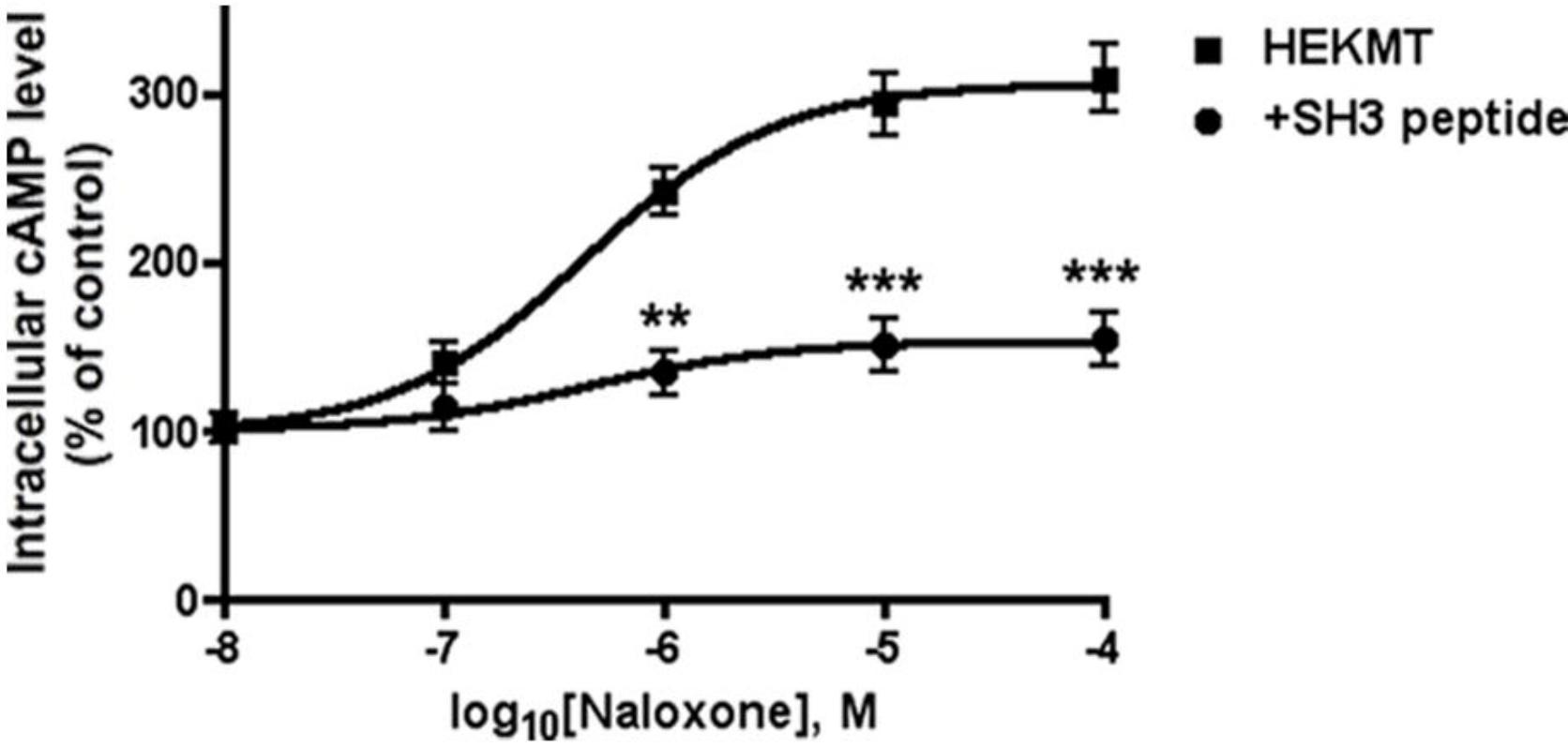
with $G\alpha_{i2}$ (Zhang et al., 2006) and causes the inhibition of adenylyl cyclase activity and decrease of intracellular cAMP level. Under the prolonged treatment, Src kinase is recruited by OPRM1 signal complex and at where it is activated. The activated Src phosphorylates the OPRM1 at Tyr^{336}. The phosphorylated Tyr^{336} serves as a docking site to recruit Grb/SOS/Ras and Raf-1, which converts the OPRM1 from a classical G_i/G_o-coupled receptor into an RTK-like entity. The activated Raf-1 eventually phosphorylates and activates the AC isozymes, most likely AC5/6, resulting in AC superactivation. By doing so, the OPRM1 can lead to an alternative signaling pathway that is initiated by the same agonist but is different depending on the duration of agonist exposure.

Figure 1

A.


Intracellular cAMP level
(% of control)
300
200
100
0
-8
-7
-6
-5
-4
log10[Naloxone], M
**
***
***
HEKMT
+SH3 peptide

B.

