Gi/o-coupled receptors compete for signaling to adenylyl cyclase in SH-SY5Y cells and reduce opioid-mediated cAMP overshoot

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Abbreviations
SNC80 ((+)-4-[(αR)-α-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-N,N-diethylbenzamide), DPDPE ([D-Pen2,5]-enkephalin), DAMGO ([D-Ala2, N-Me-Phe4, Gly5-ol]-enkephalin), N/OFQ (nociceptin/orphanin FQ), 8-OH-DPAT (8-hydroxy-2-(di-n-propylamino)tetralin hydrochloride), CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH2), ICI 174,864 (N, N-diallyl-Tyr-Aib-Aib-Phe-Leu-OH), J113397 (1-[(3R,4R)-1-cyclooctylmethyl-3-hydroxymethyl-4-piperidyl]-3-ethyl-1,3-dihydro-2H-benzimidazol-2-one), [35S]GTPγS (guanosine-5’-O-(3-[35S]thio)triphosphate), MOR (mu-opioid receptor), DOR (delta-opioid receptor), NOPr (nociceptin/orphanin FQ peptide receptor), α2AR (alpha2-adrenergic receptor), CB1 (cannabinoid receptor 1), 5-HT1A (serotonin 1A receptor), AC (adenylyl cyclase), PTX (pertussis toxin), GPCR (G protein-coupled receptor), IBMX (3-isobutyl-1-methylxanthine), ANOVA (analysis of variance), LC (locus coeruleus)
Abstract

Organization of G protein-coupled receptors and cognate signaling partners at the plasma membrane has been proposed to occur via multiple mechanisms including membrane microdomains, receptor oligomerization and protein scaffolding. Here, we investigate the organization of six types of Gi/o-coupled receptors endogenously expressed in SH-SY5Y cells. The most abundant receptor in these cells was the mu-opioid receptor (MOR), activation of which occluded acute inhibition of adenylyl cyclase (AC) by agonists to delta-opioid (DOR), nociceptin/orphanin FQ peptide (NOPr), alpha2-adrenergic (α2AR), cannabinoid (CB1) and serotonin 1A (5-HT1A) receptors. We further demonstrate that all receptor pairs share a common pool of AC. The MOR agonist DAMGO ([D-Ala², N-Me-Phe⁴, Gly⁵-ol]-enkephalin) also occluded the ability of DOR agonist to stimulate G proteins. However, at lower agonist concentrations and at shorter incubation times when G proteins were not limiting, the relationship between MOR and DOR agonists was additive. The additive relationship was confirmed by isobolographic analysis. Chronic co-administration of MOR and DOR agonists caused cAMP overshoot that was not additive, suggesting that sensitization of AC mediated by these two receptors occurs by a common pathway. Furthermore, heterologous inhibition of AC by agonists to DOR, NOPr and α2AR reduced the expression of cAMP overshoot in DAMGO-dependent cells. However, this cross-talk did not lead to heterologous tolerance. These results indicate that multiple receptors could be tethered into complexes with cognate signaling proteins and that access to shared AC by multiple receptor types may provide a means to prevent opioid withdrawal.
Introduction

Opioid receptors are members of the G protein-coupled receptor (GPCR) family and signal via activation of adenylyl cyclase (AC)-inhibitory (G_i/o) GTP-binding proteins. It has been suggested that the probability of opioid receptor/G protein interaction is enhanced by compartmentalization in the membrane (Alt et al., 2001), allowing rapidity of GPCR signal propagation (Hur and Kim, 2002). Various modes of organization in the plasma membrane have been proposed to describe these compartments, including dimerization of receptors (George et al., 2000; Gomes et al., 2004; Jordan et al., 2003; Rios et al., 2004; Rios et al., 2006; Wang et al., 2005), membrane microdomains (Allen et al., 2007) or protein scaffolds (Hall and Lefkowitz, 2002). However, mathematical modeling of experimental findings supporting compartmentalization has claimed that these data can be explained by a collision coupling model (Stickle and Barber, 1992; Tolkovsky and Levitzki, 1978) without the need to invoke compartments (Brinkerhoff et al., 2008).

Compartments also prevent interactions between two proteins by constraining cross-talk and/or sharing of effector molecules, thus leading to signaling specificity. In NG108-15 cells, muscarinic receptors and delta-opioid receptors (DOR) did not share G proteins with alpha2-adrenergic receptors (α2AR), as measured by agonist binding (Graeser and Neubig, 1993). In this scenario, co-administration of agonists for separately compartmentalized receptors would result in an additive response as each receptor type activated its own pool of effectors. Thus, in N18TG2 neuroblastoma cells, agonists to endogenous DOR and cannabinoid (CB1) receptors activated G proteins in an additive manner (Shapira et al., 2000). On the other hand, in SH-SY5Y cells, co-administration of a mu-opioid receptor (MOR) agonist and a DOR agonist produced the same level of G protein activation as the MOR agonist alone, indicating that MOR
and DOR activate the same G proteins (Alt et al., 2002). Similarly, DOR and CB₁ receptors
cotransfected in COS-7 cells shared G proteins (Shapira et al., 2000) and MOR and α₂AR
endogenously expressed in SH-SY5Y cells were observed to access the same AC enzymes
(Lameh et al., 1992).

The conflicting data on DOR and CB₁ receptor competition in N18TG2 and COS-7 cells
can potentially be explained by differences in the level of expression of receptors. At high
density, receptors compete for a limiting pool of G proteins, whereas, at low receptor
concentrations, G proteins are in excess and agonists for two receptor types activate G proteins in
an additive manner regardless of compartmentalization (Brinkerhoff et al., 2008). However, at
low receptor levels, artificially reducing G protein number (using pertussis toxin; PTX) did not
increase competition (Graeser and Neubig, 1993; Shapira et al., 2000), suggesting that receptor
number is more predictive of competition than G protein number (Brinkerhoff et al., 2008).

Competition between only two GPCR types would be observed if the receptors were able
to freely diffuse along the cell membrane to access all available G proteins, or if they were
corralled together, i.e. in a membrane microdomain, by scaffolding proteins or by dimerization.
By considering competition between multiple receptor types, the chance of all receptors sharing
the same compartment decreases, and it should therefore be easier to differentiate between
receptors that are somehow constrained together and those that are not. The goal of the
experiments presented here was to determine the degree of competition or effector sharing
between multiple inhibitory GPCRs endogenously expressed in SH-SY5Y cells and the
consequences of this competition for signaling to AC. We show that agonist-occupied MOR can
access all AC available to these other G₁₆₀-coupled GPCRs suggesting a lack of
compartmentalization and/or the presence of complexes containing multiple receptors.
Moreover, depending on the level of receptor expression, agonists at non-MOR GPCRs are able to attenuate the cAMP overshoot observed following withdrawal from exposure to a chronic MOR agonist, thus suggesting a mechanism for the prevention of opioid withdrawal.
Materials and Methods

