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Design and Functional Characterization of a Novel, Arrestin-Biased Designer G Protein-Coupled Receptor

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ABBREVIATIONS: ACh, acetylcholine; arr2, arrestin-2; arr3, arrestin-3; CNO, clozapine-N-oxide; DREADD, designer receptor exclusively activated by designer drug; GPCR, G protein-coupled receptor; HA tag, hemagglutinin tag; GRK, G protein-coupled receptor kinases; Luc, *Renilla* luciferase 8; [^{3}H]-NMS, N-[^{3}H]-methylscopolamine; OXO-M, Oxotremorine-M; RASSL, receptor activated solely by synthetic ligand; V-arr2, Venus-arrestin-2; V-arr3, Venus-arrestin-3.

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ABSTRACT

Recently, mutational modification of distinct muscarinic receptor subtypes has yielded novel designer G protein-coupled receptors (GPCRs) that are unable to bind acetylcholine (ACh), the endogenous muscarinic receptor ligand, but can be efficiently activated by clozapine-N-oxide (CNO), an otherwise pharmacologically inert compound. These CNO-sensitive designer GPCRs (alternative name: designer receptors exclusively activated by designer drug, DREADDs) have emerged as powerful new tools to dissect the in vivo roles of distinct G protein signaling pathways in specific cell types or tissues. As is the case with other GPCRs, CNO-activated DREADDs do not only couple to heterotrimeric G proteins but can also recruit proteins of the arrestin family (arrestin-2 and -3). Accumulating evidence suggests that arrestins can act as scaffolding proteins to promote signaling through G protein-independent signaling pathways. To explore the physiological relevance of these arrestin-dependent signaling pathways, the availability of an arrestin-biased DREADD would be highly desirable. In this study, we describe the development of an M_3 muscarinic receptor-based DREADD (Rq(R165L)) that is no longer able to couple to G proteins but can recruit arrestins and promote ERK1/2 phosphorylation in an arrestin- and CNO-dependent fashion. Moreover, CNO treatment of MIN6 insulinoma cells expressing the Rq(R165L) construct resulted in a robust, arrestin-dependent stimulation of insulin release, directly implicating arrestin signaling in the regulation of insulin secretion. This newly developed arrestin-biased DREADD represents an excellent novel tool to explore the physiological relevance of arrestin signaling pathways in distinct tissues and cell types.

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Introduction

G protein-coupled receptors (GPCRs) represent a superfamily of cell surface receptors that regulate the activity of virtually all physiological functions. Characteristically, GPCRs are activated via binding of extracellular ligands which trigger conformational changes that allow the receptors to interact with and activate specific classes of heterotrimeric G proteins. The activated GPCRs are rapidly phosphorylated by G protein-coupled receptor kinases (GRKs; Pierce et al., 2002). In most cases, the phosphorylated receptors bind to members of the arrestin protein family (arrestin-2 and -3; also known as β-arrestin-1 and -2), a process which interferes with receptor/G protein coupling and promotes GPCR internalization by targeting the receptors to clathrin-coated pits (Pierce et al., 2002).

However, during the past decade, it has become increasingly clear that arrestin-2 and -3 can serve as adaptor proteins that transduce signals to multiple effector pathways (Rajagopal et al., 2010; Shukla et al., 2011). Although some progress has been made in delineating the physiological functions of arrestin signaling pathways in various tissues, much remains to be learned about the in vivo physiological relevance of arrestin-mediated signaling. Importantly, such information is not only of theoretical interest, but could also be exploited for the development of novel classes of clinically useful drugs, including arrestin-biased agonists (Rajagopal et al. 2010; Shukla et al., 2011).

To facilitate studies aimed at elucidating the physiological roles of arrestin-/2/3-dependent signaling, we set out to develop a designer GPCR that can recruit arrestins after binding of an exogenous ligand but that is no longer able to activate heterotrimeric G proteins. During the past few years, clozapine-N-oxide (CNO)-sensitive designer GPCRs have emerged as highly useful tools to dissect the in vivo roles of distinct G protein signaling pathways in specific cell types or tissues (Armbruster et al., 2007; Alexander et al., 2009; Guettier et al., 2009). Structurally, these novel receptors (alternative names: designer receptors exclusively activated by designer drug (DREADDs) or second-generation receptors activated solely by synthetic ligand (RASSLs)) are muscarinic acetylcholine (ACh) receptors that contain two point mutations within the orthosteric binding pocket (Armbruster et al., 2007; Guettier et al., 2009). As a result of these mutations, these

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engineered receptors are unable to bind ACh, the endogenous muscarinic receptor ligand, but can be efficiently activated by CNO, an otherwise pharmacologically inert compound (Armbruster et al., 2007; Guettier et al., 2009). CNO stimulation of DREADDs leads to the activation of distinct classes of heterotrimeric G proteins as well as the recruitment of arrestin-2 and -3 (Alvarez-Curto et al, 2011; Nakajima and Wess, unpublished results), in a fashion similar to that observed with endogenous GPCRs.

In the present study, we describe the generation and functional characterization of a novel M_3 muscarinic receptor-based DREADD containing a point mutation within the highly conserved DRY motif (Rq(R165L)) that lacks the ability to activate heterotrimeric G proteins (CNO treatment has no effect on the levels of conventional second messengers) but still retains the ability to recruit arrestin-2 and -3 in a CNO-dependent fashion. Rq(R165L)-dependent arrestin recruitment was functionally relevant, as demonstrated in ERK1/2 phosphorylation assays carried out with cultured HEK293T cells. Moreover, we found that CNO treatment of Rq(R165L)-expressing mouse insulinoma cells (MIN6 cells) stimulated insulin release in an arrestin-dependent fashion. This latter finding strongly supports the notion that this newly developed arrestin-biased DREADD represents a powerful new tool to study the physiological relevance of arrestin-dependent signal cascades in distinct tissues and cell types.

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Materials and Methods

Drugs and Plasmids. CNO was obtained from the NIH as part of the Rapid Access to Investigative Drug Program funded by the NINDS. Acetylcholine chloride, oxotremorine-M ammonium iodide (OXO-M), atropine sulfate, and forskolin were from Sigma-Aldrich (St. Louis, MO). [^{3}H]-N-methylscopolamine ([^{3}H]-NMS: 79-83 Ci/mmol) was purchased from PerkinElmer Life Sciences (Downers Grove, IL)

The coding sequence of Rq, an M_3 muscarinic receptor-based DREADD (Guettier et al., 2009), including an N-terminal hemagglutinin (HA) epitope tag, was amplified by PCR and inserted into the pcDNA3.1 (-) vector (Invitrogen, Carlsbad, CA) using the *EcoR*I and *BamH*I sites present in the polylinker. To generate a mutant version of Rq that was unable to couple to G proteins, we generated the Rq(R165L) construct containing the R165L point mutation, using the QuikChange site-directed mutagenesis kit (Invitrogen). For BRET studies, we generated plasmids (vector backbone: pcDNA3.1 (+)) coding for receptors in which the C-terminus of Rq or Rq(R165L) was fused to the coding sequence of *Renilla* luciferase 8 (Luc), yielding Rq-Luc and Rq(R165L)-Luc, respectively. These plasmids were obtained by using a strategy similar to the one we employed for generating M3-Luc (McMillin et al., 2011). The correctness of all coding regions was verified by sequencing. Plasmids coding for rat GRK2 and Venus-tagged versions of arrestin-2 and -3 (V-arr2 and V-arr3, respectively) were kindly provided by Drs. Diaz Gimenez and Vsevolod Gurevich (Vanderbilt University) (Vishnivetskiy et al., 2011). The mammalian expression plasmid coding for the wild-type M_3 muscarinic receptor (rat) has been desribed previously (Schöneberg et al., 1995)

Cell Culture and Transfections. COS-7 cells and HEK293T cells were obtained from ATCC and cultured according to established protocols. MIN6 cells were a kind gift from Dr. Abner Notkins (NIDCR, NIH) and were cultured as described (Ishihara et al., 1994). COS-7 cells were transfected with plasmid DNA using Lipofectamine and Plus reagent (Invitrogen) according to the manufacturer's protocol. HEK293T and MIN6 cells were transfected as described below.

[^{3}H]-NMS Radioligand Binding Studies. [^{3}H]-NMS saturation and ACh and CNO inhibition binding assays were performed using membranes prepared from transfected COS-7 cells, as

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described in detail previously (Li et al., 2005; McMillin et al., 2011). In all inhibition binding studies, a fixed concentration of [^{3}H]-NMS (20 nM) was used. ACh and CNO IC_{50} values were converted to K_i values using the Cheng-Prusoff equation. Data were analyzed by using GraphPad Prism 4.0 software (GraphPad Software Inc., San Diego, CA).

Calcium Mobilization Assay. About 48 hr after transfection, cells were incubated with increasing concentrations of CNO, and changes in intracellular calcium levels were determined using FLIPR technology (Molecular Devices, Sunnyvale, CA). All measurements were carried out in 96-well plates, as described in detail previously (Li et al., 2007; McMillin et al., 2011). CNO concentration-response curves were analyzed by using Prism 4.0 software.

