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Dopamine Receptor D4 Internalization Requires a Beta-Arrestin and a Visual Arrestin

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Abstract

Purpose—The G-protein coupled receptor (GPCR) Dopamine Receptor D4 (DRD4) plays an essential role in cAMP regulation and gap junctional coupling in the photoreceptors, where DRD4 expression is under circadian control. Previous *in vitro* transfection studies of human DRD4 desensitization have reported that DRD4 is not internalized upon dopamine stimulation when beta-arrestin is co-transfected with DRD4. We hypothesized that visual arrestins, ARR1 and ARR4, play a modulatory role in DRD4 desensitization in the photoreceptors.

Methods—To test this hypothesis, immunohistochemistry analysis of mouse retinas was used to determine the cellular localization of beta-arrestins and DRD4 in photoreceptors. *In vitro* studies were performed in HEK293T cells transiently transfected with human DRD4 and arrestins. First, co-immunoprecipitation experiments were executed to test protein-protein interactions and to investigate the effect of dopamine stimulation. Second, immunohistochemistry analysis was implemented to study DRD4 internalization and translocation of ARR4.

Results—Immunohistochemistry studies of mouse retinas confirmed the expression of beta-arrestin 2, ARR1 and ARR4, as well as DRD4 in mouse cone photoreceptor inner segments.

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Co-immunoprecipitation experiments revealed a dopamine-dependent protein-protein interaction between human DRD4 and ARR4. *In vitro* internalization experiments showed that no detectable internalization of DRD4 was observed with any single arrestin co-transfected. However, a dopamine-dependent internalization of DRD4 was observed with three out of six sets of two arrestins co-transfected with DRD4. Each of these pairs of arrestins contained one visual arrestin and one beta-arrestin, and no internalization was observed with either two visual arrestins or two beta-arrestins. Additional time-course experiments revealed that *in vitro*, ARR4 translocates to co-localize with DRD4 at the plasma membrane in response to 30 minutes of dopamine stimulation.

Conclusions—The results have functional implications and we hypothesize that the desensitization and internalization in photoreceptors are synergistically mediated by both visual and beta-arrestins. These results are additionally unique because they demonstrate for the first time that at least one G-protein coupled receptor, DRD4, requires two arrestins for desensitization and internalization, and opens up the possibility that other G-protein coupled receptors may require more than one arrestin for desensitization and/or internalization.

Keywords

Dopamine receptor D4; Visual Arrestin; Beta Arrestin; Cone Arrestin; Photoreceptor; Retina

1. Introduction

Dopamine is a hormone and neurotransmitter that has important functions in many aspects of neuronal signaling, including vision. Dopamine acts as a ligand for five different G protein-coupled receptors (GPCRs), named Dopamine Receptor (DR) D1 through D5 (O'Dowd, 1993; Gingrich and Caron, 1993; Seeman and Van Tol, 1994). The dopamine receptors are classified into two groups that have opposite downstream effects in the cells in which they are expressed. The D1-like subgroup consists of DRD1 and DRD5, which activate adenylate cyclase (AC), thereby increasing the concentration of cyclic AMP (cAMP) in the cell. The D2-like subgroup, including DRD2, DRD3, and DRD4, acts to inhibit AC, effectively decreasing cAMP concentration in the cell.

One of the dopamine receptors with high retinal expression in rodents and humans is DRD4 (Cohen et al., 1992; Matsumoto et al., 1995; Suzuki et al., 1995; Bai et al., 2008; Klitten et al., 2008; Kim et al., 2010). Retinal and pineal gland DRD4 mRNA expression oscillates daily in a circadian pattern, with the highest expression during subjective night and lowest expression during subjective day (Humphries et al., 2002; Fukuhara and Tosini, 2008; Bai et al., 2008; Klitten et al., 2008; Kim et al., 2010). Its physiological action in photoreceptors is also circadian, although it counter-intuitively contributes to daytime phenotypes instead of nighttime. DRD4 contributes to the decrease in cAMP during the day (Jackson et al., 2011) and, in combination with the adenosine 2A receptor (A2AR), decreases in gap junctional coupling during the day (Li et al., 2009; Li et al., 2013). It is hypothesized that the action of DRD4 in the photoreceptors is inhibited in the nighttime by G_s-coupled GPCRs, such as A2AR, but that this inhibition is over ruled in the daytime by the increase in retinal DA, in spite of the lower DRD4 expression during the day. There is no reported evidence of circadian regulation of DRD4 expression in other cell types (Kim et al., 2010), so we

hypothesize that there is a unique mechanism for DRD4 desensitization and sequestration in the photoreceptors and pinealocytes.

Because the dopamine receptors are GPCRs, they undergo the classical GPCR activation and desensitization mechanisms. In this study, we focused on the desensitization mechanism, in which ligand binding leads to G-protein coupled receptor kinase (GRK) phosphorylation of serine and threonine residues of the intracellular loops or carboxy-terminal tail of the receptor, followed by arrestin binding. There are two β-arrestins, β-ARR1 and β-ARR2 (Lohse et al., 1990; Attramadal et al., 1992). The binding of an arrestin often, but not always, leads to recruitment of endocytic machinery and internalization of the receptor for degradation or recycling (reviewed in (Lefkowitz and Shenoy, 2005)). The desensitization of the D2-like receptors has been studied, and DRD2 and DRD3 are internalized after dopamine stimulation, as long as a β-arrestin is present (Kim et al., 2001; Namkung and Sibley, 2004). The most recent research on DRD4 showed that it is able to bind β-arrestin, but it is not internalized (Spooren et al., 2010).

The previous work on DRD4 desensitization has investigated the role of the β-arrestins because of their ubiquitous expression and interaction with many different GPCRs (Spooren et al., 2010; Cho et al., 2010). However, there are two other arrestin proteins expressed in mammalian tissues. These are the visual arrestins, ARR1 (also called S-antigen or 48kDa protein) (Wacker et al., 1977; Kuhn et al., 1984; Pfister et al., 1985; Shinohara et al., 1987) and ARR4 (also called cone arrestin, X-arrestin, or *ARR3* in the NCBI gene nomenclature) (Murakami et al., 1993; Craft et al., 1994). The visual arrestins have a distinct expression pattern. In rodents, ARR1 is highly expressed in the photoreceptor rods and cones, and in pinealocytes (Craft et al., 1990). ARR4 is not present in rods and is highly expressed in cone photoreceptors and pinealocytes. Two studies have verified the presence of the β-arrestins in the retinal photoreceptors, as well (Nicolas-Leveque et al., 1999; Cameron and Robinson, 2014). Although DRD4 is highly expressed in photoreceptors (Cohen et al., 1992; Klitten et al., 2008; Li et al., 2013), ARR1 and ARR4 were not included in the previous *in vitro* studies of DRD4 desensitization.

In this study, we sought to determine whether either of the two visual arrestins, ARR1 or ARR4, may play a role in DRD4 desensitization *in vitro*.

2. Materials and Methods

2.1 HEK293T cell culture and transfection

HEK293T/17 (HEK293T) cells were purchased from ATCC (Manassas, VA). They were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C, 5% CO_2 and used for experiments below 15 passages. HEK293T were transiently transfected with FuGENE 6 transfection reagent (Promega) using a 3 μg DNA: 1 μL Fugene ratio for 48 hrs before they were harvested for co-immunoprecipitation (co-IP) or fixed and stained for immunohistochemistry (IHC). For experiments with two plasmids co-transfected, they were added in a 1:1 receptor:arrestin ratio. For experiments with three plasmids co-transfected, the amount of each arrestin plasmid was decreased by half, so that the final ratio of receptor:arrestin:arrestin was 2:1:1.