**Materials**- $[^3]H$CP 55,940 (5-(1,1-dimethylheptyl)-2-[(1R,2R,5R)-5-hydroxy-2-(3-hydroxypropyl)cyclohexyl]-phenol), $[^3]H$DAMGO ([D-Ala$^2$, N-Me-Phe$^4$, Gly$^5$-ol]-enkephalin), $[^3]H$DPDPE ([D-Pen$^{2,5}$]-enkephalin), $[^3]H$diprenorphine, $[^3]H$nociceptin/OFQ, $[^3]H$UK14,304, $[^3]H$yohimbine, and $[^35]S$GTP$\gamma$S (guanosine-5'-O-(3-$[^35]S$thio)triphosphate) were obtained from Perkin-Elmer Life Sciences (Boston, MA). SNC80 ((+)-4-[(αR)-α-(2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-N,N-diethylbenzamide) was obtained from the Narcotic Drug and Opioid Peptide Basic Research Center at the University of Michigan (Ann Arbor, MI). DAMGO, DPDPE, naloxone, CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH$_2$), nociceptin/orphanin FQ (nociceptin/OFQ), UK 14304, clonidine, forskolin and IBMX (3-isobutyl-1-methylxanthine) were from Sigma-Aldrich (St. Louis, MO). CP 55,940 and WIN 55212-2 were from Cayman Chemical (Ann Arbor, MI). ICI 174,864 (N, N-diallyl-Tyr-Aib-Aib-Phe-Leu-OH) and J113397 (1-[(3R,4R)-1-cyclooctylmethyl-3-hydroxymethyl-4-piperidyl]-3-ethyl-1,3-dihydro-2H-benzimidazol-2-one) were from Tocris Bioscience (Ellisville, MO). Retinoic acid was obtained from Calbiochem (La Jolla, CA). Pertussis toxin (PTX) was from List Biological Laboratories (Campbell, CA). Tissue culture media, fetal bovine serum and trypsin were from Invitrogen (Carlsbad, CA). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO) and were of analytical grade.

**Cell culture**- Human neuroblastoma SH-SY5Y cells, a subclone of SK-N-SH cells, were obtained from ATCC (Manassas, VA), grown in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) at 37°C in 5% CO$_2$, and used within passages 34 – 44 from subcloning to maintain consistent neuroblast properties between experiments. All
experiments were performed in SH-SY5Y cells differentiated with 10 μM retinoic acid for 4 – 7 days prior to assay.

Radioligand binding assays- Membranes were prepared from retinoic acid-differentiated SH-SY5Y cells. Cells were rinsed with phosphate buffered saline (150 mM NaCl, 0.61 mM Na₂HPO₄, 0.38 mM KH₂PO₄, pH 7.4), resuspended in warm harvesting buffer (150 mM NaCl, 20 mM HEPES, 0.68 mM EDTA, pH 7.4) and centrifuged at 500 x g in an IEC Centra CL2 centrifuge (Thermo Scientific; Waltham, MA) with a swinging bucket rotor (IEC 215). Cells were resuspended in ice-cold 50 mM Tris-HCl buffer, pH 7.4, homogenized with a Tissue Tearor (Biospec, Inc; Bartlesville, OK) for 20 s at setting 4, and centrifuged at 27,000 x g in a Beckman (Brea, CA) J2-21 Centrifuge (JA-20 rotor). The crude membrane pellet was then resuspended in Tris buffer, homogenized for 10 s at setting 2 and centrifuged as above. Final membrane pellets were resuspended in 50 mM Tris-HCl buffer, pH 7.4, aliquoted and stored at -80°C. Protein concentration was measured using the Bradford assay.

Receptor density was determined by incubating membranes (50 μg) for 60 min at 25°C with shaking in 50 mM Tris-HCl buffer, pH 7.4 buffer containing saturating concentrations of radiolabeled ligand as follows: 12 nM [³H]DAMGO or 4 nM [³H]diprenorphine in the presence of 1 μM ICI174864 for MOR, 16 nM [³H]DPDPE or 1 nM [³H]naltrindole (NTI) for DOR, 1 nM [³H]nociceptin/OFQ for nociceptin/orphanin FQ peptide receptor (NOPr), 15 nM [³H]UK 14304 or 10 nM [³H]yohimbine for α₂AR or 6 nM [³H]CP 55,940 for CB₁. Non-specific binding was determined with unlabeled naloxone (MOR and DOR), J113397 (NOPr), UK 14304 (α₂AR) or WIN 55212-2 (CB₁). All plasticware was pre-coated with Sigma Cote (Sigma-Aldrich, St. Louis, MO) and 0.1% BSA was included for [³H]CP 55,940 binding. Assays were stopped by rapid filtration through GF/C filters presoaked in 0.1% polyethyleneimine using a Brandel
harvester (MLR-24, Gaithersburg, MD) and rinsed three times with ice-cold 50 mM Tris-HCl wash buffer, pH 7.4. Dried filters were saturated with EcoLume liquid scintillation cocktail (MP Biomedicals, Solon, OH) and radioactivity was counted in a Wallec 1450 MicroBeta (Perkin-Elmer, Waltham, MA).

**Stimulation of [35S]GTPγS binding** - Membranes were prepared from retinoic acid-differentiated SH-SY5Y cells, as described for radioligand binding assays. In some experiments, cells were treated overnight with agonist (SNC80 or DAMGO) or for 24 h with PTX (100 ng/ml) prior to membrane preparation.

Membranes (50 μg protein) were incubated with 0.1 nM [35S]GTPγS for 60 min (unless otherwise indicated) at 25°C, with or without various concentrations of SNC80 and/or DAMGO in [35S]GTPγS binding buffer (50 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 100 mM NaCl, 1 mM EDTA, 2 mM dithiothreitol and 30 μM GDP). Membranes with bound [35S]GTPγS were collected on GF/C filters (Whatman, Middlesex, UK) using a Brandel harvester (MLR-24, Gaithersburg, MD) and rinsed three times with cold wash buffer (50 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 100 mM NaCl). Bound radioactivity was determined by liquid scintillation counting as described in radioligand binding methods.

**Cyclic AMP accumulation assays** - For inhibition of AC, SH-SY5Y cells were plated in 24-well plates (5 x 10⁵ cells / well) and differentiated with 10 μM retinoic acid 4 days prior to assay. Cells were incubated with 1 μM of the indicated agonist(s) in the presence of 5 μM forskolin and 1 mM IBMX in DMEM/10% FBS for 10 min at 37°C. The assay was stopped by replacing the media with 1 ml ice-cold 3% perchloric acid. After at least 30 min at 4°C, a 400 μl aliquot of sample was neutralized with 2.5 M KHCO₃ and centrifuged at 13,000 x g. Cyclic AMP was measured from the supernatant using a [3H]cAMP assay system (GE Healthcare,
Buckinghamshire, UK) following the manufacturer’s instructions. Inhibition of cAMP formation was calculated as percent inhibition of forskolin-stimulated cAMP accumulation in the absence of opioid agonist.

For AC sensitization experiments, differentiated SH-SY5Y cells were incubated overnight in the presence or absence of DAMGO and/or DPDPE in DMEM/10% FBS at 37°C. Drug-containing media was replaced with media containing 5 μM forskolin, 1 mM IBMX and 1 μM MOR antagonist CTAP for DAMGO-treated cells, 30 μM forskolin, 1 mM IBMX and 1 μM DOR antagonist ICI 174,864 for DPDPE-treated cells, or 5 μM forskolin, 1 mM IBMX and 100 μM opioid antagonist naloxone for chronic DAMGO and DPDPE-treated cells, to precipitate cAMP overshoot. In some experiments, DAMGO-containing media was replaced with media containing 5 μM forskolin, 1 mM IBMX, 1 μM MOR antagonist CTAP and 1 μM G_{i/o}-coupled receptor agonist. After 10 min at 37°C, the assay was stopped with ice cold 3% perchloric acid and cAMP accumulation was quantified as described above. Overshoot was calculated as either percent cAMP overshoot or as a percent of forskolin-stimulated cAMP accumulation in the absence of opioid agonist.