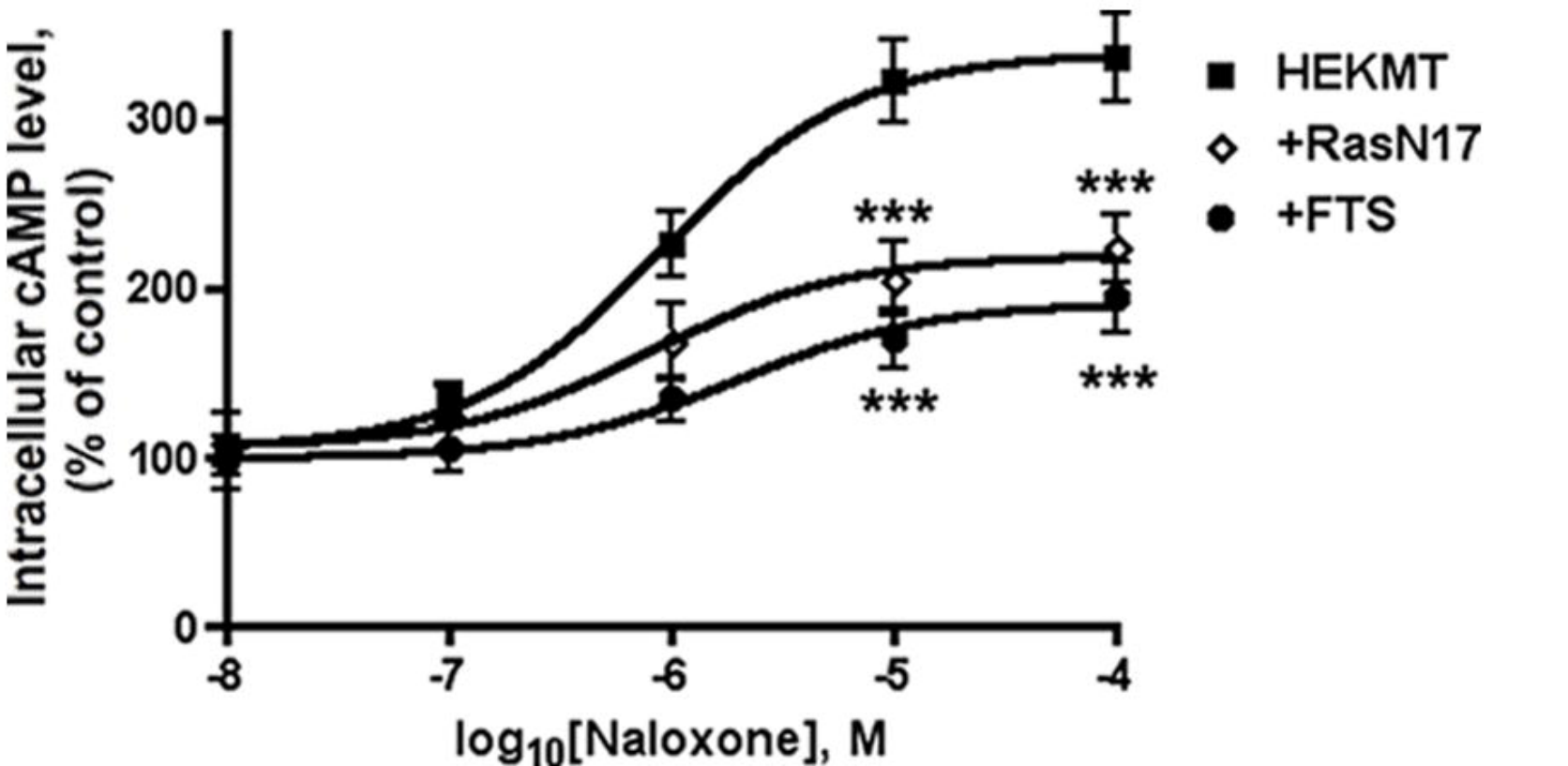

Intracellular cAMP level,
(% of control)
300
200
100
0
-8
-7
-6
-5
-4
log10[Naloxone], M
***
***
***
HEKMT
+RasN17
+FTS