2.2 Mammalian expression plasmids

The DRD4-encoding plasmids used were pcDNA3-HA-DRD4.4, and pFLAG-DRD4.4 (Oak et al., 2001; Van Craenenbroeck et al., 2005). They each express a common variant of human DRD4, along with an human influenza hemagglutinin-derived peptide (HA-) or FLAG-tag for labeling and verification. HA-tagged rat β-arrestin 1 and β-arrestin 2 (pcDNA3-barr1-HA [#14693], pcDNA3-barr2-HA [#14692]), and FLAG-tagged rat β-adrenergic receptor 2 (pcDNA3-FLAG-B2AR [#14697]) expression plasmids (Tang et al., 1999; Luttrell et al., 1999) were obtained from AddGene (Cambridge, MA). Human Arrestin 1 and Arrestin 4 cDNA plasmids were also used (pCI-hSAG, p-noEGFP-hCAR [derived from pEGFP-hCAR]) (Li et al., 2002; Li et al., 2003).

2.3 Mice

All animals were treated and protocols were approved by USC IACUC according to the guidelines established by the Institute for Laboratory Animal Research. Breeders for C57BL/6J and *Drd4*$^{-/-}$ (strain B6.129P2-*Drd4*^{tm1Dkg}/J) (Rubinstein et al., 1997) mice were obtained from Jackson Laboratory (Bar Harbor, ME). They were bred and their offspring were reared in 12 hr light/12 hr dark cycling light conditions until sacrifice. Mice were sacrificed in the dark at circadian time (CT) 0, before the lights were turned on. Other mouse strains used for immunohistochemistry (IHC) and immunoblot analysis include *Arr1*$^{-/-}$ (Xu et al., 1997) (generously provided by Dr. Jeannie Chen, University of Southern California), *Arr4*$^{-/-}$ (created and described in detail in (Nikonov et al., 2008) supplement), *Arr*-DKO (*Arr1*$^{-/-}$*Arr4*$^{-/-}$) (Nikonov et al., 2008), β-*arr1*$^{-/-}$ (also called *Arrb1*$^{-/-}$or *Arr2*$^{-/-}$) (Conner et al., 1997) and β-*arr2*$^{-/-}$ (also called β*arr2-KO* or *Arr3*$^{-/-}$) (Bohn et al., 1999) (eyes generously provided by Drs. Eugenia Gurevich, Vanderbilt University and Robert Lefkowitz, Duke University). Eyes were enucleated and eyecups were processed for IHC or retinas were dissected from eyes and stored at −80°C for immunoblot analysis.

2.4 Co-Immunoprecipitation (co-IP) and immunoblot analysis

For co-IP, transfected HEK293T (see section 2.1) were incubated for 30 min in DMEM with 10% FBS (regular medium), either with or without 10 μM DA. Cells were placed on ice and harvested by aggressive pipetting with ice-cold phosphate buffered saline solution (PBS), then centrifuged at 4°C and the supernatant was aspirated. Cell pellets were frozen at −80°C until ready to use. Cell pellets were resuspended and lysed in radio-immunoprecipitation assay (RIPA) buffer (Rondou et al., 2008) containing cOmplete protease inhibitor cocktail (Promega) and the phosphatase inhibitor β-glycerophosphate. The RIPA buffer with inhibitors was also used for all washes. 0.5 μL rabbit polyclonal anti-hARR4 antibody Luminaire founder-human Cone Arrestin (LUMIf-hCAR) (Zhang et al., 2003) was added to each tube of cleared lysate and tubes were rotated at 4°C for 3 hrs. Then, 20 μL Protein A magnetic beads (Life Technologies) were added to each sample and incubated at 4°C overnight while rotating. The beads were washed with ice-cold RIPA buffer three times before eluting bound proteins by incubating at 37°C (n=2). The opposite co-IP, using anti-HA antibody, was also performed (n=1).

Total cell lysate and co-IP eluate were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride

(PVDF) membranes (Millipore). Anti-HA (1:1000 dilution, Cell Signaling) and LUMIf-hCAR (1:20,000 dilution) primary antibodies were used in conjunction with horse radish peroxidase (HRP)-conjugated secondary antibodies (1:10,000 dilution, Bio-Rad). Hi-Blot Chemiluminescence kit (Denville) was used for detection with film.

2.5 Immunohistochemistry

Mouse retinas—Mouse eyes were enucleated and immediately fixed using published methods (Zhu et al., 2002). They were fixed in 4% paraformaldehyde (PFA) for 10 min (for anti-DRD4 antibody N-20) or 60 min (for the anti-arrestin antibodies), washed with PBS, and incubated in 30% sucrose overnight. Eyes were embedded in optimal cutting temperature (OCT) medium, frozen, and cut into 20 μm sections (for N-20) or 10 μm sections (for the anti-arrestin antibodies). Retina sections were blocked with normal donkey serum in PBS (N-20) or Chemiblocker (Millipore) (anti-arrestin antibodies), and antibodies were diluted in PBS. Retinas were incubated with an anti-DRD4 primary antibody (N-20 anti-DRD4, Santa Cruz #31480 at 1:100 dilution), anti-β-ARR1 and β-ARR2 (A2CT at 1:500 dilution) (Wei et al., 2003), anti-mARR4 (LUMIj-mCAR at 1:2,000 dilution) (Zhu et al., 2002), anti-SAG/ARR1 (monoclonal D9F2 at 1:20,000 dilution) (Donoso et al., 1990), or anti-β-ARR2 (monoclonal H-9 Santa Cruz #13140 at 1:500 dilution). The corresponding secondary antibodies were anti-goat AlexaFluor 488, anti-mouse AlexaFluor 488, anti-rabbit AlexaFluor 488, anti-rabbit AlexaFluor 568 or anti-mouse AlexaFluor 568 (Life technologies). Slides were mounted with Vectashield hard-set mounting medium with 4’,6-diamidino-2-phenylindole (DAPI) (Vector Labs), and visualized using confocal fluorescent microscopy (Zeiss LSM 510 or LSM 710).

Image analysis was performed using Zen 2012 software (Carl Zeiss Microimaging, Thernwood, NY). The intensity profile was generated by the Zen profile module for DRD4 signal and ARR4 signal.

HEK293T—Cells were seeded onto glass slides in multi-well plates and given 24 hrs to adhere. For N-20 anti-DRD4 labeling in HEK293T, 48 hrs after transfection (Section 2.1), cells were rinsed with PBS and fixed using 4% PFA in PBS. Cells were then blocked using Blotto (3% Milk, 1 mM $CaCl_2$ in tris-buffered saline solution (TBS)), or permeabilized and blocked using Blotto-T (Blotto plus 0.01% Triton-X100). FLAG-DRD4.4 was labeled with M1 anti-FLAG antibody (Sigma) and N-20 anti-DRD4 antibody (Santa Cruz Biotechnologies) together in PBS. Secondary antibodies used were anti-mouse AlexaFluor 568 and anti-goat AlexaFluor 488, respectively (Life Technologies).

For internalization experiments, 48 hrs after transfection (Section 2.1), media was removed and cells were incubated with anti-FLAG primary antibody (Sigma) at 4°C for 1 hr. DMEM with 10% FBS, with or without 10 μM DA (or 10 μM isoproterenol for B2AR), was added to cells and they were incubated for the appropriate time (15 min to 60 min) at 37°C, 5% CO_2, then immediately fixed in 4% PFA in PBS. After fixation, cells were permeabilized with Blotto-T.

Preliminary experiments indicated that 30 min of DA stimulation was optimal for internalization of DRD4, so this time point was used for all internalization experiments.

For hARR4 translocation experiments, cells were also labeled with anti-hARR4 antibody, LUMIf-hCAR (Zhang et al., 2003), and multiple DA stimulation times (15 min to 60 min) were used to demonstrate and detect the cellular translocation of ARR4 over time. After rinsing with PBS, cells were labeled with fluorescent secondary antibodies (anti-mouse AlexaFluor 488 and, for hARR4 translocation, anti-rabbit AlexaFluor 568, each at 1:500 dilution), mounted with Vectashield hard-set mounting medium with DAPI (Vector Labs), and visualized using confocal fluorescent microscopy (Zeiss LSM 510 or LSM 710).