Isobologram analysis - An isobologram for agonists (SNC80 and DAMGO) with different maxima and therefore a variable potency ratio was constructed based on the following equation (Tallarida, 2006):

\[
B = B_{50} \left( \frac{B_{50}}{E_b \left( 1 + \frac{A}{a} \right)} \right) - 1
\]

The parameters for the equation were based on values from the individual concentration-effect curves for DAMGO and SNC80. As the more efficacious drug in these experiments, DAMGO was assigned as Drug B and SNC80 as Drug A (Tallarida, 2006). B_{50} and A_{50} represent the EC_{50}.
of DAMGO and SNC80, respectively. \( E_b \) and \( E_c \) represent the maximal effect (fmol \(^{35}\text{S}\)GTP\(\gamma\)S bound/mg) produced by DAMGO and SNC80, respectively. The equation was solved for either \( a \) or \( b \) (concentration of SNC80 or DAMGO in nM, respectively) at the concentration of DAMGO that produced 50% of its maximal effect (\( B_i \)). Therefore, in this case \( B_i = B_{50} \). The derived \((a, b)\) coordinates were fit to exponential one phase decay in GraphPad Prism 5 (San Diego, CA) to produce the line of additivity. Concentration-effect curves for DAMGO were then obtained in the presence of set concentrations of SNC80 (1, 5, 10, 20 or 30 nM). The concentration of DAMGO, when in combination with SNC80, needed to produce 50% of its maximum effect was determined and plotted on the isobologram as mean ± S.E.M. for 3 separate experiments. Since the error for all points overlaps the line of additivity they were assumed to not be statistically different than the line and no further statistical analysis was conducted.

**Western blot** – SH-SY5Y membranes (40 \( \mu \)g) were mixed with sample buffer (63 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.008% bromophenol blue, 50 mM DTT), separated by SDS-PAGE on a 10% polyacrylamide gel and transferred to nitrocellulose membranes (Pierce Biotechnology, Rockford, IL) for western blotting. G proteins were detected with rabbit anti-\( \text{Go}_o \) (Santa Cruz Biotechnology, Santa Cruz, CA) and mouse anti-\( \text{Go}_{i2} \) (Millipore, Billerica, MA). As a loading control, tubulin was detected with mouse anti-\( \alpha\)-tubulin (Sigma-Aldrich, St. Louis, MO). Above antibodies were diluted 1:1000 in 5% non-fat dry milk (\( \text{Go}_o \) and tubulin) or 1% bovine serum albumin (\( \text{Go}_{i2} \)) prepared with TBS-0.05% Tween 20. Secondary antibodies used were goat anti-mouse HRP or goat anti-rabbit HRP (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:10,000 in 5% non-fat dry milk in TBS-0.05% Tween 20. SuperSignal West Pico chemiluminescent substrate (Pierce Biotechnology, Rockford, IL) was used to detect immunoreactivity. Immunoreactive band densities were quantified using ImageJ software.
(NIH), normalized to tubulin loading control and presented as fold over vehicle (mean ± S.E.M. for 3 separate experiments).

Statistical Analysis- All data were analyzed using GraphPad Prism 5 software (San Diego, CA). Data points represent at least three separate experiments in duplicate and are presented as mean ± standard error of the mean (S.E.M.), unless otherwise noted. The addition of single agonist concentrations was analyzed by one-way ANOVA with Bonferroni’s post-hoc test. Percent competition values were compared to 100 by one-sample t-test. Effects on agonist responses at various concentrations were analyzed by 2-way ANOVA with Bonferroni’s post-hoc test. EC50 values were calculated from individual concentration-effect curves using non-linear three parameter log [agonist]-response curve fit analysis in GraphPad Prism and compared for statistical significance by unpaired, two-tailed Student’s t-test. For all tests significance was set at p < 0.05.
Results

**G\(_i\)-coupled receptors expressed in SH-SY5Y cells**

Human neuroblastoma SH-SY5Y cells were differentiated with retinoic acid (10 μM for 4 – 7 days) to produce a neuronal-like phenotype. Differentiation increased MOR density from 232 ± 33 fmol/mg protein to 305 ± 42 fmol/mg protein, as identified by the specific MOR agonist \(^{[3]H}\)DAMGO, and increased the level of AC inhibition by DAMGO, as reported previously (Zadina et al., 1994). In differentiated SH-SY5Y cells, agonists for the following receptors were shown to inhibit AC: MOR, DOR, NOPr, \(\alpha_2AR\), CB\(_1\) and 5-HT\(_{1A}\) (Figure 1a). However, the ability of a maximal concentration (1 μM) of these agonists to inhibit AC was not equal. The most effective agonist was the MOR agonist DAMGO, followed closely by the DOR agonist SNC80 and the NOPr agonist nociceptin/OFQ. The following agonists had similar activity, but caused significantly less inhibition than DAMGO: UK 14304 (\(\alpha_2AR\)), clonidine (\(\alpha_2AR\)), CP 55,9140 (CB\(_1\)), 8-OH-DPAT (5-HT\(_{1A}\)). All of the agonists used are commonly regarded as full agonists, except clonidine and 8-OH-DPAT, which display partial agonist activity in certain assays. However, in this assay, clonidine caused the same degree of cAMP inhibition as the full \(\alpha_2AR\) agonist UK 14304.

The endogenous expression level of the above identified receptors was determined in membranes from differentiated SH-SY5Y cells using maximal concentrations of selective agonist radioligands for each receptor. Receptor densities are listed in Table 1 and follow a similar rank order as the ability of selective agonists for each receptor to inhibit AC. Although the agonist radioligands will preferentially recognize receptors in a high-affinity conformation, the binding experiments were performed in the absence of guanine nucleotides and Na\(^+\) ions to shift the equilibrium towards high-affinity receptor conformations. To confirm this we compared...
antagonist binding at MOR, DOR and $\alpha_2$AR (Table 1). At MOR and DOR similar values were obtained suggesting all receptors were in the high affinity state in the absence of Na$^+$ ions and GTP$\gamma$S. For the $\alpha_2$AR receptors there was a difference in agonist and antagonist binding indicating that approximately 20% of the receptors were in a low affinity state. Agonist binding at CB$_1$, and NOPr was reduced by 89% and 90% respectively (n = 2) in the presence of Na$^+$ ions and GTP$\gamma$S confirming these ligands were recognizing mostly high affinity sites.

**$G_{i/o}$-coupled receptors compete for AC**

To evaluate the level of interaction between MOR and other $G_{i/o}$-coupled receptors in SH-SY5Y cells, maximal concentrations of $G_{i/o}$-coupled receptor agonists were combined with a maximal concentration of DAMGO. When added to DAMGO, none of the agonists were able to inhibit AC to a greater degree than DAMGO alone (Figure 1b), indicating that MORs are able to access and inhibit the same AC enzymes as other $G_{i/o}$-coupled receptors. We next addressed whether agonists to $\alpha_2$AR, CB$_1$ or 5-HT$_{1A}$ receptors, which produced lower maximal inhibition than DAMGO, would compete with each other for AC. Even these less efficacious agonists were not able to inhibit AC to any greater extent in combination than when applied alone (Figure 1c).