BRET (Arrestin Recruitment) Studies. To monitor receptor-mediated arrestin-2 and -3 recruitment, we followed a protocol similar to that described by Klewe et al. (2008). COS-7 cells were seeded onto 6-well plates at a density of 3 x 10^5 cells/well. About 24 hr after plating, cells were co-transfected with plasmids coding for Rq-Luc or Rq(R165L)-Luc (200 ng), GRK2 (200 ng), and V-Arr2 or V-Arr3 **(**800 ng). In control samples, vector DNA (pcDNA3.1 (-), 800 ng) was added instead of the arrestin plasmids. After 48 hr, cells were trypsinized, transferred to microcentrifuge tubes, and centrifuged at 110 x g for 5 min at room temperature. Cell pellets were resuspended in 900 µl of PBS supplemented with glucose (1 mg/ml), ascorbic acid (1 mM), and EDTA-free complete protease inhibitor (Roche Applied Science, Indianapolis, IN). Subsequently, 45-µl aliquots were added to individual wells of a white opaque 96-well plate and incubated with increasing concentrations of CNO for 45 min at room temperature (PerkinElmer Life Sciences). Total fluorescence was first measured via excitation at 485 nm and monitoring emission at 535 nm. Coelenterazine-h (Promega, Madison, WI) was then added to each well at a final concentration of 5 µM and emissions were measured at 530 and 480 nm. Total luminescence was subsequently measured in the absence of filters. All measurements were performed using the Mithras LB 940 plate reader (Berthold Technologies, Oak Ridge, TN). The BRET ratio was defined as the ratio of emission at 530 nm to emission at 480 nm after the addition of coelenterazine-h. BRET signals are given as NetBRET ratios calculated by subtracting baseline BRET ratios obtained in the absence of V-Arr2 or V-Arr3 from BRET ratios obtained in the presence of V-Arr2 or V-Arr3 (at any given

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CNO concentration). CNO $BRET_{50}$ and $BRET_{max}$ values were obtained by using GraphPad Prism 4.0 software.

siRNA-Mediated Arrestin-2/3 Knockdown. Knockdown of arrestin-2 or -3 expression was achieved by using the following siRNAs (Ahn et al., 2003): arrestin-2, 5'-AAAGCCUUCUGCGCGGAGAAU; arrestin-3, 5'-AAGGACCGCAAAGUGUUUGUG (duplexes; Ambion, Austin, TX). The negative control siRNA used in these experiments was identical to the one employed by Ahn et al. (2003). For arrestin knockdown studies, HEK293T or MIN6 cells (~1 x 10^6 cells) were seeded into 6-well plates. About 24 hr (HEK293T cells) or 48 hr later (MIN6 cells), cells were co-transfected with 0.8 μg receptor DNA (Rq or Rq(R165L)) and 120 pmol of arrestin-2, arrestin-3, or control siRNA. HEK293T cells were co-transfected using GeneSilencer transfection reagent (Gene Therapy Systems, San Diego, CA), as previously described (Ahn et al., 2003). MIN6 cells were co-transfected by employing Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen). About 48 hr after transfection, cells were subjected to various assays (see below). We used the same arrestin siRNAs for HEK293T and MIN6 cells (human sequences described by Ahn et al., 2003), since the corresponding mouse sequences differ at only one or two positions from the human siRNA sequences used.

Western Blotting (ERK1/2 Phosphorylation) Studies. HEK293T cells (~3 x 10^5 cells) were seeded into 12-well plates. About 24 hr later, cells were transfected (see previous paragraph) and incubated for another 24 hr. After a 5–6 hr starvation period in serum-free medium (Wei et al., 2003), cells were treated with increasing concentrations of CNO for 2 min at 37 ^oC. Subsequently, the medium was removed, and 1 x Laemmli sample buffer was added to each well. Whole cell lysates were sonicated, resolved on Novex 10% Tris/glycine polyacrylamide gels (Invitrogen), and transferred to nitrocellulose membranes for immunoblotting. Phosphorylated ERK1/2 (pERK1/2), total ERK1/2, and arrestin-2 and -3 were detected via immunoblotting using the following primary antibodies: rabbit monoclonal anti-phospho-p44/42 (pERK, 1:2,000, Cell Signaling, Beverly, MA), rabbit polyclonal anti-p44/42 (total ERK1/2, 1:2,000, Cell Signaling), and mouse monoclonal anti-arrestin-2 and -3 (1:2,000, BD Biosciences, San Jose, CA). HRP-labeled secondary antibodies (Cell Signaling) were used to detect the primary antibodies via chemiluminescence. Chemiluminescent

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detection was performed using the SuperSignal Western Pico reagent or SuperSignal Extended Dura reagent (Thermo Scientific, Rockford, IL). Immunoreactive bands were quantified by densitometry using ImageJ software (NIH).

cAMP Assay. MIN6 cells were transfected with the Rq(R165L) construct as described above. About 48 hr later, cells were trypsinized, collected by centrifugation, and resuspended in PBS containing glucose (1 mg/ml), and EDTA-free complete protease inhibitor (Roche Applied Science, Indianapolis, IN) at a density of 1 x 10^6 cells/ml. Subsequently, 10 µl aliquots were added to 200 µl PCR tubes and incubated with the same volume (10 µl) of increasing concentrations of CNO or forskolin for 25 min at 37 ^oC. The incubation mixtures were then transferred into white bottom 384-well plates (~5,000 cells/well), and cells were lysed to determine drug-dependent changes in cAMP levels using a FRET-based cAMP detection technique (cAMP dynamic 2 kit; Cisbio Bioassays, Bedford, MA) according to the manufacturer's protocol.

Insulin Release Assay. MIN6 cells that had been co-transfected with different combinations of plasmid DNAs and siRNAs (see above) were seeded into 96-well plates (~1 x 10^5 cells/well). About 48 hr later, the cells were incubated with increasing concentrations of CNO at 37 °C for 1 hr in Krebs–Ringer bicarbonate/HEPES buffer containing 16.7 mM glucose. Insulin release was determined by measuring insulin concentrations in the incubation medium using an insulin ELISA kit (Crystal Chem Inc., Downers Grove, IL). CNO concentration-response curves were analyzed using GraphPad Prism 4.0 software.

Statistics. Data are expressed as means ± SEM for the indicated number of observations. For comparisons between two groups, the paired or unpaired Student's *t*-test (two-tailed) was used, as appropriate. For multiple comparisons, the one-way analysis of variance (ANOVA) was applied. A p value of <0.05 was considered statistically significant.

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Results and Discussion

Identification of an M_3 Receptor-based DREADD Unable to Couple to G_q. We recently generated and characterized a rat M_3 muscarinic receptor-based DREADD that selectively couples to G proteins of the G_q family (Rq; Guettier et al., 2009). For the sake of simplicity, we refer to this receptor as 'Rq' throughout this manuscript.

Many studies have shown that mutational modification of an arginine residue that is highly conserved among class A GPCRs (R3.50 according to the Ballesteros-Weinstein GPCR numbering system; part of the highly conserved DRY motif) abolishes or drastically reduces agonist-dependent GPCR activation (http://www.gpcr.org/7tm/). Consistent with this observation, the recently solved crystal structure of an agonist-β_2-adrenergic receptor-G_s complex indicates that R3.50 contacts key regions of the G protein α-subunit that are predicted to be critical for productive receptor/G protein coupling (Rasmussen et al., 2011).

We previously reported that a mutant M_3 receptor (rat) containing the $R165L^{3.50}$ point mutation virtually lost its ability to stimulate muscarinic agonist-induced inositol phosphate production in transfected COS-7 cells (Li et al., 2005). In contrast, this mutation did not interfere with agonist and antagonist binding affinities and receptor expresssion levels (Li et al., 2005). To examine whether this point mutation also disrupted signaling mediated by the Rq designer receptor, we generated the Rq(R165L) mutant receptor. In agreement with the data reported by Li et al. (2005), CNO treatment of Rq(R165L)-expressing COS-7 cells had no significant effect on intracellular calcium levels ($[Ca^{2+}]_i$), as studied via FLIPR technology (Fig. 1A). In contrast, CNO induced robust, concentration-dependent increases in $[Ca^{2+}]_i$ in Rq-expressing COS-7 cells (Fig. 1A). The E_{max} value for this response amounted to ~75% of the E_{max} value observed with COS-7 cells expressing the wild-type M_3 muscarinic receptor (rat; Schöneberg et al., 1995) following stimulation with the full muscarinic agonist, oxotremorine-M (OXO-M; Fig. 1B).

Radioligand binding studies carried out with membranes from transfected COS-7 cells confirmed that the R165L point mutation had little effect on [^{3}H]-NMS and CNO binding affinities and [^{3}H]-NMS B_{max} values (Table 1). This observation indicates that the inability of Rq(R165L) to

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stimulate increases in $[Ca^{2+}]_i$ was not due to receptor misfolding and/or lack of CNO binding. Moreover, like Rq, the Rq(R165L) receptor failed to bind ACh, the endogenous muscarinic receptor agonist (Table 1). Since Rq selectively couples to G proteins of the G_q family (Armbruster et al., 2007; Guettier et al., 2009; Alvarez-Curto et al., 2011) and activation of this class of G proteins triggers pronounced increases in $[Ca^{2+}]_i$, our data indicate that the Rq(R165L) construct lacks the ability to activate G_q.

BRET (Receptor-Arrestin Recruitment) Assays Carried Out With Transfected COS-7 Cells. We next used BRET technology to examine whether Rq(R165L) was still able to recruit arrestins in a CNO-dependent fashion. BRET techniques are widely used to monitor GPCR/arrestin interactions with high sensitivity in live cells (Hamdan et al., 2005; Klewe et al., 2008; Kocan and Pfleger, 2011; Alvarez-Curto et al., 2011; Vishnivetskiy et al., 2011).