2.6 Quantification and statistical analysis

Image analysis of co-IP results was performed using Image Studio Software (v. 3.1.4; LI-COR Biosciences, Lincoln, NE). Uniformly-sized rectangles were drawn around each band, with background measurements of 3 pixels on all sides of each rectangle. The mean intensity of the image within each rectangle was measured, and the mean intensity of the background measurements was automatically subtracted from this measurement by Image Studio. The background-subtracted intensities were compared to the control, which was the sample that included DRD4, ARR4, but no DA stimulation (set as 1). The ratio of each band's intensity to the control (relative intensity) was plotted on the charts in Figure 4.

Quantification of internalization experiments was done by counting the total number of DRD4-positive cells in each image and the number of cells with clearly internalized receptors. Counts from each of four experiments were averaged and the standard deviation was calculated. The baseline was calculated by taking the average percent of cells with internalized receptors of all of the unstimulated samples. Results were reported relative to the baseline, and column statistics were performed using GraphPad Prism (La Jolla, CA) to determine whether each stimulated sample is significantly different from the baseline ($p<0.05$).

Quantification of colocalization experiments was performed using a similar method. The total number of cells in each image expressing both DRD4 and ARR4 was counted, and the number of cells displaying overlap of the two signals was also counted. The percentage of cells displaying overlap was calculated for each image (n=3 to 9 for each condition). The baseline was calculated by taking the average percent of cells with DRD4 and ARR4 overlap in all of the unstimulated (no DA) samples. Results were reported and column statistics were performed as for the internalization analysis above.

3. Results

3.1 β-arrestins and visual arrestins are co-expressed in mouse retinas

It is well-documented that visual arrestins, ARR1 and ARR4, are present at high concentrations in mammalian photoreceptors (Pfister et al., 1985; Broekhuyse et al., 1985; Whelan and McGinnis, 1988; Craft et al., 1990; Craft et al., 1994; Zhu et al., 2002; Nikonov et al., 2008). The presence and expression level of β-arrestins in the photoreceptors has been studied in three reports (Nicolas-Leveque et al., 1999; Concepcion, 2007; Cameron and Robinson, 2014). First, we confirmed that the anti-β-arrestin antibody we used, A2CT (Wei

et al., 2003), recognizes both β-ARR1 and β-ARR2 using immunoblot analysis of mouse retinas (Figure 1A).

Immunohistochemical analysis of retinal sections was performed to verify that the β-arrestin proteins can be detected in mouse photoreceptors. Consistent with published results, our IHC demonstrates that β-arrestin 2 is present in the photoreceptor layer of the mouse retina (Figure 1B), and also highly expressed in the outer plexiform layer, inner nuclear layer, inner plexiform layer, and ganglion cell layer (data not shown). Using retinas from β-*arr1*$^{-/-}$ and β-*arr2*$^{-/-}$ mice, we determined that β-arr2 is more highly expressed than β-arr1 in the outer plexiform layer, outer nuclear layer, and inner and outer segment layers. The retinas from the β-*arr2*$^{-/-}$ mouse have a greatly decreased signal compared to WT (C57Bl/6J), while the retinas from the β-*arr1*$^{-/-}$ are similar to WT in immunological staining intensity (Figure 1B). These results confirm that β-ARR2 is present in the photoreceptor cell layer. There is a basal immunofluorescent signal in the β-*arr2*$^{-/-}$ mouse retina, and this may be due to the presence of β-ARR1. However, it is difficult to assess whether this signal is specific for β-ARR1 or a non-specific background signal. Therefore, β-ARR1 may also be present in the same layers of the retina as β-ARR2, although β-ARR2 is more highly expressed in the photoreceptor layers.

Further IHC studies demonstrated that the visual arrestins (ARR1 and ARR4) colocalize with β-ARR2 in the photoreceptor layer of WT mouse retinas (Figure 2). Figure 2A–C demonstrates the cellular localization of ARR1 and ARR4 in a light-adapted mouse retina. ARR1 is present in rods and cones (Figure 2A), while ARR4 is present only in cones (Figure 2B). The immunoreactive signals overlap in the cone photoreceptors (Figure 2C). The immunoreactive signal of β-ARR2 co-localizes with ARR4 in mouse cones (Figure 2D–F), particularly in the pedicle of the cone and in the cone inner and outer segments (Figure 2F insets). The immunoreactive signals of both β-arrestins (β-ARR1/β-ARR2) overlap with the signal of ARR1 in mouse rod outer segments and cone pedicles (Figure 2I insets).

3.2 DRD4 is expressed in mouse cone photoreceptors

We confirmed the immunoreactive expression of a DRD4 protein in the photoreceptor regions where visual arrestins are highly expressed. Previously, DRD4 cellular localization has been studied through the use of *in situ* hybridization studies to determine the location of DRD4 mRNA in rodent retinas (Klitten et al., 2008; Kim et al., 2010; Li et al., 2013). This is likely because the antibodies used to identify DRD4 protein expression have been ineffective and/or non-specific (Van Craenenbroeck et al., 2005; Bodei et al., 2009). Recent evidence suggests that one anti-DRD4 antibody, N-20, is specific for DRD4 in mouse retinas, because of its reduced signal in *Drd4*$^{-/-}$ retina sections compared to wild-type (for mouse details, see section 2.3) (Deming et al., 2015a) (in press). Figure 3A–F also demonstrates the specificity of N-20 for human DRD4 in transfected HEK293T cells. The top row shows the specific overlap of the N-20 immunoreactive signal (green) with the FLAG tag signal (red) in HEK293T transfected with FLAG-DRD4.4. N-20 does not demonstrate immunoreactive staining in non-transfected HEK293T. Using the N-20 antibody, we confirmed the expression of DRD4 protein in mouse photoreceptor inner segments (Figure 3G). The DRD4 signal (green) overlaps with the ARR4 signal (red) in

mouse cone inner segments. Supplementary Figure S1 includes an intensity profile of a cross-section of this image to further demonstrate the overlap of the DRD4 and ARR4 signal. DRD4 also appears to be present in rod photoreceptor inner segments, based on the immunofluorescence pattern observed in the inner segment layer between the cones.

The specificity of the DRD4 antibody was determined by comparison of control C57Bl/6J mouse retinas with *Drd4*$^{-/-}$ mouse retinas. The Santa Cruz Biotechnologies antibody N-20 gave the most reliable results out of six tested. In transfected HEK293T, N-20 clearly and specifically labels human DRD4 using IHC (Figure 3A–F) and immunoblot analysis (Deming et al., 2015a) (in press). In immunoblot applications using homogenized mouse retinas, no specific band for DRD4 was detectable using this antibody. After multiple rounds of IHC protocol optimization, a specific and repeatable signal was observed in retinas from wild-type mice compared to *Drd4*$^{-/-}$. The differing results between the two applications may be due to the different state of the DRD4 protein (tissue homogenized and denatured for immunoblot, versus fixed and frozen for IHC), or different buffers and conditions needed for the two different detection methods.

3.3 Co-IP of DRD4 with ARR4

Co-IP studies were done to determine if DRD4 interacts directly with visual arrestins when co-expressed in HEK293T and if this interaction is dependent on DA stimulation. Co-IP using an antibody recognizing human DRD4, followed by immunoblot analysis, showed that ARR4 was pulled down along with HA-DRD4.4, suggesting a protein-protein interaction between ARR4 and DRD4 (Figure 4A) (n=1). This specific binding interaction is only seen when both plasmids encoding DRD4 and ARR4 were co-transfected into the HEK293T, and when the cells were stimulated with dopamine (DA) for 30 min.

The interaction was verified by the reverse co-IP, using an anti-human ARR4 antibody followed by immunoblot analysis with an anti-HA antibody, and similar results were obtained (Figure 4B) (n=2). Again, no detectable immunoreactive proteins from the pulldown were observed without DA stimulation. The intensities of the bands in the images were quantified using image analysis software, and the results of each co-IP are presented in Figure 4C and D. These results are in contrast to the pulldown of β-ARR2 with DRD4, which occurs with or without DA stimulation (Spooren et al., 2010). Co-IP with ARR1 was also tested with the appropriate controls and an ARR1-specific antibody, but no detectable pulldown was observed with or without DA stimulation (data not shown).