The extent of competition between receptors for AC can be calculated using the following equation (Brinkerhoff et al., 2008):

$$\text{% competition} = \left[ \frac{(R_1 + R_2) - R_{1+2}}{(R_1 + R_2) - \max(R_1, R_2)} \right] \times 100$$

where $R_1$ and $R_2$ are two different receptor types giving a theoretical maximum response ($R_1 + R_2$), which is compared to the experimentally determined effect of $R_{1+2}$ and the effect of the most
efficacious agonist alone \(\text{max}(R_1,R_2)\). If two receptors do not compete, the theoretical additive and experimental additive will be equivalent so there will be zero % competition. When there is complete (100%) competition between two receptors, the experimental addition of both agonists does not increase the response over the most efficacious agonist alone. Using the data from experiments in SH-SY5Y cells described above, complete competition was observed for all receptor pairs (Table 2). In addition, the DOR agonist SNC80 or the NOPr agonist nociceptin/OFQ occluded responses from the other, less effective agonists, resulting in competition that was not significantly different from 100 % (Table 3). Therefore, at maximal agonist concentrations, G\(_{i/o}\)-coupled receptors, including the opioid receptors, compete for a shared pool of AC.

**MOR and DOR competition for G proteins**

Interactions between receptors could occur at the level of AC or G protein. To evaluate this we studied the interaction between MOR and DOR for G proteins as measured by binding of \(^{35}\text{S}\)GTP\(\gamma\)S. Maximum concentrations of DAMGO stimulated a greater degree of \(^{35}\text{S}\)GTP\(\gamma\)S binding than SNC80, consistent with their relative degrees of AC inhibition and the greater expression of MOR compared to DOR in these cells (Figure 2a). When added together, DAMGO and SNC80 stimulation of \(^{35}\text{S}\)GTP\(\gamma\)S binding was similar to binding stimulated by DAMGO alone (\(p > 0.05\) comparing DAMGO to DAMGO/SNC80 by one-way ANOVA with Bonferroni’s post-test) and significantly less than the theoretical additive (\(p < 0.01\) comparing \(R_1 + R_2\) to DAMGO/SNC80 by one-way ANOVA with Bonferroni’s post-test) (Figure 2a), giving a percent competition between DAMGO and SNC80 of 88 ± 2 %, similar to the level of
competition between these two agonists for AC. These results indicate significant sharing of G proteins between MOR and DOR in differentiated SH-SY5Y cells.

It has been proposed that MOR and DOR heterodimerize and that these oligomers can activate PTX-resistant G proteins (George et al., 2000). However, in the differentiated SH-SY5Y cells used in our experiments, [35S]GTPγS binding stimulated by the combination of DAMGO and SNC80 was completely eliminated by PTX treatment (Figure 2b; PTX 100 ng/ml, 24 h), indicating that the combination of agonists still signals through PTX-sensitive Goαi/o proteins. In addition, PTX did not alter spontaneous [35S]GTPγS binding (Figure 2b). Similarly, AC inhibition by the combination of DAMGO and SNC80 was also blocked by 24 h pretreatment with PTX (data not shown).

Isobolographic analysis of interactions between MOR and DOR agonists

Combinations of maximally effective concentrations of agonists resulted in less than additive effects and predict competition for a common effector; however, at lower agonist concentrations it should be possible to identify additive, sub-additive and synergistic interactions. To this end, the concentration dependence of DAMGO to stimulate [35S]GTPγS binding was determined in the presence of a sub-maximal concentration of SNC80. Addition of 30 nM SNC80 with DAMGO did not significantly change the potency of DAMGO to stimulate [35S]GTPγS binding (Figure 3a; EC50 of DAMGO alone = 121 ± 32 nM, EC50 of DAMGO + 30 nM SNC80 = 64 ± 12 nM, p > 0.05), and at maximal concentrations of DAMGO the level of [35S]GTPγS binding was similar in the presence or absence of SNC80 (at 1 μM and 10 μM DAMGO, p > 0.05 by two-way ANOVA with Bonferroni’s post-test) and significantly less than the theoretical additive (at 10 μM DAMGO, p < 0.05 by two-way ANOVA with Bonferroni’s
post-test; Figure 3a). However, at lower concentrations of DAMGO, combination with 30 nM SNC80 was similar to the theoretical additive (Figure 3a), indicating an additive interaction between MOR and DOR agonists until G proteins become limiting, at which point DAMGO is occlusive.

Additive actions of agonists or deviations from additivity can be observed graphically using an isobologram (Tallarida, 2006). An isobologram for combinations of DAMGO and SNC80 that produced 50% of the maximal DAMGO effect was constructed based on values from the individual concentration-effect curves for DAMGO and SNC80 using the equation described in Methods (Tallarida, 2006). The line of additivity is not linear because DAMGO and SNC80 have different maxima and therefore a variable potency ratio. 

The binding of [35S]GTPγS is time-dependent with a t1/2 of approximately 20 min for DAMGO or SNC80-stimulated binding. Therefore, at time points shorter than 20 min, G proteins should not be limiting. When [35S]GTPγS binding was measured after a 10 or 20 min incubation, the combination of maximal concentrations of DAMGO and SNC80 was similar to the theoretical additive (Figure 3c) with competition between receptors only 12 ± 8.6 % after 10 min, increasing to 33 ± 11% after 20 min. These results are in agreement with an additive
interaction of these agonists at less than saturating concentrations when G proteins are not limiting.

_Heterologous inhibition of AC prevents opioid receptor-mediated cAMP overshoot_

Chronic administration of opioid agonists and other G<sub>1/o</sub>-coupled receptor agonists causes a homeostatic sensitization of AC resulting in an overshoot of cAMP production upon addition of a competitive antagonist (Watts, 2002). To determine if MOR and DOR accessed the same systems responsible for AC sensitization during chronic treatment, cells were treated overnight with the peptidic DOR agonist DPDPE (10 µM) in the presence or absence of DAMGO. DPDPE alone produced an overshoot response which was enhanced in the presence of 10 nM, but not 100 nM DAMGO (Figure 4).

Since MOR and DOR accessed the same pool of AC in both the acute and chronic opioid state, we hypothesized that overshoot of cAMP occurring upon precipitation of withdrawal from chronic MOR agonist treatment would be prevented by acute addition of a DOR agonist. To test this hypothesis, differentiated SH-SY5Y cells were treated overnight with DAMGO (100 nM) and withdrawal was precipitated with the MOR antagonist CTAP in the presence or absence of 1 µM SNC80. The addition of SNC80 attenuated the AC overshoot response (Figure 5). Moreover, this attenuation was also observed with addition of the NOPr agonist nociceptin/OFQ and the α<sub>2</sub>AR agonists UK 14304 and clonidine (Figure 5). Agonists that gave a reduced acute inhibition of cAMP (CP 55,9140 and 8-OH-DPAT) were unable to attenuate MOR-mediated cAMP overshoot.

To determine the concentration-relationship of this effect, DAMGO-mediated cAMP overshoot was precipitated by CTAP in the presence or absence of varying concentrations of
SNC80. The addition of SNC80 reduced DAMGO-mediated overshoot in a concentration-dependent manner (Figure 6a). Furthermore, SNC80 inhibited cAMP production with a similar potency in vehicle or DAMGO-treated cells (vehicle = 14.6 ± 7.8 nM, DAMGO-treated = 13.8 ± 7.2 nM). The effect of SNC80 was via DOR because MOR was blocked by the selective antagonist CTAP in both vehicle and DAMGO-treated cells, and in separate experiments 1 μM CTAP did not affect AC inhibition by 100 nM SNC80 (100 nM SNC80 = 59 ± 3 % cAMP inhibition, 100 nM SNC80 + 1 μM CTAP = 55 ± 2 % cAMP inhibition; n = 6, p > 0.05).