Figure 2

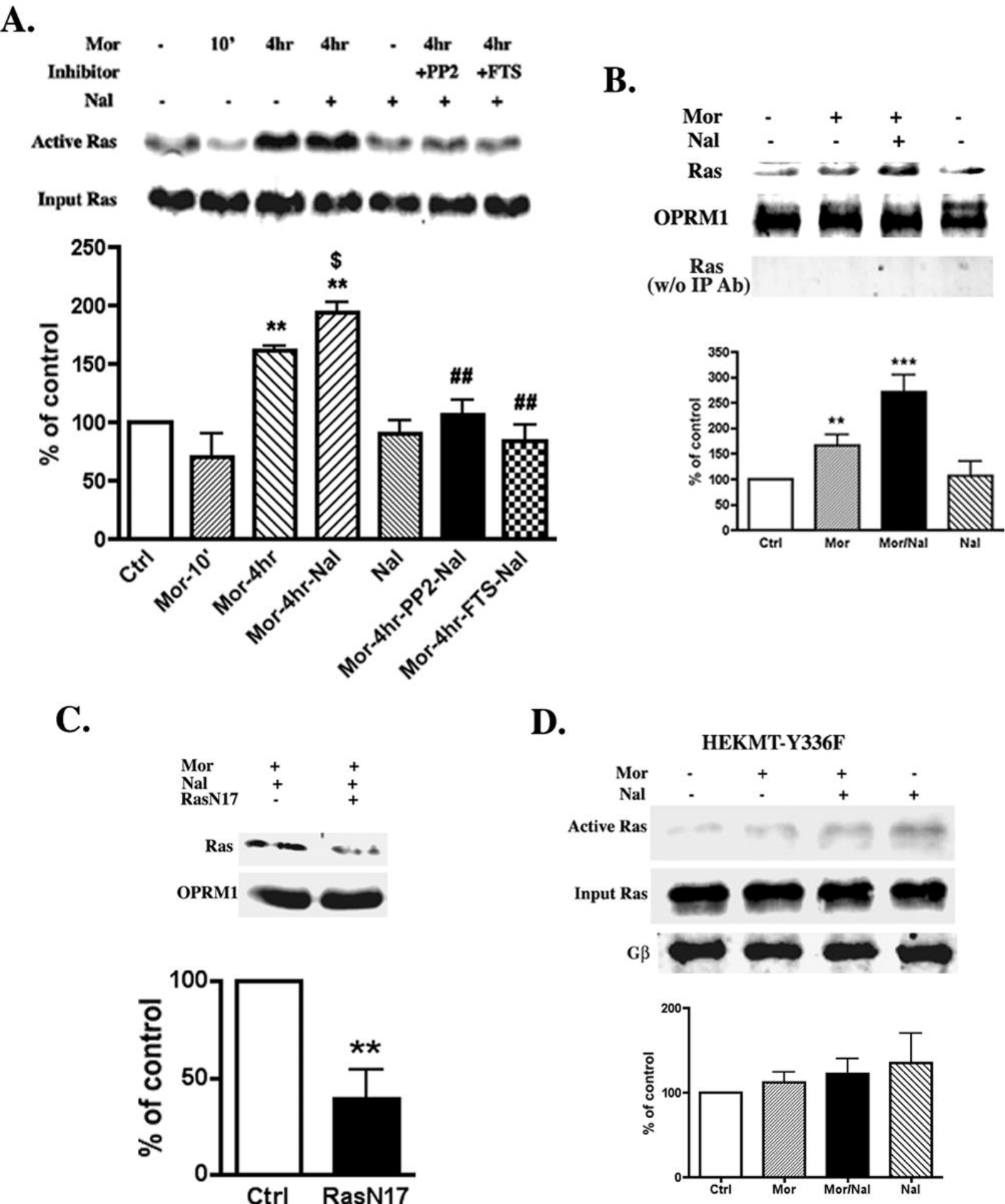
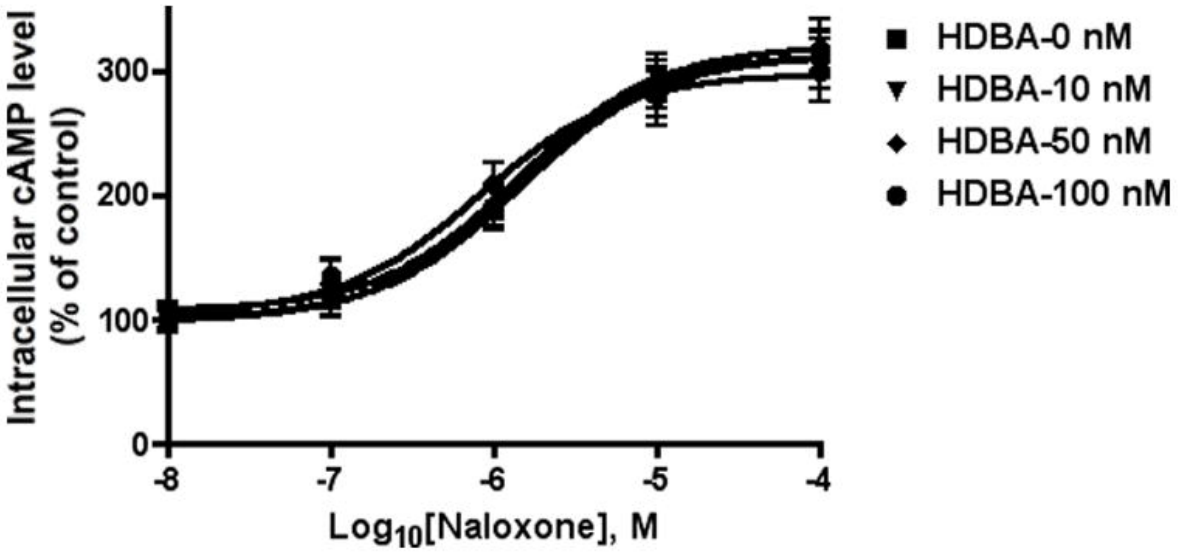
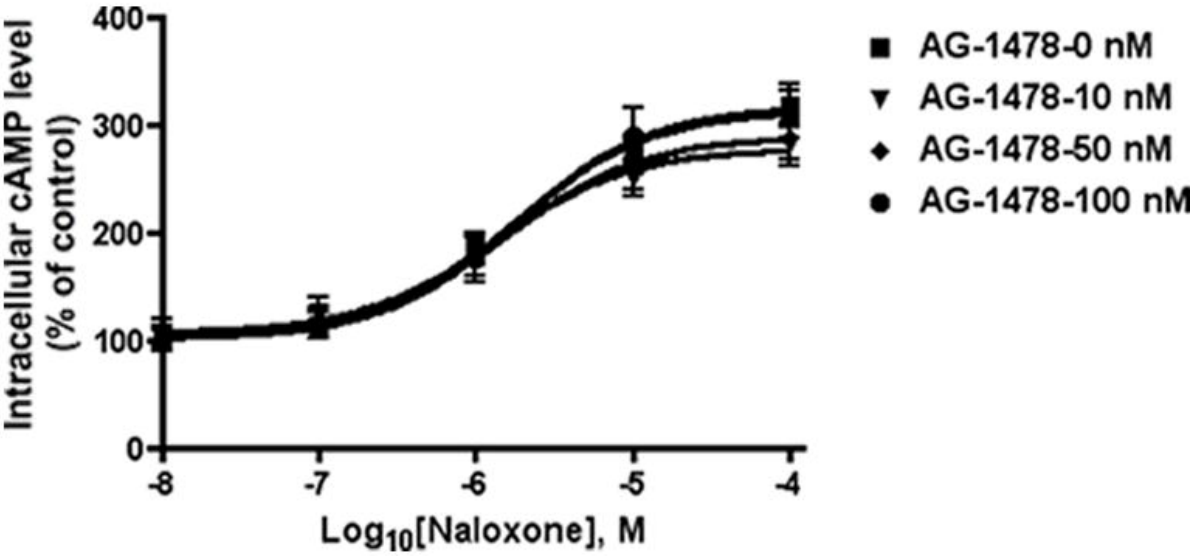

A.
Mor - 10' 4hr 4hr - 4hr 4hr
Inhibitor +PP2 +FTS
Nal - - - + + + +
Active Ras
Input Ras
% of control
250
200
150
100
50
0
**
$
**
##
##
Ctrl
Mor-10'
Mor-4hr
Mor-4hr-Nal
Nal
Mor-4hr-PP2-Nal
Mor-4hr-FTS-Nal
B.
Mor - + + -
Nal - - + -
Ras
OPRM1
Ras
(w/o IP Ab)
% of control
350
300
250
200
150
100
50
0
**
***
Ctrl
Mor
Mor/Nal
Nal
C.
Mor + +
Nal + +
RasN17 - +
Ras
OPRM1
% of control
100
50
0
**
Ctrl
RasN17
D.
HEKMT-Y336F
Mor - + + -
Nal - - + +
Active Ras
Input Ras
Gβ
% of control
200
100
0
Ctrl
Mor
Mor/Nal
Nal

Figure 3

A.