Following a protocol similar to that described by Klewe et al. (2008), we co-transfected COS-7 cells with a receptor construct containing a C-terminal *Renilla* Luciferase 8 sequence (Rq-Luc or Rq(R165L)-Luc; BRET donor), a modified version of arrestin-2 or -3 containing an N-terminal Venus tag (V-arr2 or V-arr3, respectively; BRET acceptor), and a GRK2 expression construct. Consistent with published data (Alvarez-Curto et al., 2011), CNO treatment of cells co-expressing Rq-Luc and V-arr2 or V-arr3 resulted in concentration-dependent BRET signals, indicative of CNO-dependent arrestin recruitment (Fig. 1C). We noted that CNO-dependent maximum BRET responses ($BRET_{max}$) were ~2.5-fold higher for the Rq-Luc/V-arr3 combination, as compared to the Rq-Luc/V-arr2 pair ($BRET_{max}$ values: Rq-Luc/V-arr2, 0.025 ± 0.003; Rq-Luc/V-arr3, 0.063 ± 0.007; $p<0.01$; $n = 3$).

Interestingly, Rq(R165L)-Luc-expressing cells yielded CNO-dependent BRET signals that were qualitatively similar to those observed with Rq-Luc (Fig. 1C). However, compared to Rq-Luc (see above), the interaction of Rq(R165L)-Luc with V-arr3 was characterized by a ~30% reduction in $BRET_{max}$ (0.045 ± 0.007; $n = 3$; $p<0.05$, as compared to Rq/V-arr3; Fig. 1C). The Rq(R165L)-Luc/V-arr2 combination yielded a $BRET_{max}$ value (0.026 ± 0.005; $n = 3$) that was similar to the one observed with the Rq-Luc/V-arr2 pair (Fig. 1C). CNO EC_{50} values are given in the legend to Fig. 1C.

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Taken together, these data indicate that the Rq(R165L) receptor fails to activate G_q but retains the ability to recruit arrestin-2 and -3 in a CNO-dependent fashion. Thus, Rq(R165L) represent the first arrestin-biased DREADD.

Calcium Mobilization and ERK1/2 Phosphorylation Studies Carried Out With HEK293T Cells. Many studies have shown that GPCR-dependent phosphorylation of ERK1/2 in HEK293T cells is mediated, at least in part, by arrestin-3-mediated signaling (see, for example, Wei et al., 2003). In agreement with the data obtained with COS-7 cells, CNO treatment of Rq(R165L)-expressing HEK293T cells had no significant effect on $[Ca^{2+}]_i$ but resulted in robust, concentration-dependent increases in $[Ca^{2+}]_i$ in Rq-expressing HEK293T cells (Fig. 2A). The E_{max} value for this latter response amounted to ~65% of the E_{max} value observed with OXO-M-stimulated HEK293T cells expressing the wild-type M_3 receptor (Fig. 2B).

Since the Rq(R165L) receptor retained the ability to recruit arrestins in BRET assays, we next examined whether this receptor was able to stimulate ERK1/2 phosphorylation in HEK293T cells. Specifically, we transfected HEK293T cells with plasmid DNA coding for Rq or Rq(R165L), together with either arrestin-3 siRNA or negative control siRNA. Under these experimental conditions, the use of arrestin-3 siRNA reduced arrestin-3 protein expression by ~70% (Fig. 2D). After a 2 min incubation at 37 ^oC with increasing concentrations of CNO, cells were lysed and probed for the expression of total ERK1/2 and phosphorylated ERK1/2 (pERK1/2) via immunoblotting. As shown in Fig. 2C, E, CNO stimulated ERK1/2 phosphorylation in both Rq- and Rq(R165L)-expressing cells in a concentration-dependent fashion. Consistent with the outcome of the BRET recruitment assays, CNO treatment of Rq-expressing cells resulted in more robust signals at all CNO concentrations used (~2-fold increase in pERK1/2 accumulation at 1 and 100 μM CNO, as compared to Rq(R165L); $p<0.05$). Strikingly, following treatment of cells with arrestin-3 siRNA, CNO-induced ERK1/2 phosphorylation was greatly reduced in Rq-expressing cells and virtually abolished in Rq(R165L)-expressing cells (Fig. 2E). These data indicate that Rq(R165L) is not only able to recruit arrestins but can also initiate arrestin-dependent signaling in a CNO-dependent fashion.

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While we observed pronounced arrestin-3-dependent ERK1/2 phosphorylation after only 2 min of CNO treatment, a longer CNO incubation time (45 min) was required to obtain robust BRET signals in the arrestin recruitment assays (see above). The most likely explanation for this phenomenon is that different conformational states of arrestin and/or different arrestin signaling complexes are involved in generating the signals measured in the two different assays.

Second Messenger and Insulin Release Assays Performed With MIN6 Cells. To demonstrate the usefulness of the arrestin-biased Rq(R165L) designer receptor to address biologically relevant questions, we expressed the Rq(R165L) construct, as well as Rq (for control purposes), in MIN6 cells (transfection efficiency: ~10-20%, as judged by the use of a GFP reporter plasmid; data not shown). MIN6 cells are derived from mouse pancreatic β-cells and are widely used as an in vitro model system to study the regulation of insulin release and other β-cell functions (Ishihara et al., 1993).

Since increases in $[Ca^{2+}]_i$ or intracellular cAMP levels stimulate the release of insulin from β- and insulinoma cells (Ahrén, 2009), we first examined whether CNO-dependent activation of Rq(R165L) affected the levels of these second messengers. As expected, CNO treatment of Rq-expressing MIN6 cells resulted in concentration-dependent increases in $[Ca^{2+}]_i$ (Fig. 3A). In contrast, CNO had no significant effect on $[Ca^{2+}]_i$ in Rq(R165L)-expressing MIN6 cells (Fig. 3A). The E_{max} value for Rq-mediated increases in $[Ca^{2+}]_i$ amounted to ~15% of the E_{max} value observed after OXO-M stimulation of M_3 receptors endogenously expressed by MIN6 cells (Ruiz de Azua et al., 2010; Fig. 3B). The relatively low efficacy of the Rq receptor in MIN6 cells (as compared to COS-7 and HEK293T cells) is most likely a consequence of the relatively low transfection efficiency that we observed with MIN6 cells (see previous paragraph).

Moreover, incubation of Rq(R165L)-expressing MIN6 cells with CNO did not lead to any detectable changes in cAMP levels (Fig. 3C).

To study the potential role of arrestin signaling in regulating insulin secretion, we transfected MIN6 cells with plasmid DNA coding for Rq or Rq(R165L), together with either arrestin-2 or -3 siRNA or negative control siRNA. Interestingly, in both Rq- and Rq(R165L)-expressing cells treated with control siRNA, CNO stimulated insulin release in a concentration-dependent fashion

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(Fig. 4A, B). However, treatment of both Rq- and Rq(R165L)-expressing cells with arrestin-2 or -3 siRNA led to significant reductions in insulin secretion (Fig. 4A-C). Arrestin-2 or -3 knockdown reduced but did not completely prevent insulin secretion following CNO treatment of Rq(R165L)-expressing cells. One possible explanation for this observation is that treatment of MIN6 cells with arrestin-2/3 siRNA lowered but did not abolish arrestin-2/3 protein expression (~50-70% reduction in arrestin-2/3 expression; Fig. 4D). In agreement with our findings, several recent studies suggest that activation of arrestin-dependent signaling pathways can promote insulin release from pancreatic β-cells or insulinoma cells (Sonoda et al., 2008; Kong et al., 2010).

In conclusion, we developed a new CNO-sensitive designer GPCR (Rq(R165L)) that fails to activate G proteins but is able to recruit arrestins and promote arrestin-dependent signaling. Importantly, when expressed in a β-cell line, activation of Rq(R165L) resulted in arrestin-dependent insulin release, highlighting the potential usefulness of this newly developed, arrestin-biased DREADD to study the physiological roles of arrestin signaling pathways. It should be noted that arrestin-biased GPCRs have been described previously (Wei et al., 2003; Shenoy et al., 2006). However, the Rq(R165L) construct offers the great advantage that it can only be activated by administration of an exogenous ligand (CNO) that is otherwise pharmacologically inert. Studies with mice (or other experimental animals) expressing this arrestin-biased DREADD in a tissue-specific fashion should provide detailed novel information about the potential physiological relevance of arrestin-dependent signal pathways.

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Authorship Contributions

Participated in research design: Nakajima, Wess

Conducted experiments: Nakajima

Contributed new reagents or analytic tools: Nakajima

Performed data analysis: Nakajima

Wrote or contributed to the writing of the manuscript: Nakajima, Wess

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Footnote

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Figure Legends

Fig. 1. Biochemical studies carried out with transfected COS-7 cells. A, B, Calcium assays. COS-7 cells transiently expressing the Rq or Rq(R165L) receptors (A) or the wild-type rat M_3 muscarinic receptor (B) were incubated with increasing concentrations of CNO or OXO-M, respectively (Vector = pcDNA). Drug-induced changes in $[Ca^{2+}]_i$ were measured via FLIPR. Panel (A) shows data from a representative experiment (two additional experiments gave similar results). Panel (B) shows pooled data from three independent experiments (means ± SEM). All assays were carried out in duplicate. The CNO EC_{50} value at the Rq receptor was 28.2 ± 7.7 nM, and the OXO-M EC_{50} value at the wild-type M_3 receptor was 2.33 ± 0.31 nM, respectively (means ± SEM; n = 3). AU, arbitrary units. C, BRET (arrestin recruitment) studies. COS-cells co-expressing Rq-Luc or Rq(R165L)-Luc (BRET donor), Venus-arrestin-2 or -3 (V-Arr2 or V-Arr3; BRET acceptor), and GRK2 were incubated with increasing concentrations of CNO. BRET measurements were carried out as described under Materials and Methods. BRET signals are expressed as NetBRET ratios obtained by subtracting baseline BRET ratios obtained in the absence of BRET acceptor (V-arr2 or V-arr3) from BRET ratios measured in the presence of V-arr2 or V-arr3. The panel shows data from a representative experiment, carried out in duplicate. Two additional experiments gave similar results. CNO $BRET_{50}$ values (in μM) were as follows (means ± SEM, n =3): Rq-Luc + V-Arr2, 0.15 ± 0.03; Rq-Luc + V-Arr3, 1.45 ± 1.05; Rq(R165L)-Luc + V-Arr2, 0.37 ± 0.07; Rq(R165L)-Luc + V-Arr3, 1.24 ± 0.45.