Reciprocally, DOR-mediated cAMP overshoot was attenuated in a concentration-dependent manner by DAMGO (Figure 6b). In these experiments, cells were treated overnight with DPDPE and specific DOR-mediated cAMP overshoot was precipitated using the selective DOR antagonist ICI 174,864 (1 μM) in the absence or presence of increasing concentrations of DAMGO. In addition to preventing overshoot, DAMGO inhibited AC with a similar potency in vehicle or DPDPE-treated cells (EC_{50}: vehicle-treated = 32.2 ± 12.5 nM, DPDPE-treated = 29.0 ± 7.1 nM). 1 μM ICI 174,864 did not shift the ability of DAMGO to stimulate $[^{35}S]$GTPγS binding in SH-SY5Y cells (EC_{50}: Control = 263 ± 30 nM; with ICI 174,864 = 283 ± 35 nM, p = 0.70, n = 2).

Lack of heterologous tolerance between MOR and DOR

The similar EC_{50} values for AC inhibition in opioid-treated and naïve cells for both MOR and DOR agonists suggest a lack of cross-tolerance between these two receptors in SH-SY5Y cells. To confirm this, agonist-stimulated $[^{35}S]$GTPγS binding was measured in membranes from SH-SY5Y cells that were treated overnight with vehicle, DAMGO or SNC80. DAMGO stimulated $[^{35}S]$GTPγS binding in vehicle-treated SH-SY5Y membranes in a concentration
dependent manner (Figure 7a, EC$_{50}$ = 86 ± 16 nM). Both the maximum effect (vehicle-treated = 121 ± 3.7 % stimulation; DAMGO-treated = 29 ± 2.9 % stimulation, p < 0.0001) and EC$_{50}$ (vehicle-treated = 86 ± 16 nM; DAMGO-treated = 260 ± 62 nM, p < 0.05) of DAMGO-stimulated [³⁵S]GTP$_{γ}$S binding was significantly attenuated in membranes from cells treated overnight with 1 μM DAMGO, indicating the development of tolerance (Figure 7a). In comparison, SNC80-stimulated [³⁵S]GTP$_{γ}$S binding was similar in membranes from vehicle or DAMGO-treated cells (Figure 7b, vehicle-treated EC$_{50}$ = 29.8 ± 12.5 nM; DAMGO-treated EC$_{50}$ = 34.6 ± 10.8 nM; vehicle-treated max = 53.1 ± 4.7 % stimulation; DAMGO-treated max = 56.5 ± 3.5 % stimulation). The reverse treatment paradigm produced similar results. Treatment of SH-SY5Y cells overnight with 1 μM SNC80 produced marked homologous tolerance, indicated by a significant reduction in [³⁵S]GTP$_{γ}$S binding by maximal concentrations of SNC80 (Figure 7c, vehicle-treated = 57.2 ± 3.4 % stimulation; SNC80-treated = 14.6 ± 4.8 % stimulation, p < 0.0001). However, the potency and efficacy for DAMGO to stimulate [³⁵S]GTP$_{γ}$S binding was not affected by SNC80 pretreatment (Figure 7d, vehicle-treated EC$_{50}$ = 69 ± 8.5 nM; SNC80-treated EC$_{50}$ = 79 ± 1.6 nM; vehicle-treated max = 106.7 ± 7.5 %; SNC80-treated max = 100.7 ± 5.9 %, p > 0.05).

Basal binding of [³⁵S]GTP$_{γ}$S in the absence of agonist was similar in vehicle, DAMGO or SNC80-pretreated cells (vehicle-treated = 9.8 ± 0.6 fmol/mg; DAMGO-treated = 11.6 ± 1.4 fmol/mg; SNC80-treated = 11.4 ± 0.6 fmol/mg, p > 0.05 one-way ANOVA with Bonferroni’s post-test), indicating that G proteins were unchanged by overnight agonist treatment. To confirm this, G$_{α_o}$ and G$_{α_i2}$ were identified by western blot in the membrane samples used in the above experiments. G$_{α_o}$ and G$_{α_i2}$ protein levels were not changed following overnight treatment with DAMGO or SNC80 (Figure 7e). Together these results confirm a lack of cross-tolerance
between MOR and DOR in differentiated SH-SY5Y cells, similar to previous reports in undifferentiated cells (Alt et al., 2002; Zadina et al., 1994).
Discussion

In this study, we have shown that AC inhibition by agonists to DOR, NOPr, α₂AR, CB₁ and 5-HT₁A receptors in differentiated SH-SY5Y cells was occluded by a maximal concentration of the MOR agonist DAMGO, suggesting that all these receptors compete for and inhibit the same AC enzymes. The competition, as shown by MOR and DOR, began at the G protein, was additive when G proteins were not limiting, and reached an occlusive ceiling at maximal agonist concentrations. Similar competition occurred during chronic agonist exposure such that acute administration of agonists to DOR, NOPr and α₂AR reduced the expression of AC sensitization following chronic DAMGO-treatment. However, the cross-talk between MOR and DOR did not lead to heterologous tolerance.

The rank order of AC inhibition by a maximum concentration of full agonists acting at Gᵢ/o-coupled receptors in SH-SY5Y cells was MOR > DOR ≥ NOPr > α₂AR ≥ CB₁ = 5-HT₁A. This order is mostly determined by the relative receptor expression as measured by [³H]agonist binding, which follows a similar pattern. One exception was NOPr, which was expressed at relatively low levels (30 ± 11 fmol/mg protein), yet the NOPr agonist nociceptin/OFQ inhibited AC as well as the DOR agonist SNC80 or the MOR agonist DAMGO. This suggests that NOPr is efficiently coupled to Gαᵢ/o proteins in the cells, which is consistent with evidence that NOPr displays agonist-independent constitutive activity (Beedle et al., 2004). Thus, all of these Gᵢ/o-coupled receptors shared a common pool of AC, but the proportion of the AC pool utilized by each receptor was determined by the agonist-driven activity of each receptor to inhibit AC (Figure 8). The most active agonist, the MOR agonist DAMGO, had access to the most AC and at a maximal concentration occluded effects by agonists to DOR, NOPr, α₂AR, CB₁ and 5-HT₁A receptors. Agonists to α₂AR, CB₁ and 5-HT₁A receptors inhibited AC the least and even these
receptors were not additive with each other, suggesting that the pool is always limiting even for receptors with lower expression levels.

The ability of all receptors to share AC indicates that barriers to prevent free diffusion in the membrane, such as receptor oligomers, membrane microdomains, and protein scaffolds (Allen et al., 2007; George et al., 2000; Gomes et al., 2004; Hall and Lefkowitz, 2002) do not segregate these receptors from the common pool of AC. Two alternative hypotheses could explain these results. Free access of receptors to all G proteins and AC as predicted by the collision coupling model would allow receptors to share a common pool of AC and has recently been discussed as an alternative explanation to negative cooperativity data that was attributed to dimerization (Chabre et al., 2009). However, such a scenario would seem unlikely given the evidence that MOR diffusion is restricted to sub-micrometer domains (Sauliere et al., 2006). Secondly, there could be complexes of multiple receptors isolated with signaling molecules. Most of the Gi/o-coupled receptors expressed in SH-SY5Y cells have been reported to heterodimerize with MOR, including DOR (George et al., 2000; Gomes et al., 2004), NOPr (Wang et al., 2005), α2AR (Jordan et al., 2003) and CB1 (Rios et al., 2006), while DOR has been shown to form heterodimers with α2AR (Rios et al., 2004). In addition, preformed signaling complexes containing GPCR and multiple effectors have been identified (Davare et al., 2001). In SH-SY5Y cells, such complexes could include MOR and at least five other Gi/o-coupled receptor types.