Intracellular cAMP level
(% of control)
300
200
100
0
-8
-7
-6
-5
-4
Log10[Naloxone], M
HDBA-0 nM
HDBA-10 nM
HDBA-50 nM
HDBA-100 nM

B.


Intracellular cAMP level
(% of control)
400
300
200
100
0
-8
-7
-6
-5
-4
Log10[Naloxone], M
AG-1478-0 nM
AG-1478-10 nM
AG-1478-50 nM
AG-1478-100 nM

C.

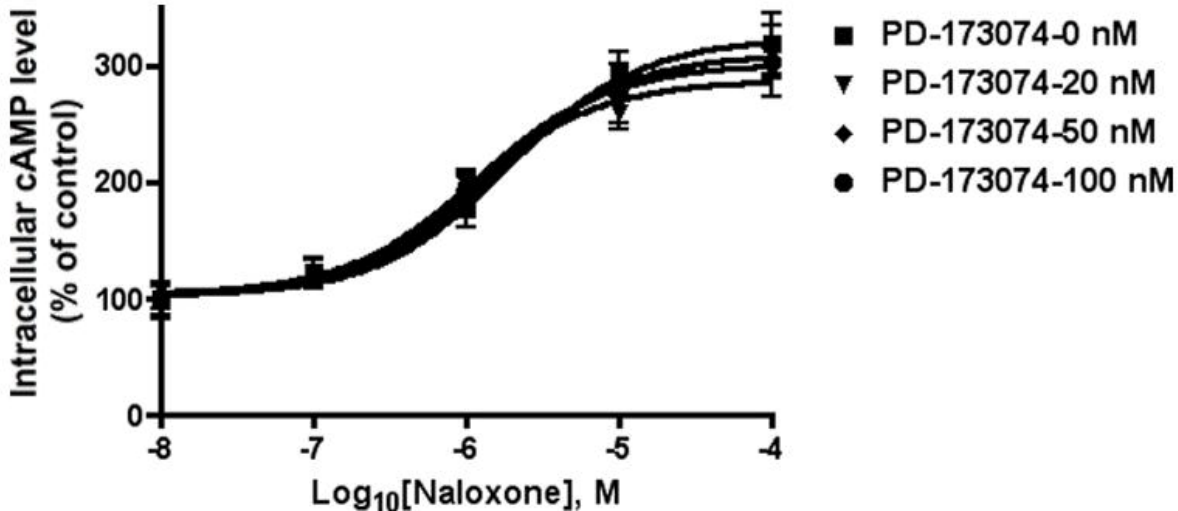

Intracellular cAMP level
(% of control)
300
200
100
0
-8
-7
-6
-5
-4
Log10[Naloxone], M
PD-173074-0 nM
PD-173074-20 nM
PD-173074-50 nM
PD-173074-100 nM