3.4 Internalization of DRD4 requires two different arrestins

Classic GPCR internalization experiments were performed to determine under which conditions, if any, DRD4 was internalized. The first set of experiments tested co-expression of human DRD4 with one arrestin at a time, including both visual and β-arrestins, and confirmed that DRD4 co-transfected with either β-ARR1 or β-ARR2 did not allow internalization (Figure 5A). This was consistent with published results (Spooren et al., 2010). We additionally co-transfected DRD4 with each of the visual arrestins, ARR1 and ARR4, and no receptor internalization was detectable when either visual arrestin was co-expressed with DRD4 (Figure 5A).

In each experiment, a positive control (β2 adrenergic receptor (B2AR) with β-ARR1, stimulated by 10 μM isoproterenol) was also included to verify that the experimental conditions allow internalization of a classically-sequestered GPCR (von Zastrow and Kobilka, 1992). B2AR was clearly internalized when stimulated with its agonist, isoproterenol (iso) (Figure 5C), indicating that the HEK293T cells we used contain the essential GPCR internalization components, and that the stimulation conditions (30 min at 37°C) allow internalization of at least one GPCR.

Next, two arrestins were co-transfected with DRD4 (β-ARR1+β-ARR2, ARR1+ARR4, β-ARR1+ARR1, β-ARR1+ARR4, β-ARR2+ARR1, or β-ARR2+ARR4). Preliminary experiments indicated that 30 min of DA stimulation (10 μM) was the optimum time needed to observe internalization, so all further experiments were done at this time point. After 30 min of DA stimulation, specific combinations of arrestins did allow DRD4 internalization (Figure 6B). Internalization is observed with only three of the six combinations: β-ARR1+ARR1, β-ARR1+ARR4, and β-ARR2+ARR4.

Visual results were confirmed by counting the number of cells with internalized receptors compared to the total number of DRD4-expressing cells in the images (Figure 5B and 6C). The three combinations of arrestins allow internalization that is significantly different from the baseline, which is set as the average values of all unstimulated cells (no DA added to identical medium for the same incubation time). Two of the combinations of arrestins allow a 6.7 fold greater number of cells demonstrating DRD4 internalization compared to non-stimulated baseline (β-ARR1+ARR4, $p<0.01$; and β-ARR1+ARR1, $p<0.05$), while β-ARR2+ARR4 is 5.8 fold greater than baseline ($p<0.01$). The other combinations of arrestins are not significantly different from the baseline.

In our experiments, we co-transfected three cDNA plasmid constructs encoding DRD4 and two arrestins at a time. Not all transfected cells will express all three proteins, but with our experimental conditions and subsequent observations, the cells with DRD4 expression usually (greater than 50%) also immunologically stained for the appropriate arrestins; however, this observation was not quantified. In our quantification of DRD4 internalization, in the positive groups about 30% of cells with DRD4 expression displayed DRD4 internalization, compared with about 5% in unstimulated groups. Compared with the positive control, the β2 adrenergic receptor (B2AR), in which about 75% of cells display internalization of the receptor, this percentage is relatively low. The lower percentage may be due to the decreased chance of co-transfecting three cDNA plasmid constructs instead of two, in addition to the more subtle endocytosis observed for DRD4 compared to B2AR.

3.5 Translocation of ARR4 to plasma membrane

An important feature of arrestin-mediated GPCR desensitization is translocation of the arrestin from the cytoplasm to the plasma membrane. To study this, we looked at the cellular immunological localization of ARR4 in transfected HEK293T cells with different durations of DA stimulation (15 to 60 min), or without DA stimulation for the same time periods. This allowed us to observe the cellular location of ARR4 over time, including whether or not it translocates to DRD4 at the cell membrane and the length of time it remains there. Without DA stimulation, ARR4 is diffusely immunologically stained within the cytoplasm

with little to no overlap with DRD4 at the plasma membrane (Figure 7A,G, and M). Without β-ARR1 or β-ARR2, there is no significant increase in the amount of DRD4 and ARR4 overlap (Figure 7A–F). When β-ARR1 or β-ARR2 is present, after 20 min of DA stimulation ARR4 appears to be localized with DRD4 at the plasma membrane (Figure 7I and O, large arrowheads), and this is prior to DRD4 internalization. At 30 min, ARR4 localizes with DRD4 at the plasma membrane (Figure 7J and P, large arrowheads) and on some, but not all, of the internalized DRD4 (Figure 7J and P, small arrowheads). After 45 or 60 min of DA stimulation, strong internalization of DRD4 is still observed (Figure 7 K,L,Q, and R, small arrowheads), but only minimal co-localization of DRD4 and ARR4 is present (Figure 7 K,L,Q, and R, large arrowheads). The co-localization of DRD4 and ARR4 at these time points appears similar to that of the cells stimulated for 15 min. Further statistical analysis (Figure 7S) determined that the only conditions with DRD4/ARR4 co-localization above baseline (calculated as the average percent of cells demonstrating co-localization of ARR4 and DRD4 in the unstimulated populations) were DRD4+β-ARR1+ARR4 and DRD4+β-ARR2+ARR4, with either 20 or 30 min of DA stimulation. All other populations were not significantly higher than baseline, although at 15, 45, and 60 min the amount of co-localization appears slightly higher when either β-ARR1 or β-arr2 is present (Figure 7S).

4. Discussion

In this study, we demonstrated that DRD4 and ARR4 interact *in vitro* in a dopamine-dependent manner, and that DRD4 is internalized after dopamine stimulation, as long as the correct combination of both a β-arrestin (β-ARR1 or β-ARR2) and a visual arrestin (ARR1 or ARR4) are expressed in the same cell. We further showed that these *in vitro* observations are potentially relevant and may be extended to systems *in vivo*, as DRD4 is expressed in mouse photoreceptor inner segments along with visual arrestins and β-ARR2.

4.1 DRD4 expression in photoreceptors with visual arrestin

In the retina, visual arrestins are co-expressed with β-ARR2 (Figure 1B and Figure 2) and may contribute to desensitization of non-opsin GPCRs. Previously, DRD4 has been shown to act through the G-protein cone transducin in mesencephalic cells (Yamaguchi et al., 1997), indicating that the cone opsins, which are also GPCRs, and DRD4 have a potential shared target of activation. We propose that they may also share components in their desensitization pathway. The activated cone opsins can be bound and desensitized by either ARR4 or ARR1 (Nikonov et al., 2008). In mouse retinas, we show that DRD4 and ARR4 are both present in the inner segments of cones (Figure 3G), which means that these two proteins may potentially interact and function together in the mouse retina.

4.2 Co-immunoprecipitation of DRD4 with ARR4 in vitro

To test the potential interaction of DRD4 with ARR4 and the other three arrestins, we set up *in vitro* experiments in which DRD4 was co-transfected with an arrestin in HEK293T cells. We showed with co-IP studies that DRD4 and ARR4 do interact when overexpressed in HEK293T cells, but only when the transfected cells are stimulated with DA (Figure 4). Co-IP studies have previously shown that DRD4 interacts with β-ARR1 and β-ARR2, but that this interaction is not dependent on DA stimulation (Spooren et al., 2010). The same

study also reported that the intracellular portion of DRD4 is phosphorylated in HEK293T, and the phosphorylation is also not dependent on DA stimulation but is instead observed constitutively (Spooren et al., 2010). DRD4 phosphorylation is likely performed by the GPCR kinase, GRK2, because GRK2 is known to phosphorylate the other D2-like receptors (Kim et al., 2001), and GRK2 is present in photoreceptors as well (de Almeida Gomes and Ventura, 2004).