Agonists for DOR, NOPr and α2AR were able to inhibit AC in the naïve and opioid-dependent state. The rank order of the effectiveness of agonists to inhibit AC remained the same in control and DAMGO-dependent cells, so that the most efficacious agonists (SNC80 and nociceptin/OFQ) significantly prevented DAMGO-mediated cAMP overshoot. One exception
was the α2AR agonist UK 14304, which was equally effective as SNC80 and nociceptin/OFQ at preventing DAMGO-mediated overshoot, but considerably less efficacious at AC inhibition in the naïve cell. An increase in α2AR density following chronic DAMGO exposure could explain the enhanced UK 14304 response. However, in rats, α2AR density in various brain regions was either decreased or unchanged following chronic morphine treatment (Smith et al., 1989).

Alternatively, the α2AR signaling system may become more efficient after chronic MOR agonist treatment possibly through opioid-induced changes in the activity of regulators of G protein signaling (RGS) proteins (Traynor, 2010).

AC sensitization occurs following chronic MOR occupation and is thought to be important for the manifestation of withdrawal (Watts, 2002). Specifically, upregulation of the cAMP/AC/protein kinase A (PKA) pathway in the locus coeruleus (LC) has been identified as a mediator of opioid dependence and withdrawal, most recently by Zachariou et al., 2008. Thus, drugs that inhibit the cAMP pathway and can counter AC sensitization, such as the Gxi/o-coupled receptor agonists presented here, would have therapeutic potential in the treatment of opioid withdrawal. For instance, the clinical utility of the α2AR agonist clonidine in opioid withdrawal has been known for some time (Gold et al., 1978) and α2AR agonists are often used “off label” to treat or prevent opioid withdrawal. Furthermore, the α2AR agonist lofexidine could become the first non-opiate FDA-approved treatment of opioid withdrawal (Yu et al., 2008).

It is thought that clonidine prevents opioid withdrawal symptoms by reversing hyperactivity of noradrenergic neurons in the LC (Aghajanian, 1978). There are two proposed mechanisms for withdrawal-induced hyperactivity of LC neurons in opioid-dependence. The first is an enhanced input of excitatory glutamate into the LC (Aston-Jones et al., 1997). The second is an intracellular sensitization mediated by upregulation of the cAMP pathway, and is
supported by data that in vitro, withdrawal-induced hyperactivity is suppressed by inhibitors of PKA and is enhanced by forskolin or an active cAMP analog (Ivanov and Aston-Jones, 2001). Thus, our findings that clonidine can heterologously inhibit cAMP and prevent DAMGO-mediated cAMP overshoot, provides a mechanism for clonidine in opioid withdrawal.

NOP and CB₁ receptors are also co-expressed with MOR in LC neurons, where NOP receptors were found to activate the same population of K⁺ channels as MOR and α₂AR (Connor et al., 1996; Scavone et al., 2010). Furthermore, intracerebroventricular injection of nociceptin/OFQ prevented naloxone-precipitated withdrawal symptoms in morphine-dependent rats, and the non-peptidic NOPr agonist Ro 64-6198 reduced the expression of morphine-withdrawal jumping in mice when administered just prior to precipitation of withdrawal (Kotlinska et al., 2000; Kotlinska et al., 2003). This is again consistent with our findings that administration of nociceptin/OFQ during antagonist-precipitated withdrawal reduced cAMP overshoot and supports an intracellular mechanism of competitive inhibition of shared AC. Although the CB₁ agonist CP 55,940 did not prevent MOR-induced cAMP overshoot in SH-SY5Y cells, this may be due to low expression levels. This is pertinent because levels of CB₁ in the brain are generally high and acute administration of cannabinoid agonists to morphine-dependent rodents prevented withdrawal symptoms including jumping, weight loss, wet dog shakes and diarrhea, although the mechanism or site of action was not determined (Hine et al., 1975).

In contrast to α₂AR, NOPr and CB₁ receptors, the role of DOR in attenuating MOR-mediated withdrawal may be less relevant in vivo. Although we have shown that DOR shares AC with MOR, and the DOR agonist SNC80 inhibits DAMGO-mediated cAMP overshoot in SH-SY5Y cells, an in vivo intracellular mechanism between MOR and DOR to prevent...
morphine-withdrawal will depend on co-expression in a single neuron. DOR is not expressed in the LC and although other brain regions are undoubtedly important in withdrawal (Christie et al., 1997), the co-expression of MOR and DOR on neurons in other regions is debatable (Scherrer et al., 2009). Furthermore, administration of the DOR agonist BW373U86 just prior to naloxone-precipitated withdrawal in morphine-dependent rats did not reduce withdrawal signs (Lee et al., 1993).

In conclusion, these studies have shown that all identified G_{i/o}-coupled receptors endogenously expressed in differentiated SH-SY5Y cells shared a common pool of AC. The interaction likely begins at the G-protein level for all receptors, as shown for MOR and DOR. At this stage, we cannot distinguish between a lack of compartmentalization and the presence of signalosomes that contain several receptor types and signaling proteins. However, biophysical data on MOR membrane diffusion would tend to support a model in which there is organization of receptors (Sauliere et al., 2006). Regardless of the model, heterologous inhibition of shared AC by DOR, NOPr and α₂AR agonists prevented the expression of cAMP overshoot in MOR agonist-dependent cells. Thus, these studies support an intracellular mechanism for the prevention of morphine-withdrawal symptoms by acute administration of α₂AR, NOPr or CB₁ receptor agonists.
Acknowledgements

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Authorship Contributions

*Participated in research design:* Levitt and Traynor

*Conducted experiments:* Levitt and Purington

*Performed data analysis:* Levitt, Purington and Traynor

*Wrote or contributed to the writing of the manuscript:* Levitt and Traynor
References:


Footnotes

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Legends for Figures

Figure 1. G\textsubscript{\(i\beta\gamma\)}-coupled receptors endogenously expressed in SH-SY5Y cells share a common pool of AC.  
A, acute inhibition of 5 \(\mu\)M forskolin-stimulated AC by 1 \(\mu\)M of the indicated agonist alone.  Agonist (receptor) = DAMGO (MOR), SNC80 (DOR), OFQ (NOPr), UK 14304 (\(\alpha_2\)AR), clonidine (\(\alpha_2\)AR), CP 55,940 (CB\(_1\)), 8-OH-DPAT (5-HT\(_{1A}\)).  ***p < 0.001 compared to DAMGO by one-way ANOVA with Bonferroni’s post-test.  
B, co-incubation with 1 \(\mu\)M DAMGO occludes inhibition by 1 \(\mu\)M of all indicated agonists.  All bars are not statistically different from 1 \(\mu\)M DAMGO alone (p > 0.05 by one-way ANOVA with Bonferroni’s post-test).  
C, lower efficacy agonists (1 \(\mu\)M) were also not additive when co-administered in the indicated pairs (p > 0.05 for all pairs when compared to the most efficacious agonist of the pair by one-way ANOVA with Bonferroni’s post-test).  Data are presented as mean ± S.E.M. (n = 4, in duplicate) of percent cAMP inhibition, where stimulation by 5 \(\mu\)M forskolin alone is represented as 0%.