Fig. 2. Signaling studies performed with transfected HEK293T cells. A, B, Calcium assays. HEK293T cells transiently expressing the Rq or Rq(R165L) receptors (A) or the wild-type rat M_3 muscarinic receptor (B) were incubated with increasing concentrations of CNO or OXO-M, respectively (Vector = pcDNA). Drug-induced changes in $[Ca^{2+}]_i$ were measured by via FLIPR. Data are presented as means ± SEM of four independent experiments, each carried out in duplicate. The CNO EC_{50} value at the Rq receptor was 0.25 ± 0.16 μM, and the OXO-M EC_{50} value at the wild-type M_3 receptor was 5.22 ± 1.48 nM, respectively. AU, arbitrary units. C-E, ERK

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phosphorylation assay. HEK293T cells were transfected with expression constructs coding for Rq or Rq(R165L), together with either arr3 siRNA or negative control siRNA (CTL). Cells were stimulated with increasing concentrations of CNO for 2 min at 37 ^{o}C. Subsequently, cell lysates were probed for the expression of total ERK1/2 and phosphorylated ERK1/2 (pERK1/2) via immunoblotting. Panel (C) shows representative Western blots. ND, no drug. Panel (D) shows representative immunoblotting data indicating that treatment of HEK293T cells with arr3 siRNA led to a strong reduction in arr3 protein expression (note that the arrestin antibody used recognized both arr2 and arr3). Panel (E) provides a summary of pERK1/2 protein levels (pooled data). In each individual experiment, band intensities in the absence of CNO were set equal to 1 (ND, no drug). Each bar represents the mean ± SEM from three or four independent experiments. *$p<0.05$, **$p<0.01$, and ***$p<0.001$ vs. the corresponding control response.

Fig. 3. Second messenger studies carried out with MIN6 cells. A, B, Calcium assays. MIN6 cells transfected with the Rq or Rq(R165L) constructs (A) or vector DNA (pcDNA) (B) were incubated with increasing concentrations of CNO or OXO-M, respectively. Drug-induced changes in $[Ca^{2+}]_i$ were measured by via FLIPR. Panel (A) shows data from a representative experiment (two additional experiments gave similar results). Panel (B) shows pooled data from three independent experiments. All assays were carried out in duplicate. The CNO EC_{50} value at the Rq receptor was 28.2 ± 7.7 nM, and the OXO-M EC_{50} value at the wild-type M_3 receptor was 1.00 ± 0.12 μM, respectively (means ± SEM; n = 3). AU, arbitrary units. B, cAMP assay. MIN6 cells transiently expressing the Rq(R165L) construct were incubated with increasing concentrations of forskolin or CNO. cAMP concentrations were measured in cell lysates by using a FRET-based cAMP kit (for details, see Materials and Methods). Data are given as means ± SEM of three independent experiments, each carried out in duplicate.

Fig. 4. Insulin release studies. MIN6 cells were transfected with expression plasmids coding for either Rq (A) or Rq(R165L) (B), together with either arrestin-2 or -3 siRNA (arr2 or arr3 siRNA) or negative control siRNA (CTL). Representative concentration-response curves are shown. Three independent experiments gave similar results. C, Pooled insulin release data obtained in three

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independent experiments. The panels shows relative insulin responses at two different CNO concentrations (CTL siRNA = 100%). *p<0.05, **p<0.01, vs. the corresponding control response. D, Arrestin levels were monitored via Western blotting using an anti-arrestin antibody that recognized both arr2 and arr3. Cells were incubated with increasing concentrations of CNO for 60 min at 37 ^{o}C in the presence of 16.7 mM glucose, and insulin secretin into the medium was determined as described under Materials and Methods. CNO EC_{50} (in μM) and E_{max} values (in ng insulin/ml) were as follows (means ± SEM, n = 3): Rq + CTL siRNA: EC_{50} = 0.55 ± 0.33, E_{max} = 43.5 ± 3.5; Rq + arr2 siRNA: EC_{50} = 4.60 ± 0.96, E_{max} = 28.1 ± 1.5; Rq + arr3 siRNA: EC_{50} = 2.98 ± 0.84, E_{max} = 39.9 ± 2.1; Rq(R165L) + CTL siRNA: EC_{50} = 2.14 ± 0.40, E_{max} = 40.3 ± 3.82; Rq(R165L) + arr2 siRNA: EC_{50} = 1.16 ± 0.88, E_{max} = 27.2 ± 0.80; Rq(R165L) + arr3 siRNA: EC_{50} = 1.33 ± 0.32, E_{max} = 26.9 ± 2.93.

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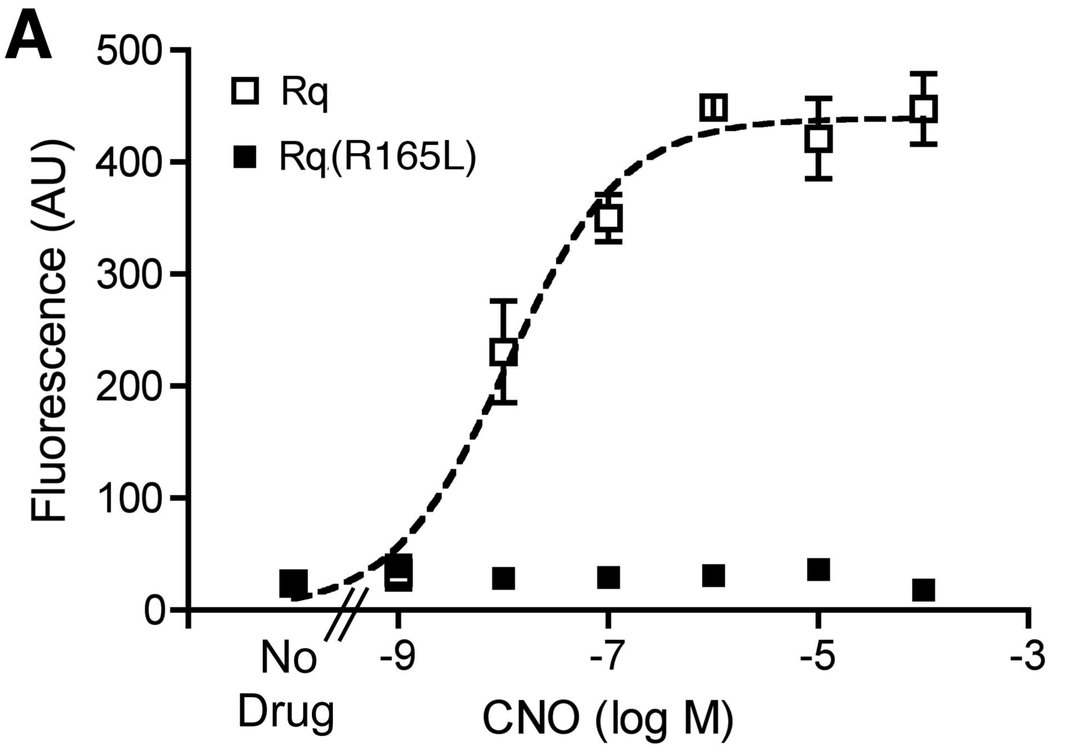
TABLE 1

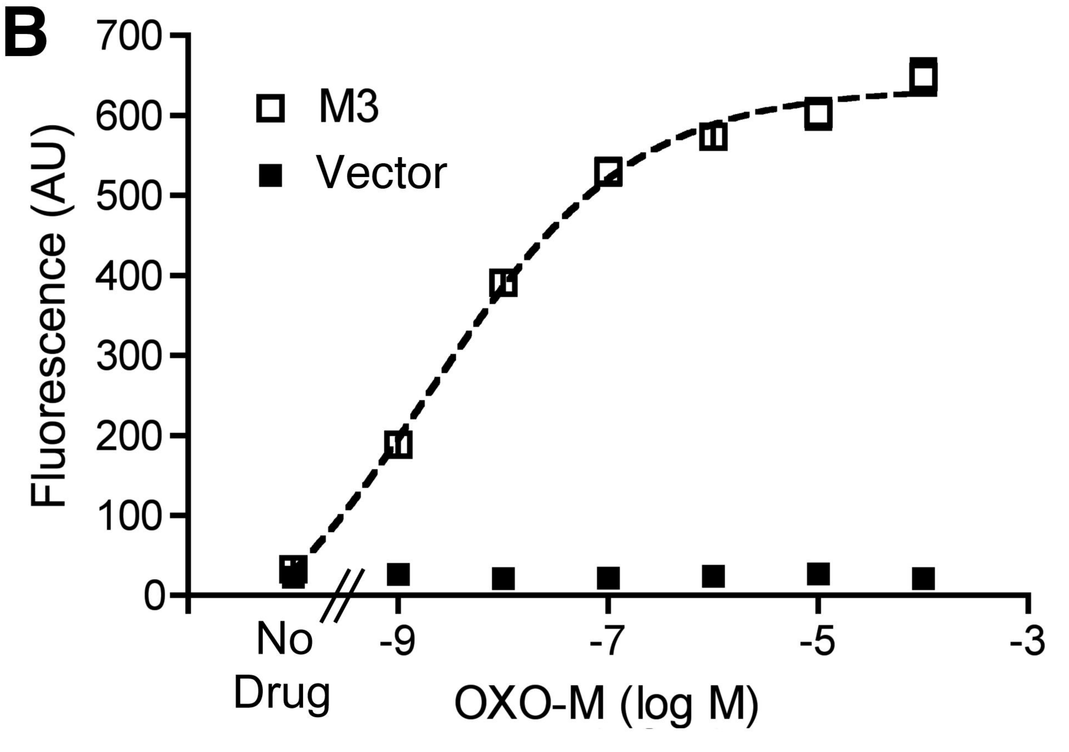
Comparison of the ligand binding properties of the Rq and Rq(R165L) constructs