The DA-independent phosphorylation and β-arrestin binding of DRD4 indicates that these processes may not be a part of the DRD4 desensitization pathway, since DRD4 was not activated by DA. In contrast to these results, we show that the binding of ARR4 is dependent on DA stimulation, indicating that it is this interaction which is specific to receptor desensitization. Because only the presence of both a β-arrestin and a visual arrestin allows detectable DA-dependent internalization, the interaction of ARR4 or ARR1 with DRD4 must be an essential step for the observed desensitization and sequestration of the receptor.

Our co-IP experiments indicate that the overexpression of a β-arrestin is not required for ARR4 binding to DRD4, although it is necessary for the observed internalization of the DA-stimulated receptor. Based on the internalization results, we repeated the co-IP with HEK293T that were co-transfected with plasmids encoding DRD4, ARR4, and β-ARR1 to determine whether ARR4 would display increased binding to DRD4 when β-ARR1 is also present. Any pulldown of DRD4 with ARR4 that may have occurred was below detection level with our immunoblot analysis. This may be because in preliminary immunoblot analysis, we observed that decreasing the amount of plasmid DNA in the transfection mixture leads to a decrease in the total amount of the corresponding protein in transfected cells (data not shown). Because of this observation we hypothesize that the amount of each arrestin expressed in the HEK293T is decreased when three plasmids are co-transfected instead of two (Section 2.1), so the amount of ARR4 may have been decreased below the detection limit in the experiments including β-ARR1. Alternatively, β-ARR1 may compete with ARR4 for the same DRD4 binding site(s), thereby decreasing the amount of ARR4 bound to DRD4 to undetectable levels.

4.3 Internalization of DRD4

In further *in vitro* experiments, we demonstrated that the overexpression of each individual arrestin with DRD4 does not allow sequestration of the receptor (Figure 5A and B). This was in contrast to our positive control, B2AR, which demonstrated robust internalization under identical conditions upon receptor activation by isoproterenol (Figure 5C). However, we were interested in the mechanism of DRD4 desensitization in photoreceptors and pinealocytes, where DRD4 expression is circadian. In these highly specialized neurons, the two β-arrestins are expressed along with the two visual arrestins. We hypothesized that a combination of arrestins would allow desensitization and sequestration of DRD4, so we tested the co-expression of two arrestins at a time with DRD4.

We were surprised by the initial and repeated results of these experiments, which clearly demonstrated the requirement for at least two different arrestins to be co-expressed for DRD4 internalization (Figure 6). Interestingly, with β-ARR1 and β-ARR2 co-expressed,

no internalization is observed. This indicates that the internalization we observe may not occur in cells not expressing visual arrestins. Additionally, combinations of either ARR1 and ARR4 or β-ARR2 and ARR1 do not display detectable internalization. Because three out of six combinations of two arrestins with DRD4 do not demonstrate internalization of DRD4, the internalization observed is not merely an artifact of arrestin overexpression. Instead, the observed specific internalization is dependent on the presence of a unique combination of arrestins and the stimulation of DA.

In any population of transiently transfected cells, there will be a group of cells that do not appear to express the transfected construct. Co-transfection of two or more mammalian expression plasmids also complicates this problem, as it can be difficult to determine which cells simultaneously express both proteins. Because of the inclusion of the appropriate controls (unstimulated populations for each group, DRD4 with each arrestin individually, and all six possible combinations of two arrestins), it is clear that the internalization observed is due to the presence and expression of specific arrestins and not differences in transfection efficiency between plasmids. In addition, the results were replicated in multiple experiments (n=4) and with multiple expression constructs for DRD4 (HA-DRD4.4, FLAG-DRD4.4) and β-arrestins (HA-βarr1, FLAG-βarr1, HA-βarr2, and FLAG-βarr2). Interestingly, when we transfected the same constructs into COS-7 cells (Gluzman, 1981; Pierce et al., 2000) and stimulated with DA, no internalization was observed for any group (data not shown). Isoproterenol-stimulated B2AR was still internalized robustly in COS-7 cells. This set of experiments in COS7 cells suggests that HEK293T cells are unique, perhaps because of their human origin or neuronal gene expression characteristics (reviewed in (Thomas and Smart, 2005)), that allows the detectable internalization of DRD4, while COS-7 cells are missing at least one critical component for DRD4 internalization.

From these experiments, it is unclear why DRD4 would require two arrestins. To our knowledge, there is no other GPCR that requires the expression of two arrestins to undergo internalization after desensitization, so DRD4 appears to be the first and perhaps a unique GPCR that requires this mechanism for desensitization and/or internalization. Since each arrestin alone is not sufficient to allow internalization of DRD4, we hypothesize that each arrestin performs a specific and perhaps sequential role in the desensitization and internalization process. Furthermore, we have shown that the presence of two β-arrestins or two visual arrestins also does not allow internalization. These cumulative observations lead us to propose a more defined hypothesis. In each cell where internalization occurs, β-ARR1 or β-ARR2 performs a similar role to one another, while visual ARR1 or ARR4 also perform a similar role that is distinct from that of a β-arrestin.

While β-ARR1 and β-ARR2 can perform unique functions outside of GPCR desensitization (reviewed in (Shenoy and Lefkowitz, 2003), they have in common an ability to bind phosphorylated GPCRs and recruit endocytic machinery to begin the process of clathrin-coated reuptake (reviewed in (Walther and Ferguson, 2013)). The abiilty of visual arrestins to take part in clathrin-coated endocytosis has not been as thoroughly investigated; however, there are no reports that ARR4 is able to interact with proteins in the endocytic machinery. On the other hand, ARR1 is able to bind and modulate N-ethylmaleimide sensitive factor (NSF) (Huang et al., 2010) and Adaptor protein 2 (AP2) (Orem et al., 2006; Moaven et al.,

2013), proteins that are essential components in the SNARE complex and the clathrin-coated endocytosis machinery, repectively.

However, visual arrestins have in common their ability to bind GPCRs and to desensitize light-activated, phosphorylated opsins in all photoreceptors (Kuhn et al., 1984; Xu et al., 1997; Mendez et al., 2000; Nikonov et al., 2008). Based on these classical studies, as well our cumulative data, we propose that the primary contribution of visual arrestin involves a critical agonist-dependent binding to DRD4 and possibly a comformational and affinity shift. Subsequently, β-arrestin, which binds to DRD4 with or without DA stimulation, may then be able to recruit essential components of the endocytic machinery once DRD4 is fully desensitized by visual arrestin, which it is unable to do without visual arrestin binding. It is also possible that β-arrestin binds one component of the internalization scaffold, such as clathrin, while visual arrestin binds another component, such as AP2. Further studies are ongoing to determine the specific role each arrestin contributes in the desensitization and internalization of DRD4.

4.4 Quantification of Internalization

There are many published methods of quantifying GPCR internalization *in vitro*. Nearly all of these methods measure the decrease in the presence of receptors on the cell membrane after internalization (reviewed in (Milligan, 2003)). In the case of DRD4 internalization, the amount of receptor that moves into the cell is small compared to the total number of receptors that are expressed on the plasma membrane. This is clear from the IHC images of DRD4 internalization, in which there is still a stronger immunofluorescent signal on the membrane compared to the signal of the internalized receptors (Figure 6B).

The quantification results displayed in Figure 5B and 6C were obtained through direct counting from IHC images obtained in four separate experiments. We counted the total number of cells with DRD4 expressed and the number of cells with DRD4 internalized. The fraction of cells with DRD4 internalized was calculated for each group, including the non-stimulated controls. The non-stimulated cells occasionally also appear to display internalization (about 5% of the cells), so it was appropriate to use these as controls to ensure that the internalization observed in the other groups was truly agonist-dependent. The average of the non-stimulated control groups was set as a baseline of 1, and all stimulated groups were compared to this baseline. The fold difference was plotted in Figure 5B and 6C.