Figure 2.  MOR and DOR share pertussis toxin-sensitive G proteins.  
A, stimulation of \([^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding in membranes from SH-SY5Y cells after 60 min incubation with 1 \(\mu\)M DAMGO or SNC80 alone or in combination (DAMGO/SNC80).  Incubation with DAMGO and SNC80 in combination (DAMGO/SNC80) did not significantly increase \([^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding more than DAMGO alone (p > 0.05 by one-way ANOVA with Bonferroni’s post-test), and stimulated significantly less \([^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding than the theoretical additive of the individual responses (R1 + R2) (***p < 0.01 by one-way ANOVA with Bonferroni’s post-test).  
B, pretreatment of SH-SY5Y cells with pertussis toxin (PTX, 100 ng/ml) for 24 h prior to membrane preparation blocked stimulation of \([^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding by 1 \(\mu\)M DAMGO, SNC80 or
the combination (DAMGO/SNC80) (***p < 0.001 by two-way ANOVA with Bonferroni’s post-test). Pertussis toxin treatment did not alter spontaneous \[^{35}\text{S}]\text{GTP} \gamma \text{S} \ binding in the absence of agonist (p > 0.05 by two-way ANOVA with Bonferroni’s post-test). Data are presented as mean ± S.E.M. (n = 3, in triplicate).

Figure 3. DAMGO and SNC80 activation of G protein is additive at concentrations or time-points when G protein is not limiting. A, concentration-dependent stimulation of \[^{35}\text{S}]\text{GTP} \gamma \text{S} \ binding in SH-SY5Y membranes following 60 min incubation with various concentrations of DAMGO alone (filled squares) or DAMGO with 30 nM SNC80 (open squares). The EC\text{50} \ of DAMGO is not significantly altered by the addition of 30 nM SNC80 (DAMGO alone = 121 ± 32 nM, DAMGO + 30 nM SNC80 = 64 ± 12 nM, p = 0.14 by two-tailed Student’s \(t\)-test). Co-incubation of DAMGO with 30 nM SNC80 produces additive \[^{35}\text{S}]\text{GTP} \gamma \text{S} \ binding similar to the theoretical additive curve (filled circles), which diverges only when DAMGO becomes occlusive at maximal concentrations (*p < 0.05 at 10 \(\mu\text{M}\) for “DAMGO + 30 nM SNC80” compared to the “theoretical addition” by two-way ANOVA with Bonferroni’s post-test; n = 4, in duplicate). B, isobologram for agonists with a variable potency ratio calculated as described in Methods. Stimulation of \[^{35}\text{S}]\text{GTP} \gamma \text{S} \ binding by DAMGO was conducted in the presence of indicated concentrations of SNC80. Concentration combinations that produced 50 % of the maximum effect of DAMGO alone are plotted from 3 separate experiments as mean ± S.E.M. Points on the line indicate additivity between DAMGO and SNC80. C, stimulation of \[^{35}\text{S}]\text{GTP} \gamma \text{S} \ binding in SH-SY5Y membranes following incubation with 1 \(\mu\text{M}\) DAMGO or SNC80 alone or in combination for 10 or 20 min, before the incubation reaches steady state. At both time-points, co-incubation with DAMGO and SNC80 (DAMGO/SNC80) is greater than DAMGO alone (*p
< 0.05 by one-way ANOVA with Bonferroni’s post-test) and similar to the theoretical additive (R1 + R2) (p > 0.05 by one-way ANOVA with Bonferroni’s post-test; n = 2, in triplicate).

Figure 4. MOR and DOR share AC during chronic agonist administration. SH-SY5Y cells were treated overnight with vehicle (open bars) or 10 μM DPDPE (hatched bars) in the presence or absence of the MOR agonist DAMGO (10 nM or 100 nM) to induce dependence. Withdrawal was precipitated with the opioid antagonist naloxone (100 μM) in the presence of 5 μM forskolin. Data are presented as mean ± S.E.M. (n = 4, in duplicate) of the percent of forskolin-stimulated cAMP, where forskolin alone is 100% and is indicated by the dashed line. Overnight incubation with DPDPE produced overshoot on its own and enhanced the overshoot produced by 10 nM, but not 100 nM DAMGO. ***p < 0.001 compared to the vehicle with the same concentration of DAMGO by two-way ANOVA and Bonferroni’s post-test.

Figure 5. DAMGO-mediated cAMP overshoot is reduced by heterologous inhibition of shared AC by agonist to DOR, NOPr or α2AR. AC sensitization was developed by incubating SH-SY5Y cells overnight with 100 nM DAMGO. To precipitate withdrawal, DAMGO-containing media was replaced with media containing 5 μM forskolin, 1 mM IBMX and 1 μM CTAP in the presence or absence of 1 μM of non-MOR agonist, as indicated, for 10 min. Data are presented as mean ± S.E.M. of percent cAMP overshoot, where stimulation by forskolin alone is represented as 0%. Three of six experiments, in duplicate, were compiled that produced > 100% DAMGO overshoot in the absence of non-MOR agonist. **p < 0.01, ***p < 0.001 compared to DAMGO overshoot without non-MOR agonist by one-way ANOVA with Bonferroni’s post-test.
Figure 6. Inhibition of cAMP by MOR or DOR agonists is similar for sensitized or nonsensitized AC. A, SH-SY5Y cells were incubated with vehicle (filled squares) or the MOR agonist DAMGO (100 nM, open squares) overnight to induce dependence. Withdrawal was precipitated with the MOR antagonist CTAP (1 μM) in the presence of 5 μM forskolin. Acute cAMP production was inhibited by including various concentrations of the DOR agonist SNC80 in the precipitating media. The concentration-response of SNC80 to inhibit cAMP was similar in control and DAMGO-dependent cells (EC50: vehicle-treated = 14.6 ± 7.8 nM, DAMGO-treated = 13.8 ± 7.2 nM, p > 0.05 by two-tailed student’s t-test). B, cells were incubated with vehicle (filled squares) or the DOR agonist DPDPE (10 μM, open squares) overnight to induce dependence. Receptor-specific withdrawal was precipitated with the DOR antagonist ICI 174,864 (1 μM) in the presence of 30 μM forskolin. Various concentrations of the MOR agonist DAMGO were included in the precipitating media to acutely inhibit cAMP production. The concentration-response of DAMGO to inhibit cAMP production was similar in control and DPDPE-withdrawn cells (EC50: vehicle-treated = 32.2 ± 12.5 nM, DPDPE-treated = 29.0 ± 7.1 nM, p > 0.05 by two-tailed Student’s t-test). Data are presented as mean pmol cAMP/mg protein ± S.E.M. (n = 3 or 4, in duplicate). cAMP produced by forskolin alone is indicated by the dashed line.