The indicated receptors were transiently expressed in COS-7 cells. [^{3}H]-NMS saturation and [^{3}H]-NMS/CNO or [^{3}H]-NMS/ACh competition binding experiments were carried out using membrane homogenates prepared from transfected COS-7 cells, as described under Materials and Methods. Data are presented as means ± SEM of at least three independent experiments, each performed in duplicate.

Receptor	[^{3}H]-NMS binding		CNO binding	ACh binding
	K_D (nM)	B_{max} (pmol/mg)	K_i (μM)	K_i (μM)
Rq	16.2 ± 3.0	16.7 ± 1.1	1.36 ± [illegible]	[illegible][a]
Rq(R165L)	14.4 ± [illegible]	12.7 ± [illegible] ± 0.14	[illegible]	[illegible][a]

[a]ND, no detectable inhibition of [^{3}H]NMS binding (20 nM) at ACh concentrations up to 0.1 mM.





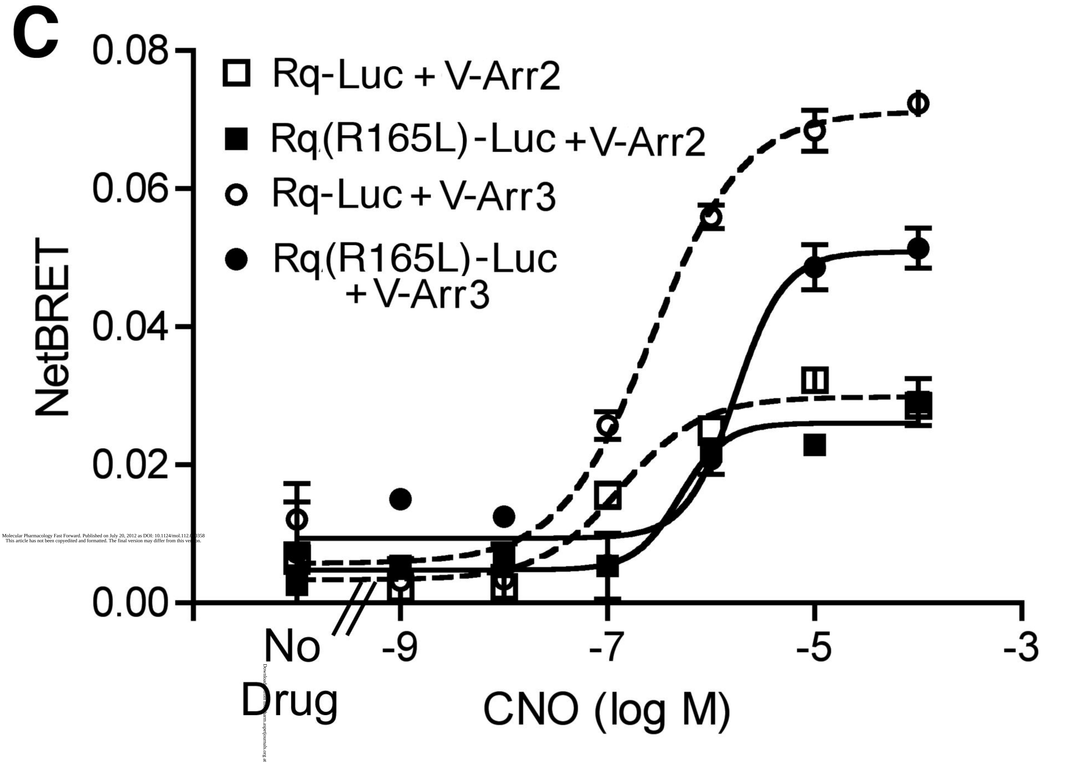
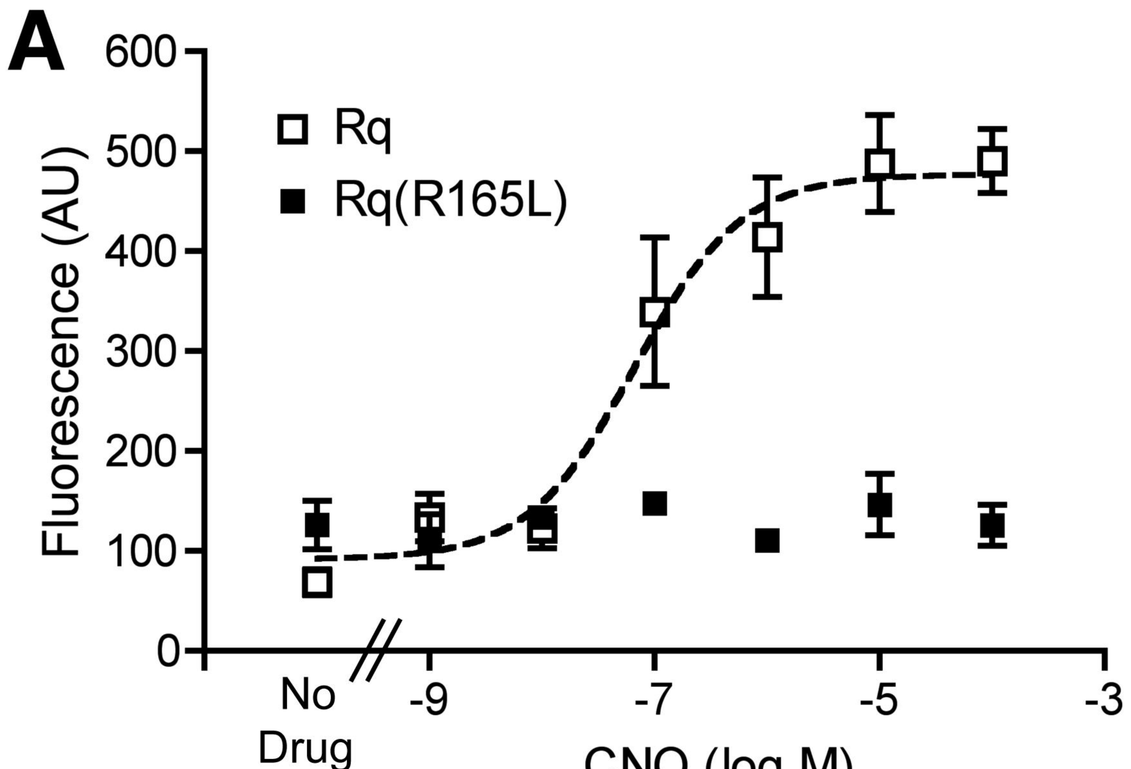
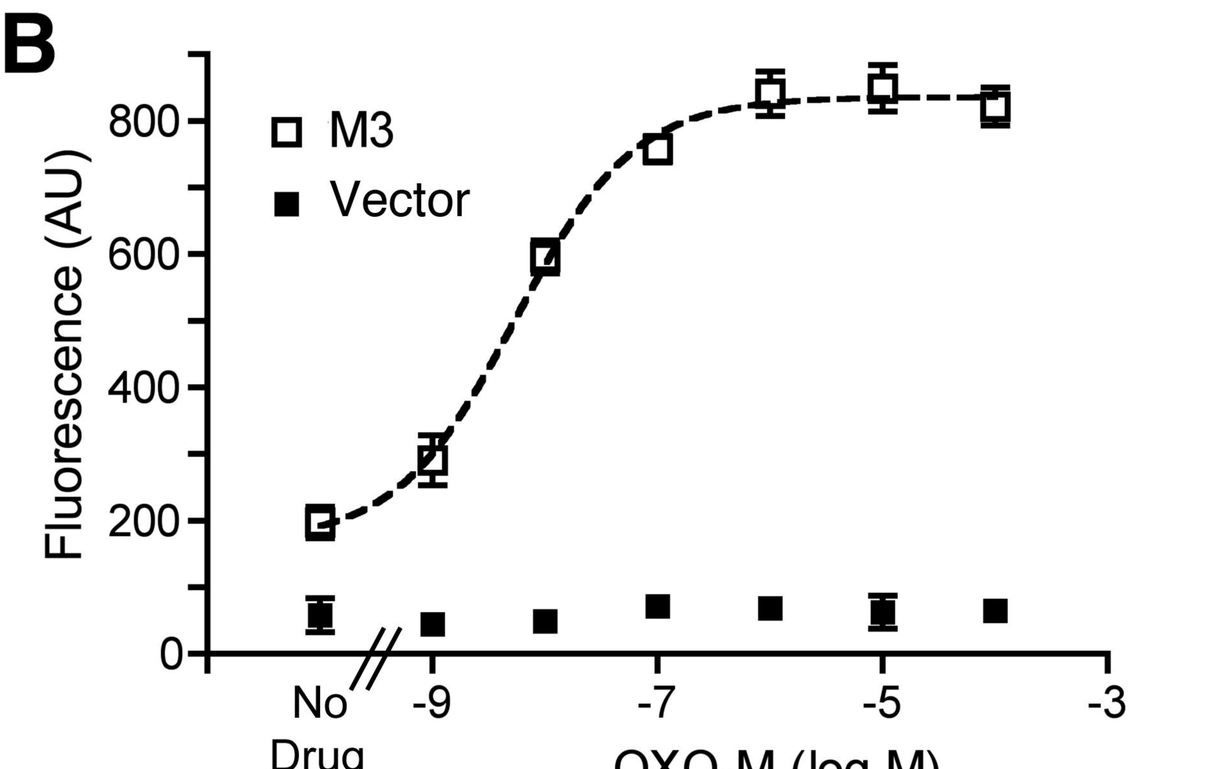
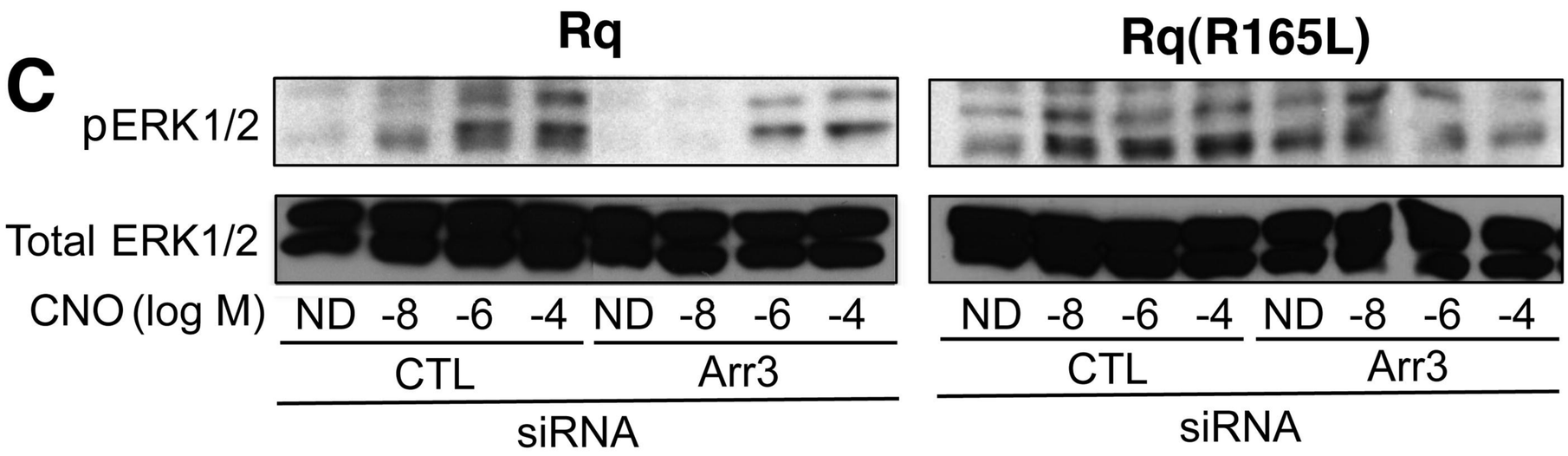
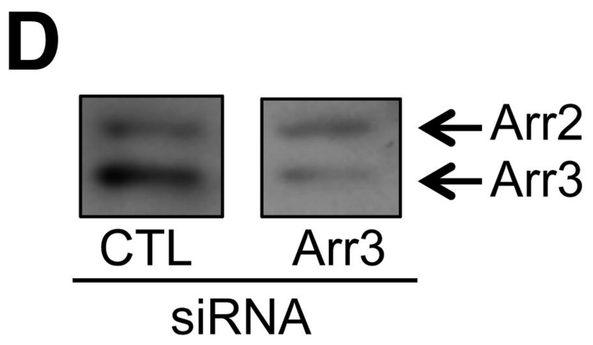