4.5 Translocation of ARR4 to plasma membrane

A key feature of arrestin-mediated GPCR sequestration is migration of arrestin from the cytoplasm to the plasma membrane in order to interact with the intracellular portions (loops or C-terminus) of the GPCR (Oakley et al., 2002). One study reported that β-ARR2 also translocates to the plasma membrane to co-localize with DRD4 in response to DA stimulation (Cho et al., 2006), but a more recent report was unable to confirm the observation (Spooren et al., 2010). With immunohistochemistry tools, we labeled DRD4 and ARR4 in co-transfected HEK293T cells to determine the subcellular localization of ARR4 with or without the presence of β-ARR1 or β-ARR2, at multiple durations after DA stimulation (Figure 7). The use of sequential DA stimulation timepoints allowed observation

of the subcellular location of ARR4 without stimulation (at 15, 20, 30, 45, and 60 min), after stimulation but before DRD4 internalization (15 and 20 min), at the peak of DRD4 internalization (30 min), and after DRD4 internalization has occurred (45 and 60 min). We noted that both DA and beta-arrestin were required for stronger co-localization of DRD4 and ARR4 in HEK293T (Figure 7). This is in contrast to our initial findings that over expression of beta-arrestin is not required for co-IP pulldown of ARR4 with DRD4 (Figure 4). The time course of ARR4 translocation we observed *in vitro* is similar to the time course of visual arrestin translocation to the photoreceptor outer segments, which occurs within 20 min of continuous light exposure (Broekhuyse et al., 1985; Whelan and McGinnis, 1988; Zhu et al., 2002; Zhang et al., 2003).

The subcellular redistribution of ARR4 is similar to the translocation of β-arrestin observed with other GPCRs, where β-arrestin translocation occurs rapidly and continues through 30 min of stimulation (Oakley et al., 2002). Many GPCRs demonstrate a faster time course for β-arrestin translocation (Oakley et al., 2002), which occurs within two min of agonist stimulation, in contrast to 15–20 min for ARR4 and DRD4. The time discrepancy may be explained by the relative rates of action of these GPCRs compared to DRD4. Many GPCRs in neurons, muscle, and other tissue require fast activation and desensitization for sensitive response to signals.

DRD4, on the other hand, has been hypothesized to be a slower-acting GPCR, based on its constitutive phosphorylation and β-arrestin binding, which may slow down its signal *in vivo* (Spooren et al., 2010). In addition, our initial hypothesis is that DRD4 sequestration is important in regulating the diurnal physiology in photoreceptors and pinealocytes, because of its unique circadian regulation in these specialized neurons. This modulation would only be required once per day, when the lights turn on, to decrease DRD4's presence on the photoreceptor membrane. The decrease in DRD4 mRNA expression and increase of a light dependent dopamine synthesis has been observed within a brief one hr window of light onset (Kim et al., 2010), so a relatively slow recruitment of ARR1 or ARR4 and subequent desensitization would not impact the overall circadian regulation of DRD4.

5. Conclusion

These experiments reveal a surprising new potential mechanism for desensitization and sequestration of GPCRs in cells where visual arrestins are highly expressed, primarily in the photoreceptors and pinealocytes.

Our results support the hypothesis that DRD4 is desensitized and internalized by a unique mechanism in the photoreceptors and pinealocytes that is dependent on a dual expression of visual and beta-arrestin. DRD4, ARR1 and ARR4 are localized to inner segments and synapses, and the ubiquitously expressed β-arrestins are also expressed in photoreceptors and pinealocytes. The agonist-dependent internalization of DRD4 is only observed *in vitro* when specific combinations of arrestins are present, and all of the combinations that work contain both a β-arrestin and a visual arrestin.

Based on previous work, activation and expression of the DRD4 signal transduction cascade in the mouse photoreceptors are critical for maintaining normal daytime high acuity vision (Jackson et al., 2012). Likewise, without the expression of ARR4 in the photoreceptors, a similar decrease in contrast sensitivity and normal visual acuity is observed (Brown et al., 2012 53:ARVO E-Abstract 760/A637; Deming et al., 2015b) (submitted for publication). We propose that the unique DRD4 desensitization process demonstrated in this work plays a role in the fine tuning of DRD4 circadian expression and downstream signaling, including gap junctional coupling and postsynaptic On-and/or Off-bipolar signal.

In addition, the discovery that two arrestins are required for the internalization of one GPCR indicates that this may also be the case for other GPCRs that were previously thought to be resistant to internalization. Further investigation of these GPCRs, particularly those which are relevant to dopamine functions, may reveal that they can be internalized when the proper arrestins are expressed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- DRD4 is expressed in mouse photoreceptors with β-arrestin and visual arrestin
- DRD4 has a dopamine-dependent interaction with cone arrestin 4 *in vitro*
- DRD4 is not internalized after DA stimulation if only one arrestin is co-expressed
- DA-stimulated DRD4 is internalized in HEK cells when visual and β-arrestin are co-expressed

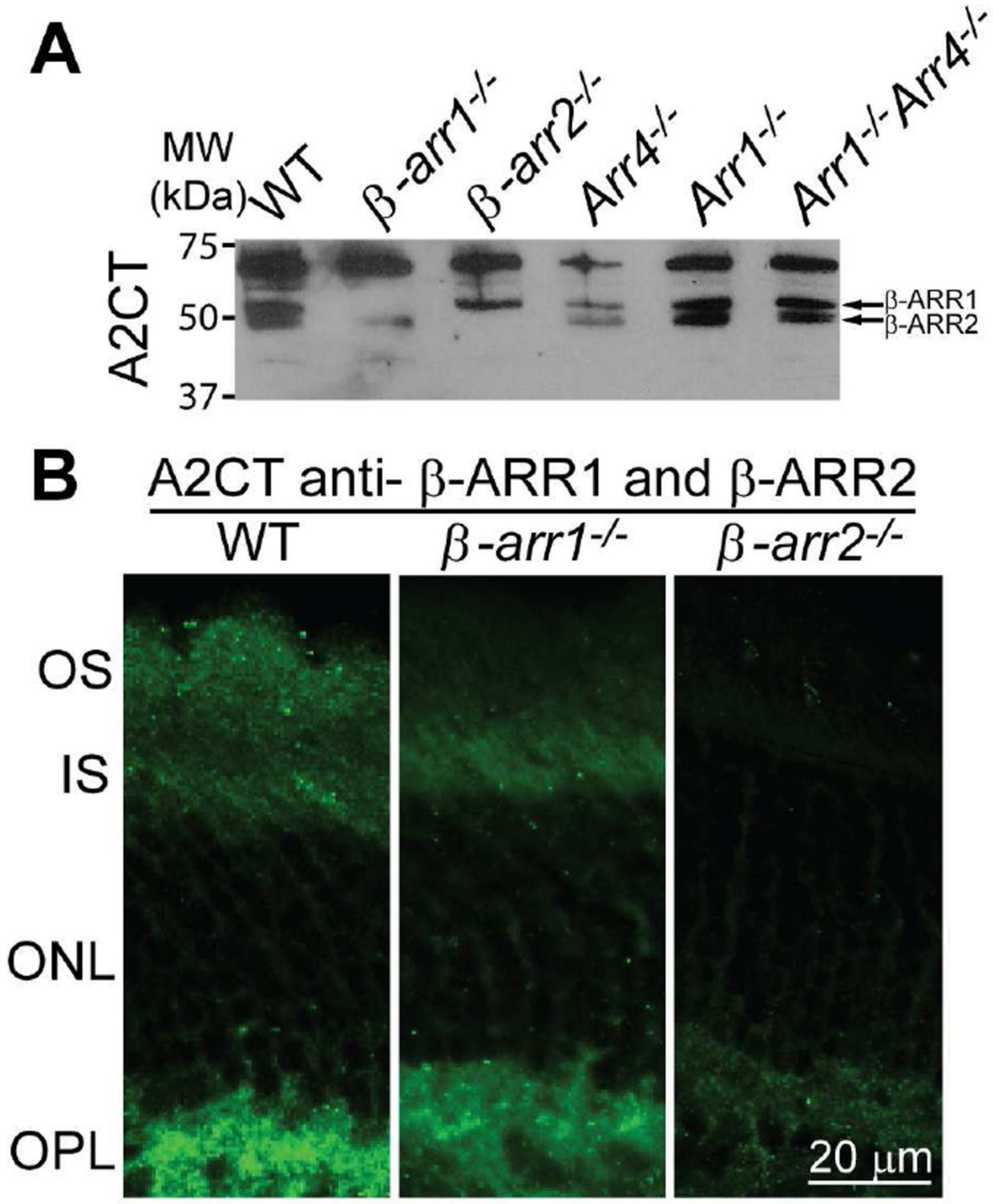


Fig 1.