Figure 4

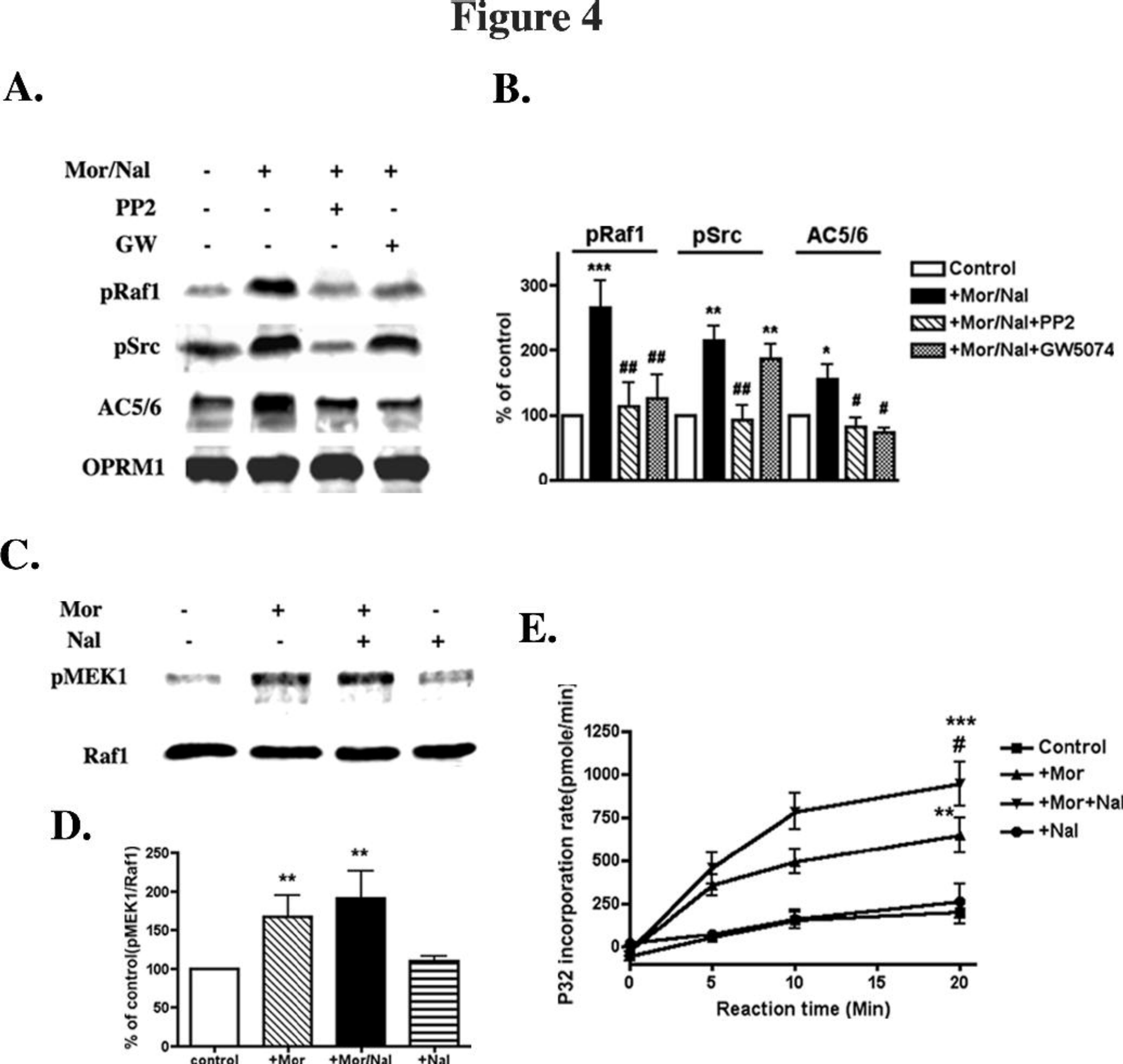

A.
Mor/Nal - + + +
PP2 - - + -
GW - - - +
pRaf1
pSrc
AC5/6
OPRM1
B.
pRaf1
pSrc
AC5/6
% of control
300
200
100
0
***
**
**
*
##
##
##
#
#
Control
+Mor/Nal
+Mor/Nal+PP2
+Mor/Nal+GW5074
C.
Mor - + + -
Nal - - + +
pMEK1
Raf1
D.
% of control(pMEK1/Raf1)
250
200
150
100
50
0
**
**
control
+Mor
+Mor/Nal
+Nal
E.
P32 incorporation rate(pmole/min)
1250
1000
750
500
250
0
***
#
**
0
5
10
15
20
Reaction time (Min)
Control
+Mor
+Mor+Nal
+Nal