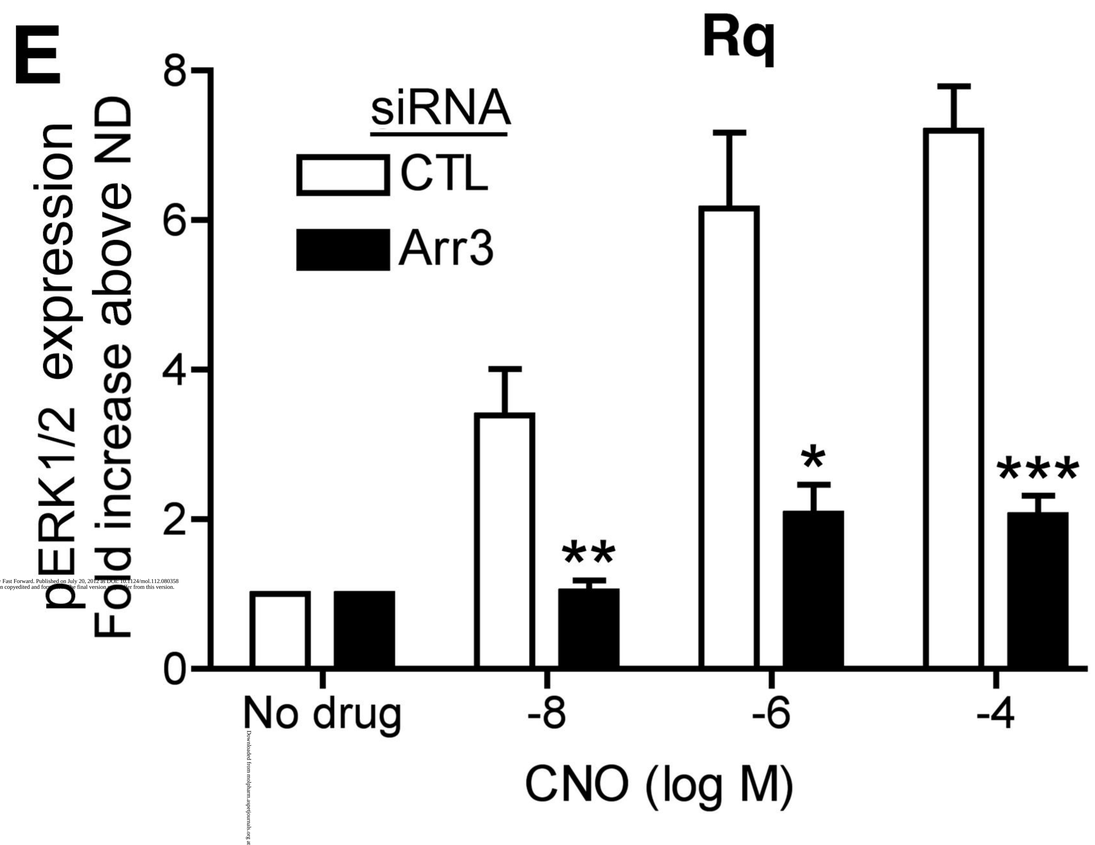
Figure 1











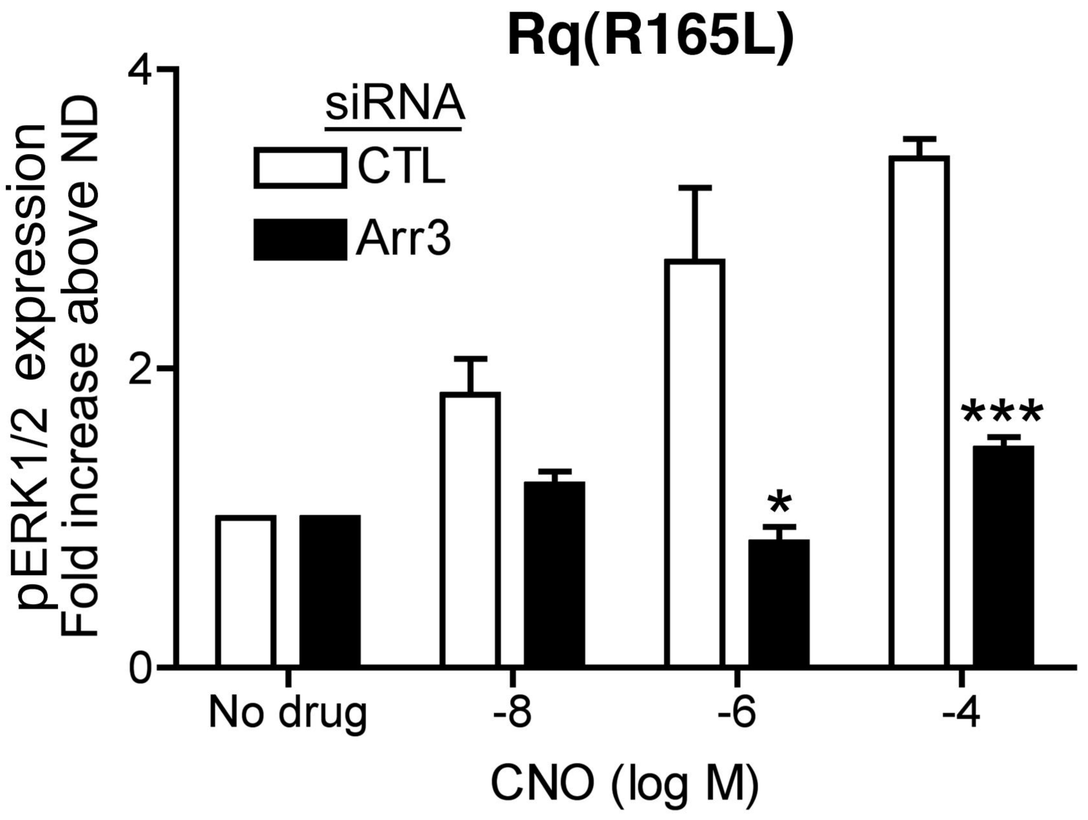
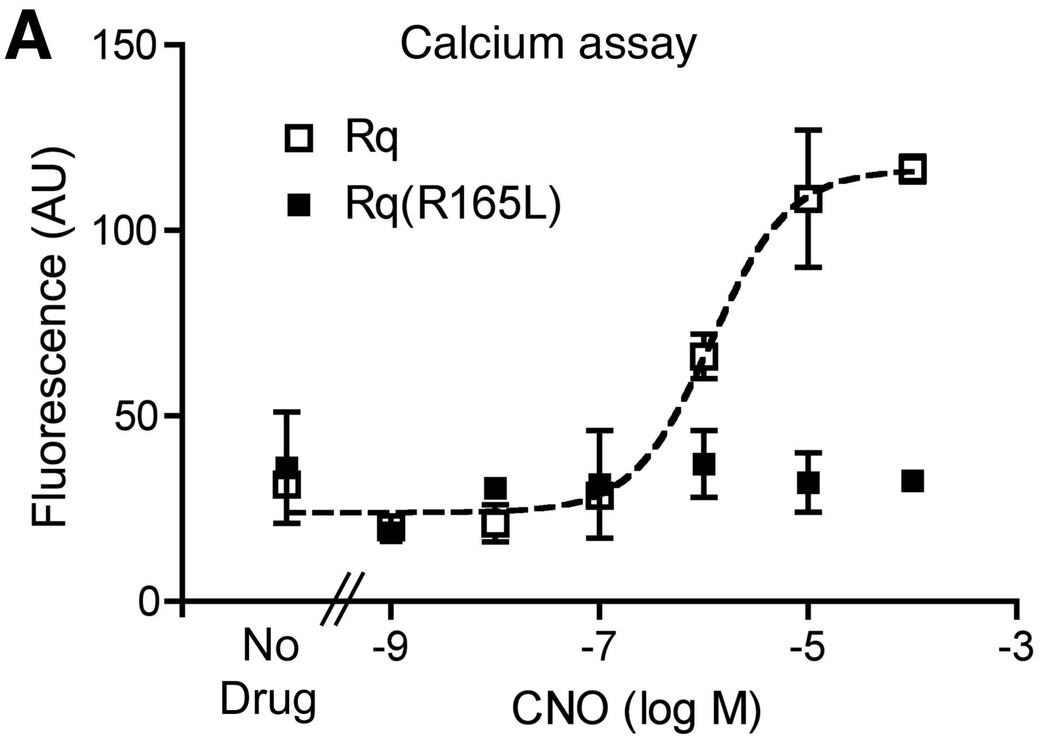
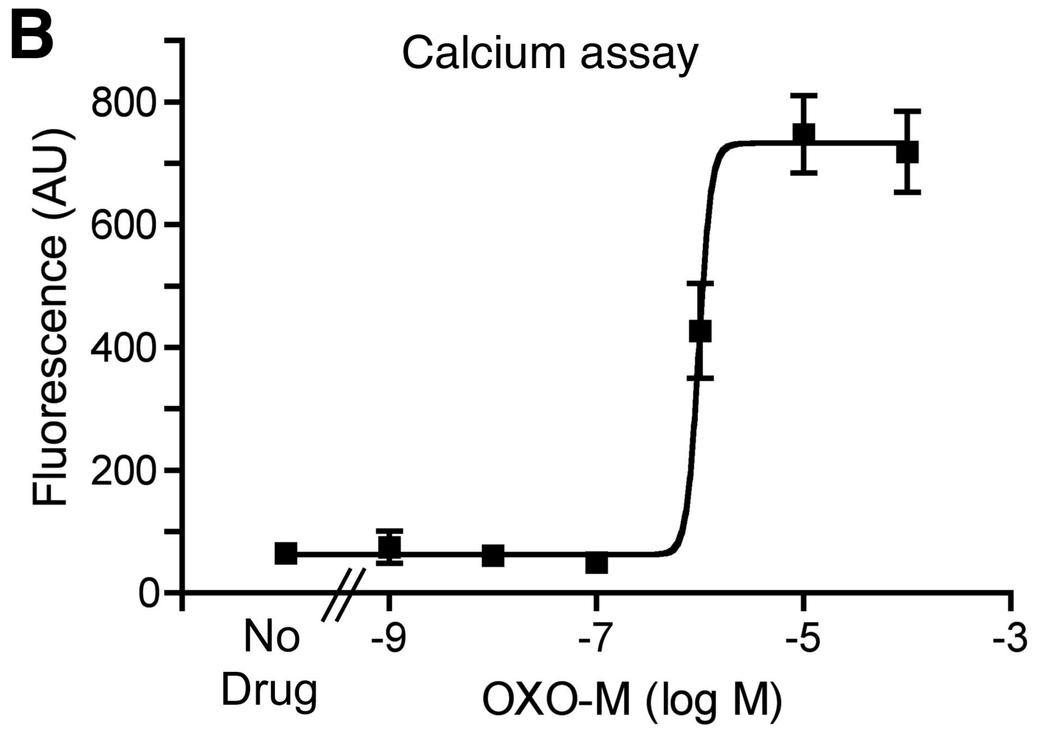