Figure 7. Lack of heterologous tolerance between MOR and DOR. SH-SY5Y cells were incubated with 1 μM of the MOR agonist DAMGO (A, B) or the DOR agonist SNC80 (C, D) for 24 h prior to membrane preparation. [35S]GTPγS binding in membranes from treated cells was stimulated by incubation for 60 min with various concentrations of DAMGO (A, D) or SNC80 (B, C). Chronic treatment with 1 μM DAMGO reduced the potency of DAMGO (EC50: vehicle-
treated = 86 ± 16 nM; DAMGO-treated = 260 ± 62 nM, p = 0.04), but not SNC80 (EC$_{50}$:
vehicle-treated = 29.8 ± 12.5 nM; DAMGO-treated EC$_{50}$ = 34.6 ± 10.8 nM, p > 0.05). Similarly,
chronic treatment with 1 μM SNC80 almost completely abolished SNC80-mediated $[^{35}\text{S}]$$\Gamma\Gamma\text{TP}\gamma\text{S}$
binding, but did not alter the potency of DAMGO-mediated $[^{35}\text{S}]$$\Gamma\Gamma\text{TP}\gamma\text{S}$ binding (EC$_{50}$:
vehicle-treated = 69 ± 8.5 nM; SNC80-treated = 79 ± 1.6 nM, p > 0.05). EC$_{50}$ statistical comparisons
were made by two-tailed Student’s $t$-test. $E$, $G\alpha_o$, and $G\alpha_{i2}$ were detected in membranes from
cells treated overnight with vehicle (DMEM, V), 1 μM DAMGO (D) or 1 μM SNC80 (S).
Immunoreactive density was quantified, normalized to tubulin loading control and compared to
vehicle-treated cells (n = 3). There was no difference in G protein levels following agonist
treatment (p > 0.05 by one-way ANOVA with Bonferroni’s post-test).

Figure 8. Schematic depicting the accessibility of Gi/o-coupled receptors to portions of the total
AC pool. The amount of the AC pool utilized by each receptor type is related to the agonist-
mediated activity of each receptor. The most active and most highly expressed receptor, MOR,
shares AC with all other receptor types. The other receptor types share AC in a manner
predicted by receptor density and/or the ability of agonist to inhibit AC such that three receptor
groups exist – one that contains MOR only, one that contains MOR, DOR and NOPr, and one
that contains MOR, DOR, NOPr, $\alpha_2\text{AR}$, CB$_1$ and 5-HT$_{1A}$. 

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Table 1. Receptor density in differentiated SH-SY5Y cells.

Receptor numbers in cell membranes were determined using selective agonist radioligands at a maximal concentration as described in Methods. Results are presented as mean fmol radioligand bound/mg protein ± S.E.M. (n = 3-6).

<table>
<thead>
<tr>
<th>Receptor Type</th>
<th>Radioligand</th>
<th>Receptor density * (fmol/mg protein ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOR</td>
<td>[3H]DAMGO</td>
<td>305 ± 42</td>
</tr>
<tr>
<td>DOR</td>
<td>[3H]DPDPE</td>
<td>191 ± 21</td>
</tr>
<tr>
<td>CB1</td>
<td>[3H]CP 55,940</td>
<td>60 ± 25</td>
</tr>
<tr>
<td>α2AR</td>
<td>[3H]UK 14304</td>
<td>35 ± 1</td>
</tr>
<tr>
<td>NOPr</td>
<td>[3H]nociceptin/OFQ</td>
<td>30 ± 11</td>
</tr>
</tbody>
</table>

*5-HT1A not tested

*Binding of maximal concentrations of antagonists for MOR ([3H]diprenorphine in the presence of the delta selective ICI 174,864), DOR ([3H]NTI) and α2AR ([3H]yohimbine) was 103 ± 8.0 %, 98 ± 11 % and 79 ± 6 % of the agonist binding, respectively.
Table 2. Competition between indicated agonists (1 μM) for acute inhibition of AC.

Percent competition was calculated as described in Results (Brinkerhoff et al., 2008) using experimental data shown in Figure 1. The percent competition from three individual experiments was compiled and is presented as mean ± S.E.M.

<table>
<thead>
<tr>
<th>Agonist R$_1$ (receptor)</th>
<th>Agonist R$_2$ (receptor)</th>
<th>% competition (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAMGO (MOR)</td>
<td>SNC80 (DOR)</td>
<td>78 ± 8</td>
</tr>
<tr>
<td>DAMGO (MOR)</td>
<td>Nociceptin/OFQ (NOPr)</td>
<td>97 ± 12</td>
</tr>
<tr>
<td>DAMGO (MOR)</td>
<td>UK 14304 (α$_2$AR)</td>
<td>115 ± 10</td>
</tr>
<tr>
<td>DAMGO (MOR)</td>
<td>Clonidine (α$_2$AR)</td>
<td>106 ± 22</td>
</tr>
<tr>
<td>DAMGO (MOR)</td>
<td>CP 55,9140 (CB$_1$)</td>
<td>121 ± 24</td>
</tr>
<tr>
<td>DAMGO (MOR)</td>
<td>8-OH-DPAT (5-HT$_{1A}$)</td>
<td>97 ± 13</td>
</tr>
<tr>
<td>UK 14301 (α$_2$AR)</td>
<td>CP 55,9140 (CB$_1$)</td>
<td>95 ± 19</td>
</tr>
<tr>
<td>8-OH-DPAT (5-HT$_{1A}$)</td>
<td>Clonidine (α$_2$AR)</td>
<td>95 ± 16</td>
</tr>
<tr>
<td>8-OH-DPAT (5-HT$_{1A}$)</td>
<td>CP 55,9140 (CB$_1$)</td>
<td>83 ± 6</td>
</tr>
</tbody>
</table>
Table 3. Competition between 1 μM SNC80 or nociceptin/OFQ and other Gi/o-coupled receptor agonists for acute inhibition of AC.

Inhibition of AC by 1 μM of agonist(s) alone or in combination was performed as described for Figure 1 and in Methods. Percent competition was calculated from three individual experiments, in duplicate, as described for Table 2.

<table>
<thead>
<tr>
<th>Agonist $R_1$ (receptor)</th>
<th>Agonist $R_2$ (receptor)</th>
<th>% competition (mean ± SEM)</th>
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</thead>
<tbody>
<tr>
<td>SNC80 (DOR)</td>
<td>Nociceptin/OFQ (NOPr)</td>
<td>79 ± 18</td>
</tr>
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<td>SNC80 (DOR)</td>
<td>UK 14304 ($\alpha_2$AR)</td>
<td>84 ± 30</td>
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<td>SNC80 (DOR)</td>
<td>Clonidine ($\alpha_2$AR)</td>
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<td>SNC80 (DOR)</td>
<td>CP 55,9140 (CB$_1$)</td>
<td>88 ± 32</td>
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<td>SNC80 (DOR)</td>
<td>8-OH-DPAT (5-HT$_{1A}$)</td>
<td>119 ± 12</td>
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<td>Nociceptin/OFQ (NOPr)</td>
<td>UK 14304 ($\alpha_2$AR)</td>
<td>108 ± 15</td>
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<tr>
<td>Nociceptin/OFQ (NOPr)</td>
<td>Clonidine ($\alpha_2$AR)</td>
<td>119 ± 11</td>
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<tr>
<td>Nociceptin/OFQ (NOPr)</td>
<td>CP 55,9140 (CB$_1$)</td>
<td>119 ± 7</td>
</tr>
<tr>
<td>Nociceptin/OFQ (NOPr)</td>
<td>8-OH-DPAT (5-HT$_{1A}$)</td>
<td>124 ± 10</td>
</tr>
</tbody>
</table>
Figure 3

A

![Graph A](image)

B

![Graph B](image)

C

![Graph C](image)
Figure 4

% of forskolin-stimulated cAMP

[Vehicle] | [10 μM DPDPE]

[DAMGO] (nM)

0 | 10 | 100

***

***

0 100
Figure 5

% cAMP overshoot

DAMGO overshoot
SNC80
OFQ
UK 14304
Clonidine
CP 559140
8-OH-DPAT

+ 1 μM agonist
Figure 6

A

Overnight treatment

- Vehicle
- 100 nM DAMGO

B

Overnight treatment

- Vehicle
- 10 μM DPDPE

pmol cAMP/μg protein

log [SNC80] (M)

log [DAMGO] (M)