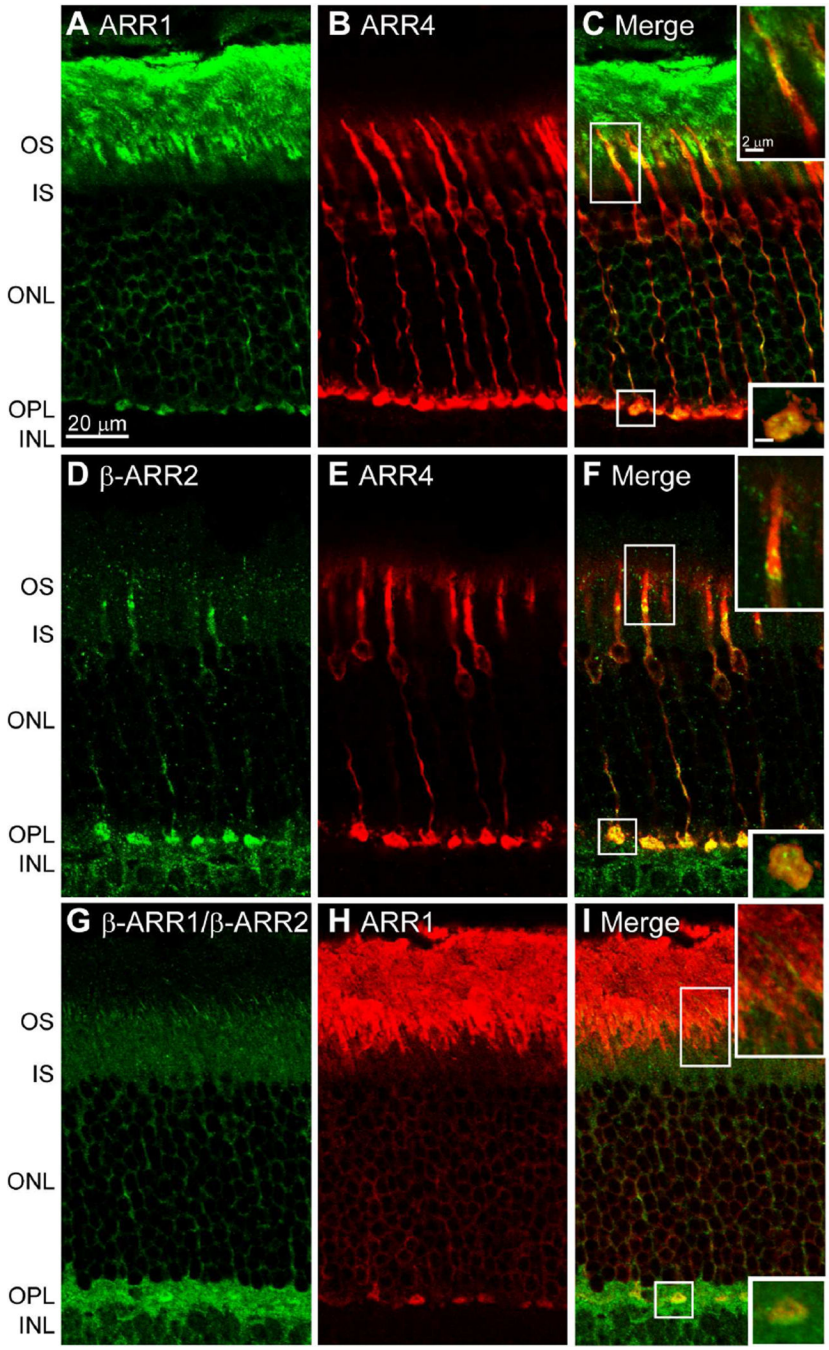


Fig 2.