Figure 5

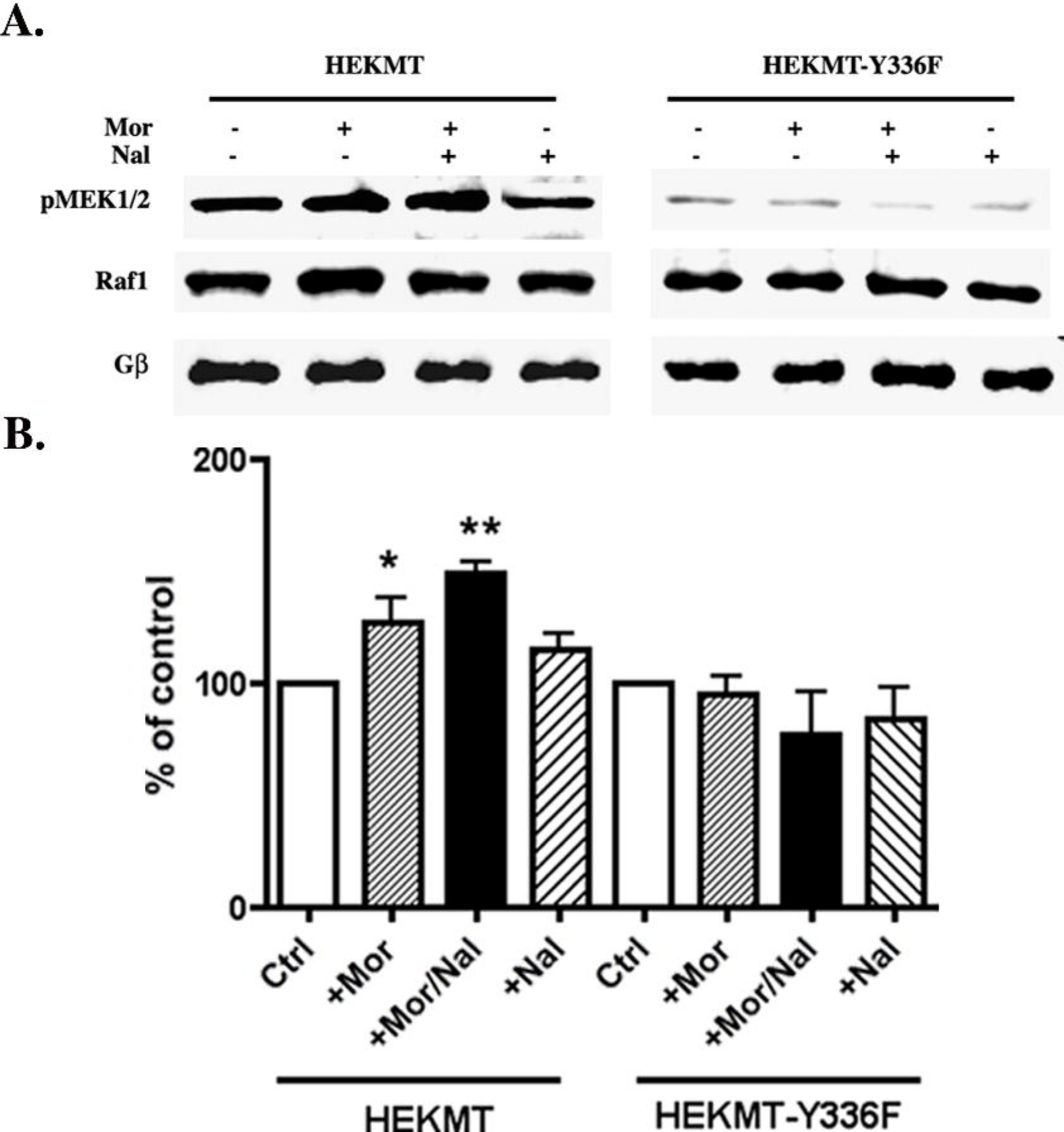

A.
HEKMT
HEKMT-Y336F
Mor
Nal
pMEK1/2
Raf1
Gβ
B.
% of control
200
100
0
*
**
Ctrl
+Mor
+Mor/Nal
+Nal