Figure 2





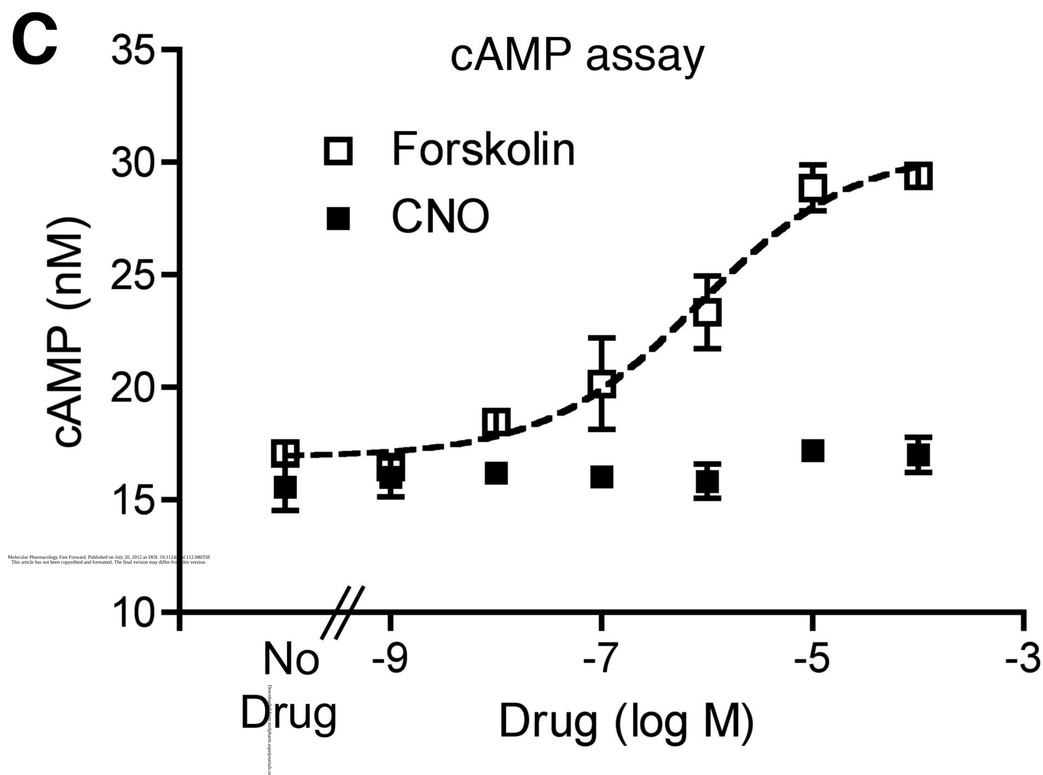
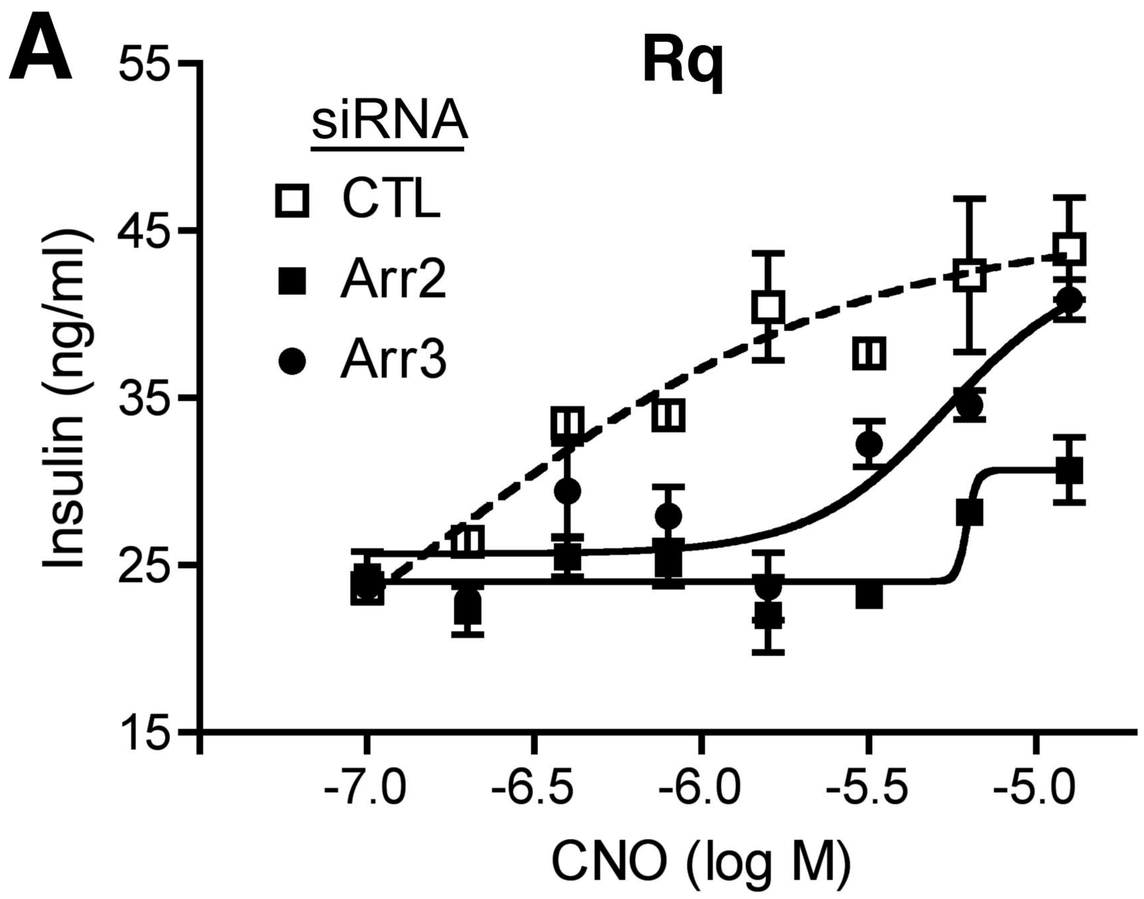
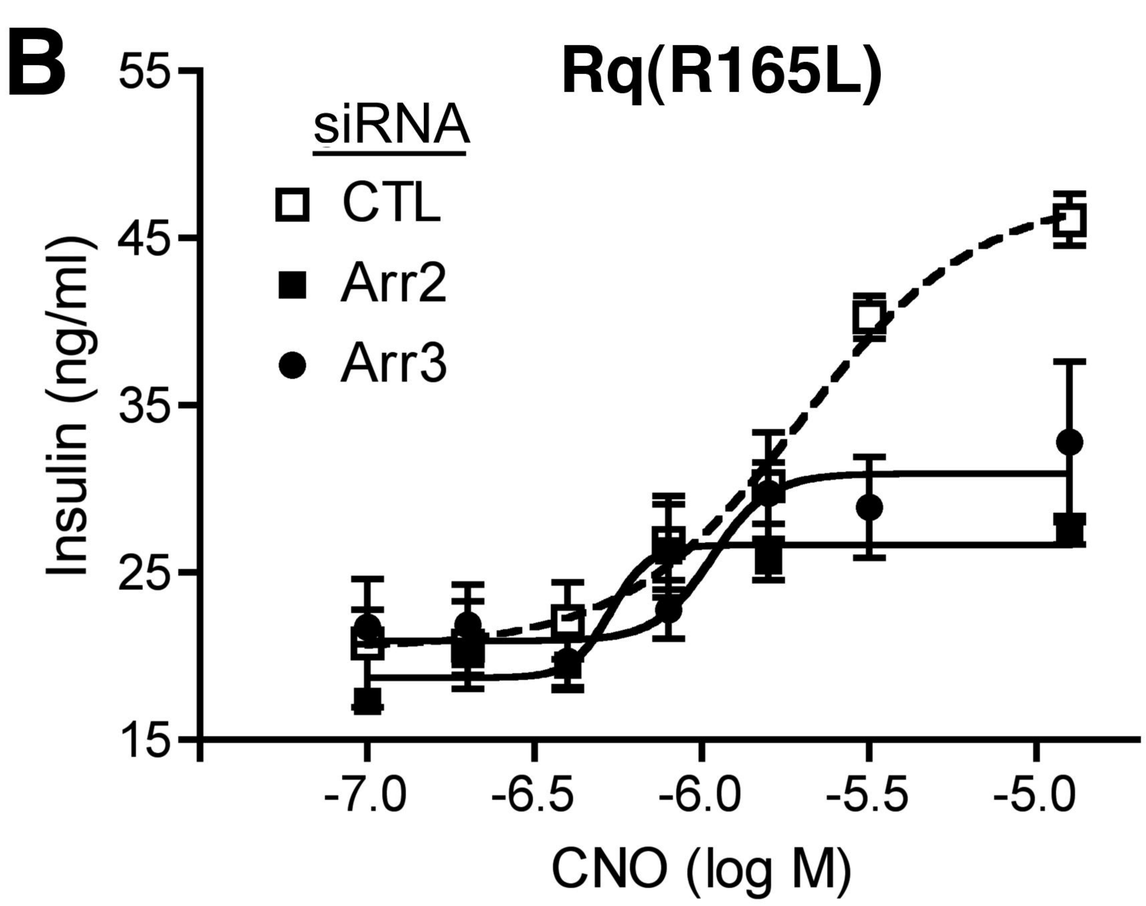
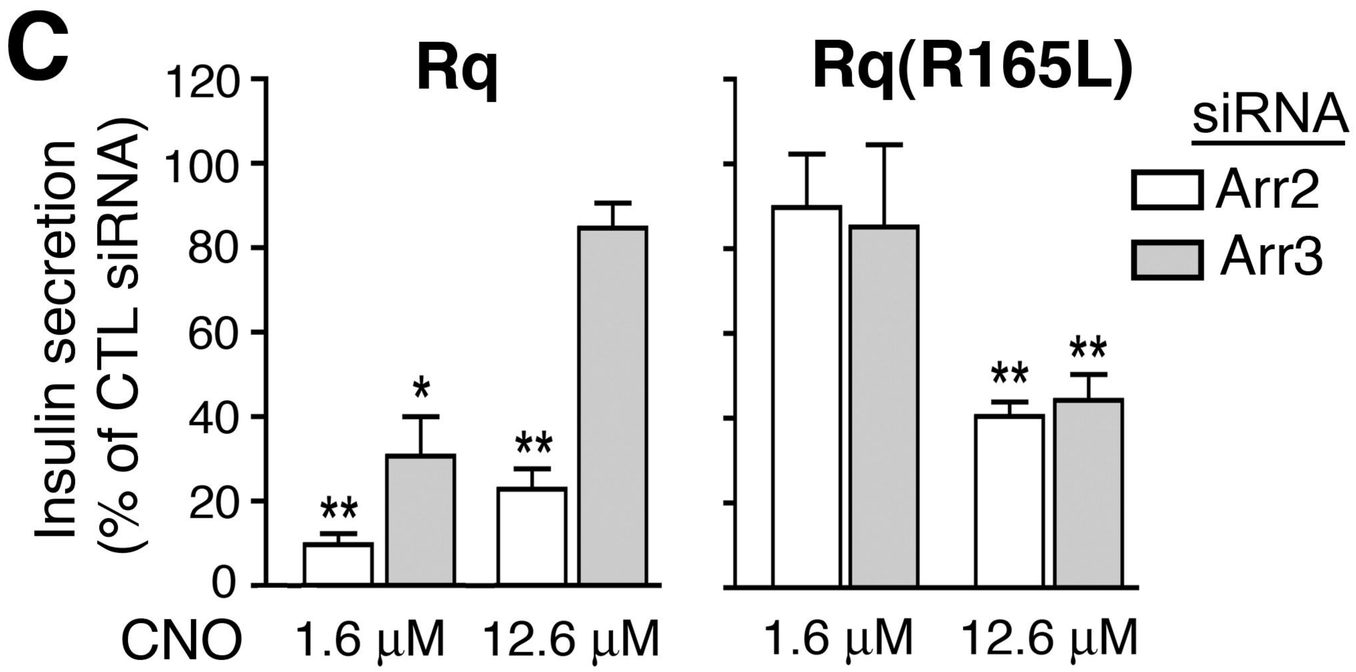


Figure 3







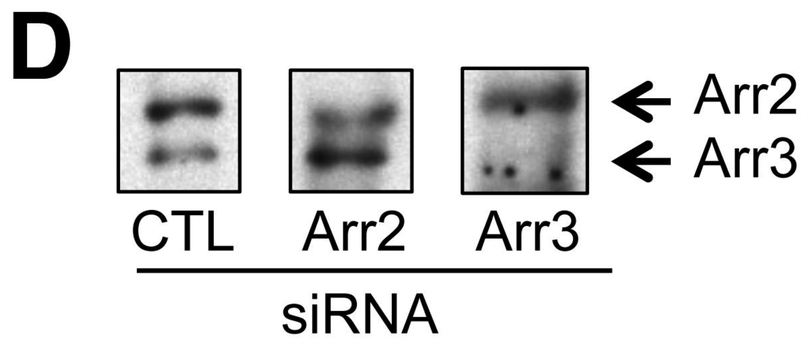


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Figure 4