HEKMT
HEKMT-Y336F

Figure 6

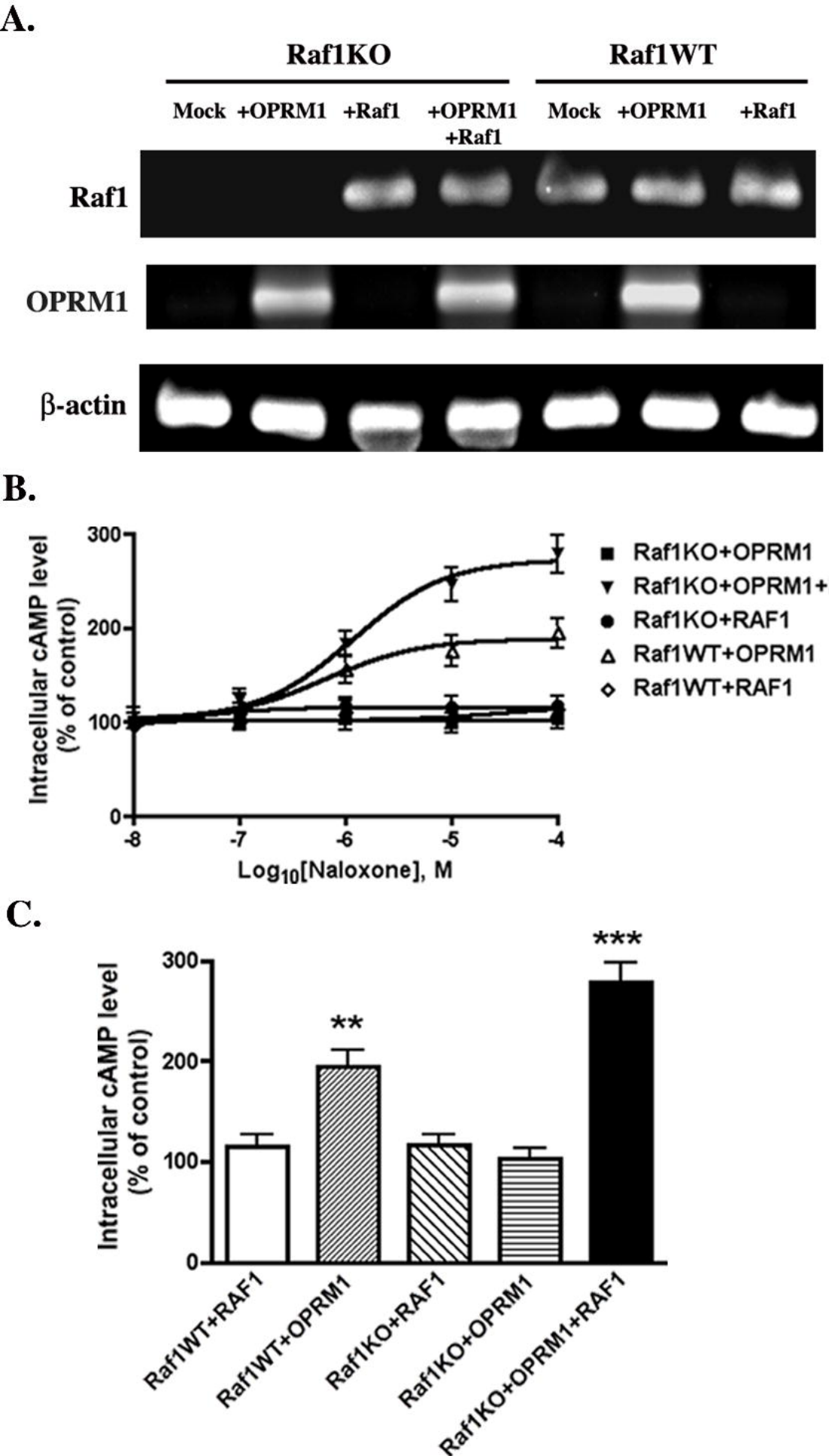
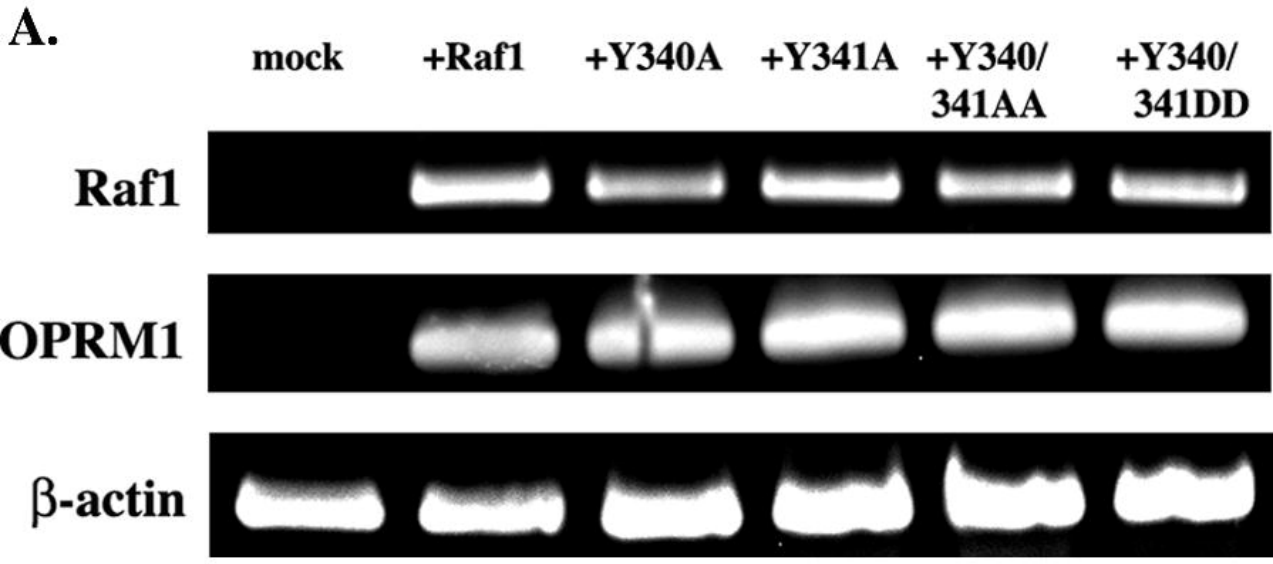
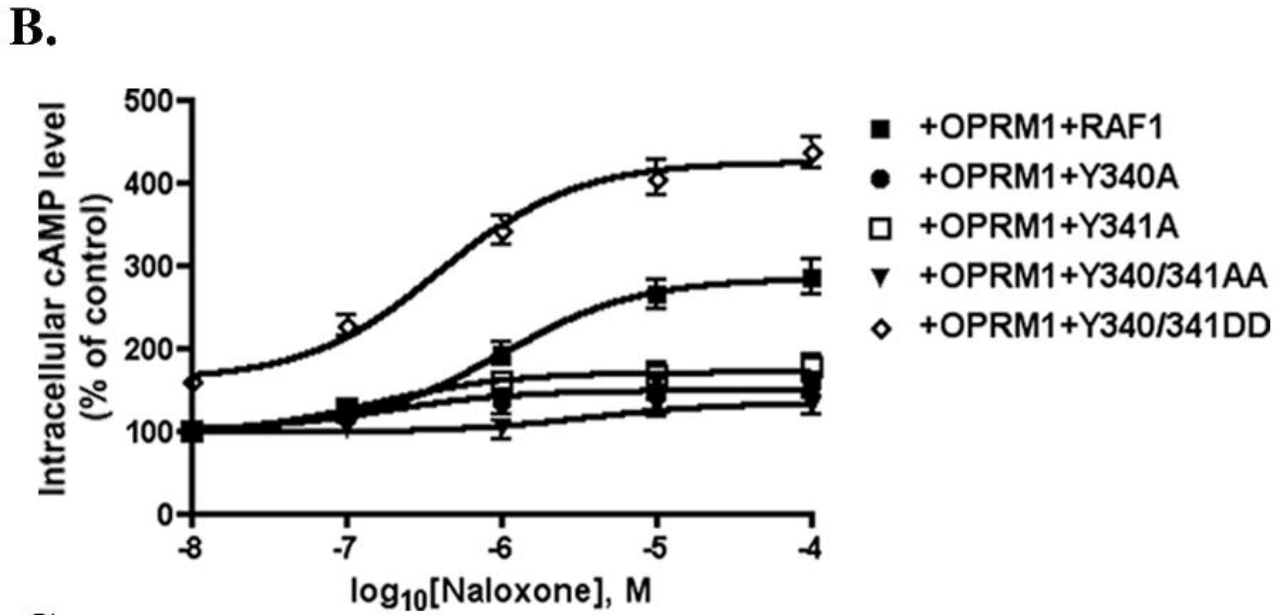

A.
Raf1KO
Raf1WT
Mock
+OPRM1
+Raf1
+OPRM1 +Raf1
Mock
+OPRM1
+Raf1
Raf1
OPRM1
β-actin
B.
Intracellular cAMP level (% of control)
300
200
100
0
-8
-7
-6
-5
-4
Log10[Naloxone], M
Raf1KO+OPRM1
Raf1KO+OPRM1+RAF1
Raf1KO+RAF1
Raf1WT+OPRM1
Raf1WT+RAF1
C.
Intracellular cAMP level (% of control)
300
200
100
0
**
***
Raf1WT+RAF1
Raf1WT+OPRM1
Raf1KO+RAF1
Raf1KO+OPRM1
Raf1KO+OPRM1+RAF1

Figure 7


A.
mock
+Raf1
+Y340A
+Y341A
+Y340/ 341AA
+Y340/ 341DD
Raf1
OPRM1
β-actin


B.
Intracellular cAMP level (% of control)
500
400
300
200
100
0
-8
-7
-6
-5
-4
log10[Naloxone], M
+OPRM1+RAF1
+OPRM1+Y340A
+OPRM1+Y341A
+OPRM1+Y340/341AA
+OPRM1+Y340/341DD

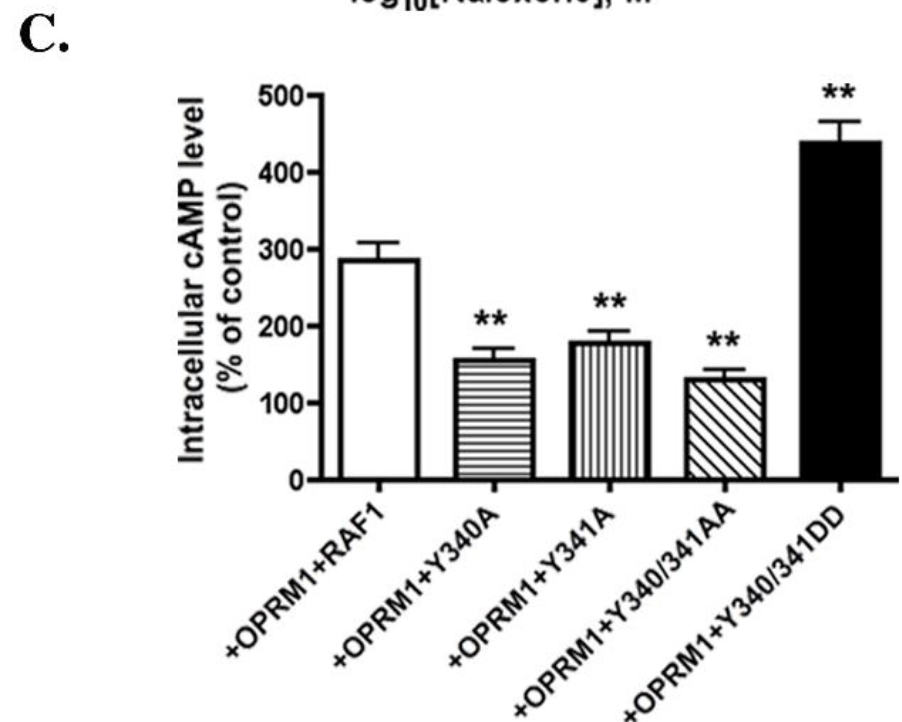
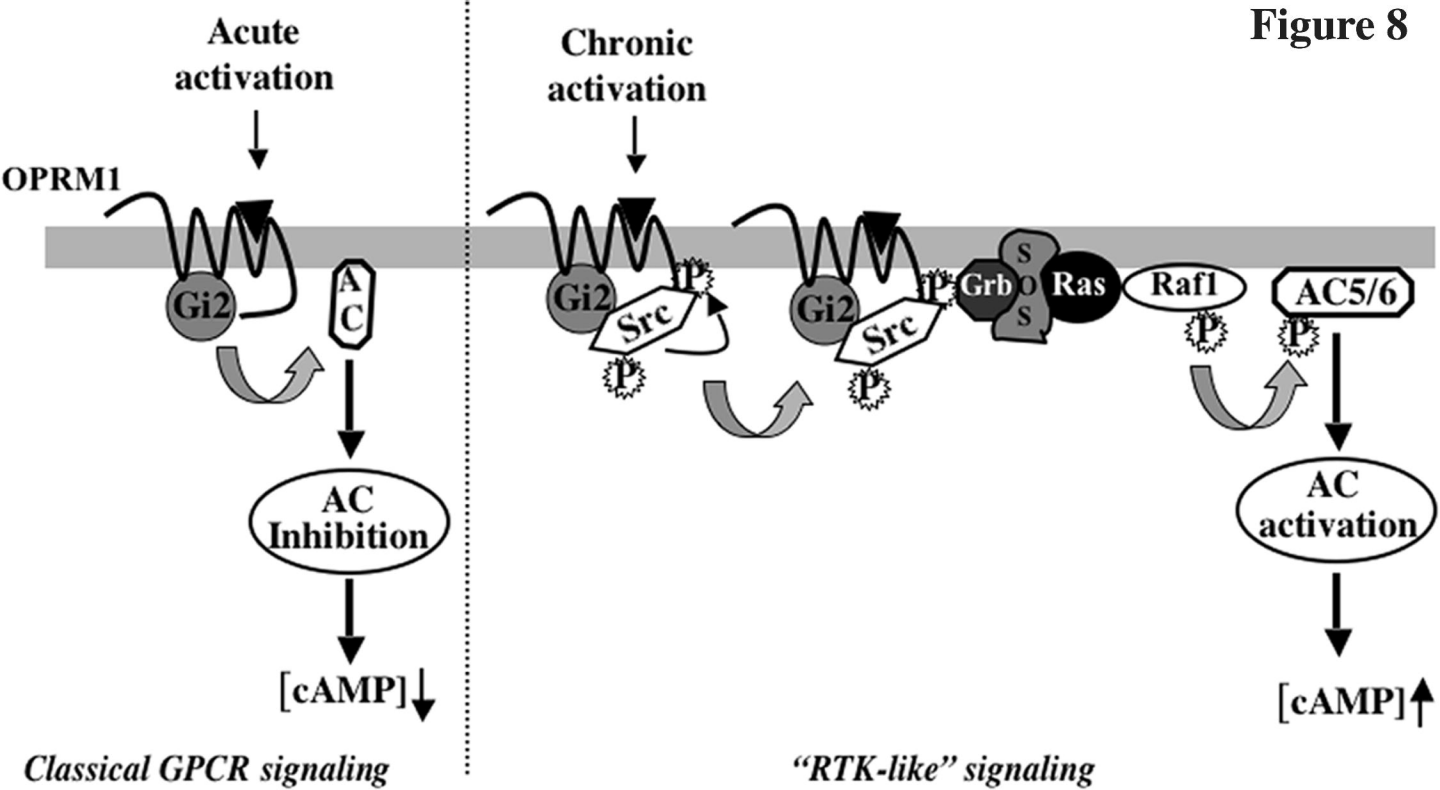

C.
Intracellular cAMP level (% of control)
500
400
300
200
100
0
**
**
**
**
+OPRM1+RAF1
+OPRM1+Y340A
+OPRM1+Y341A
+OPRM1+Y340/341AA
+OPRM1+Y340/341DD

Figure 8


Acute activation
OPRM1
Gi2
AC
AC Inhibition
[cAMP]↓
Classical GPCR signaling
Chronic activation
Gi2
Src
P
Gi2
Src
P
Grb
SOS
Ras
Raf1
AC5/6
AC activation
[cAMP]↑
"RTK-like" signaling