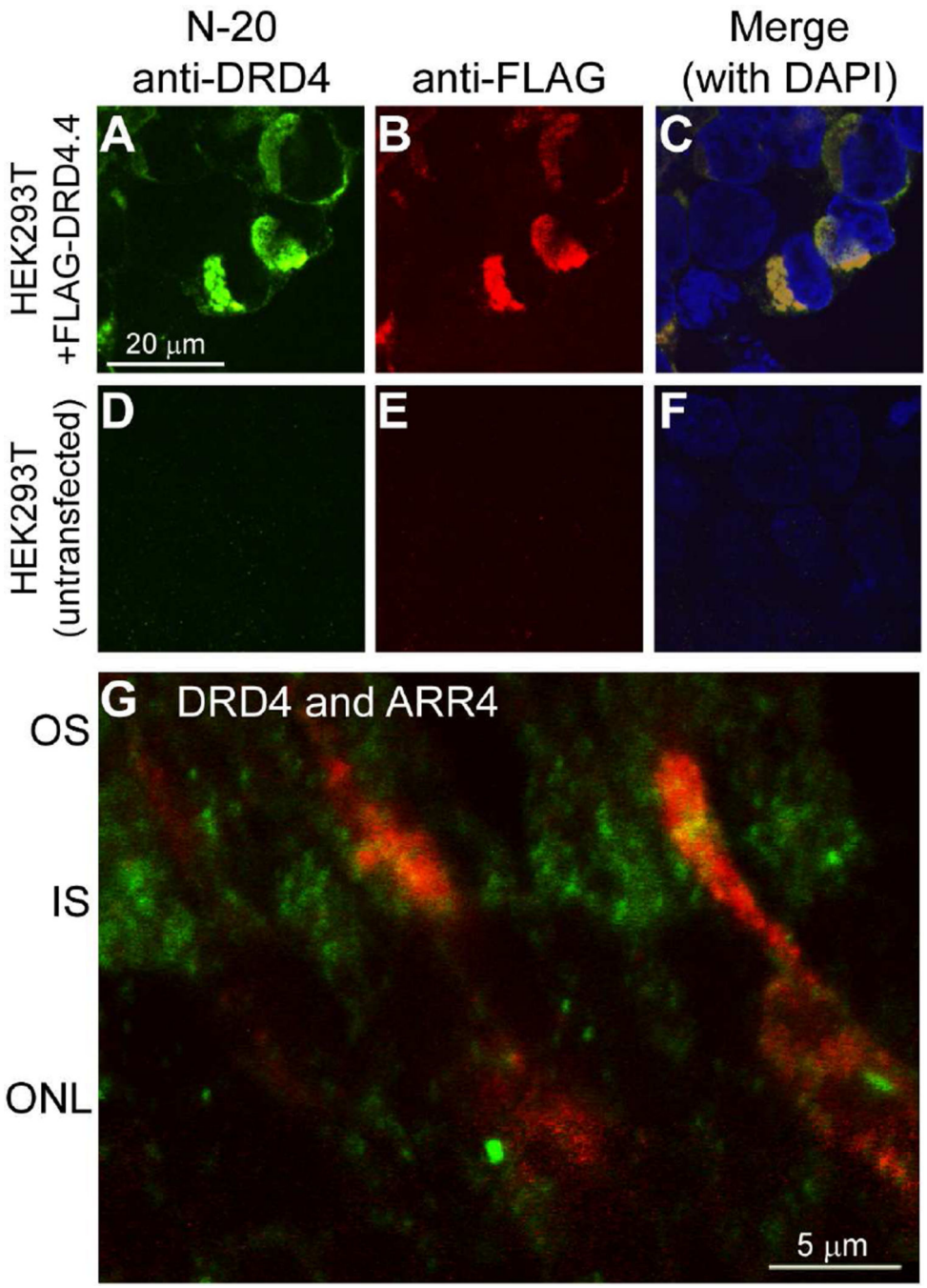
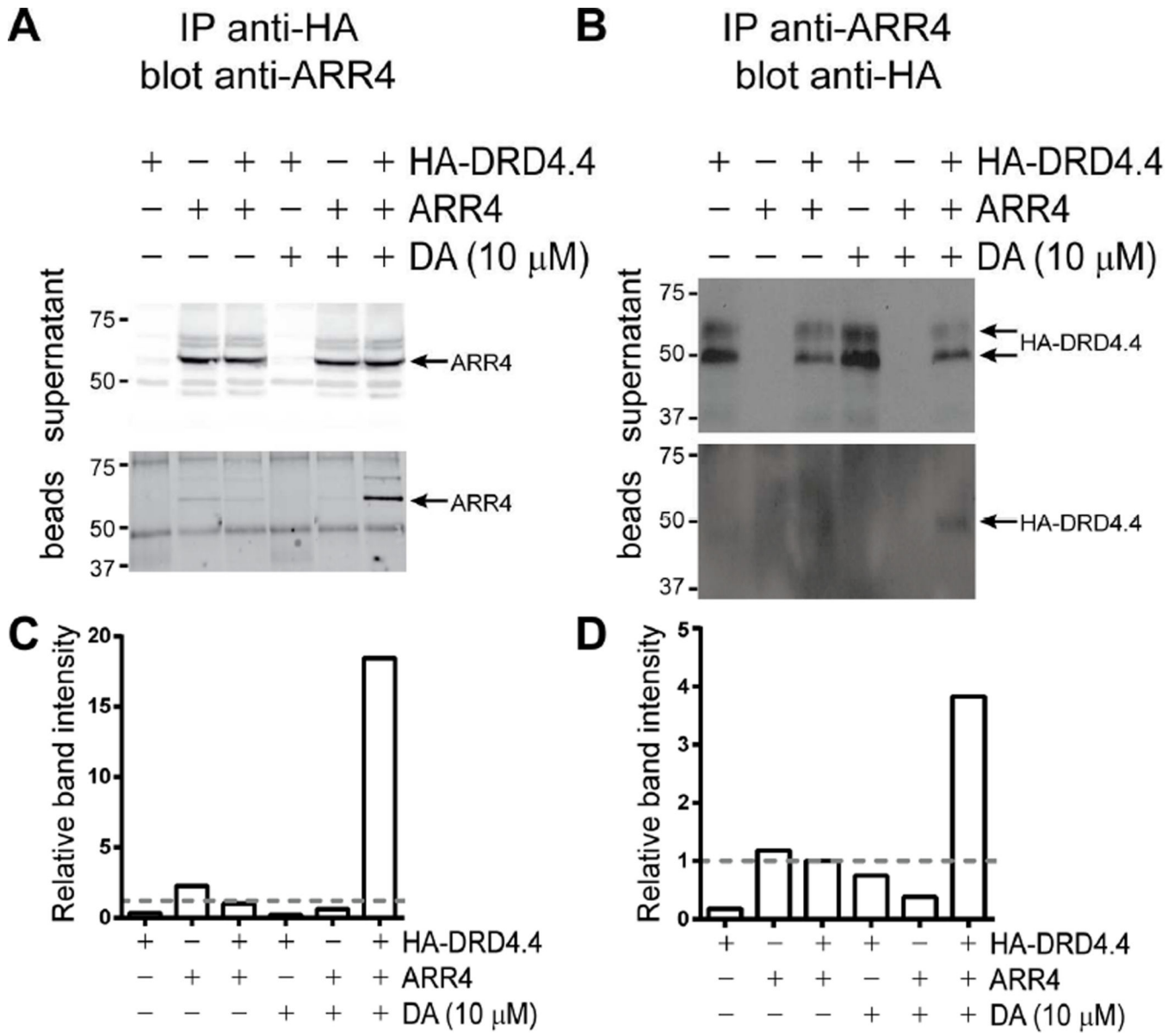


Fig 3.



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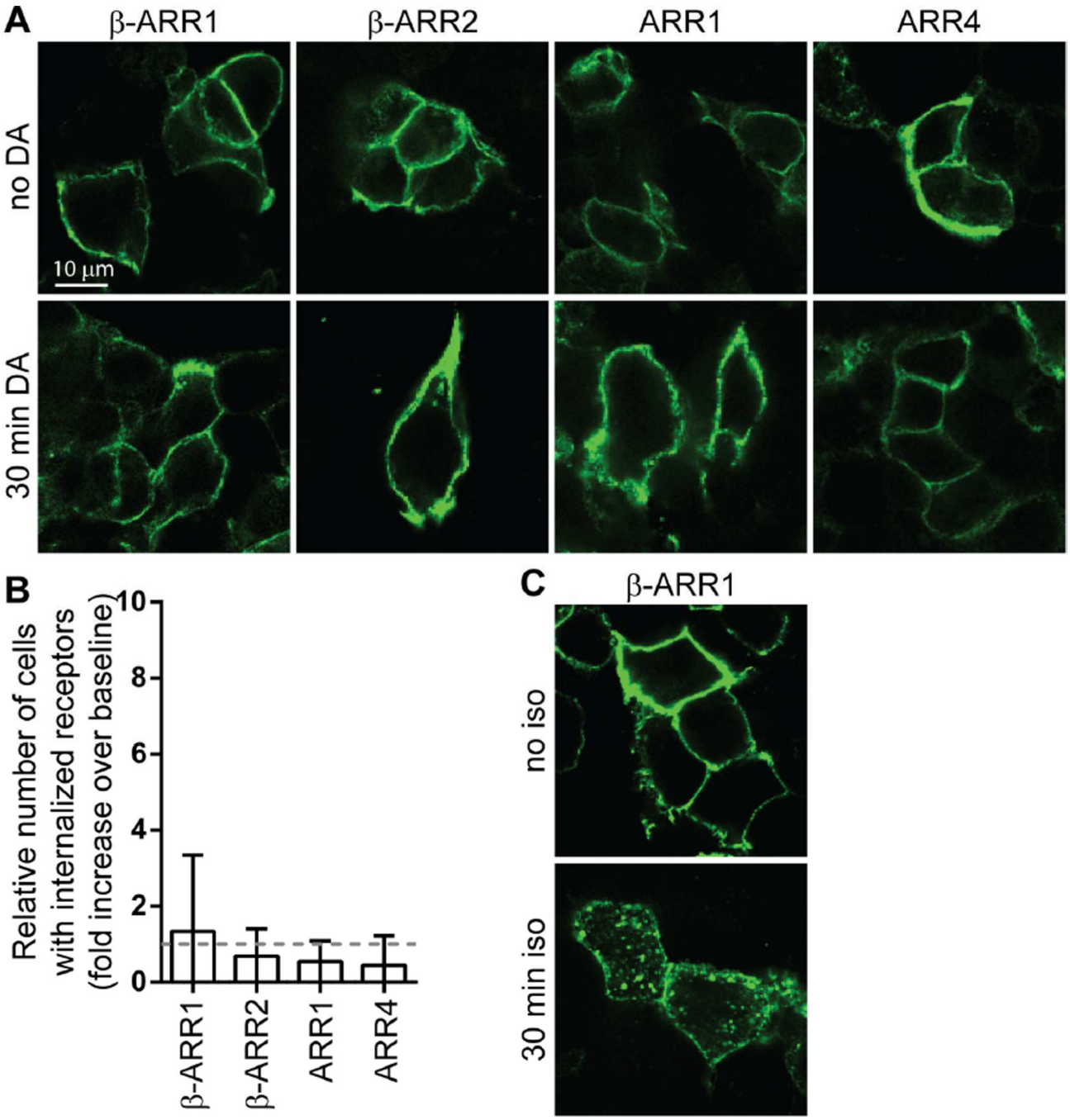

Fig 5.

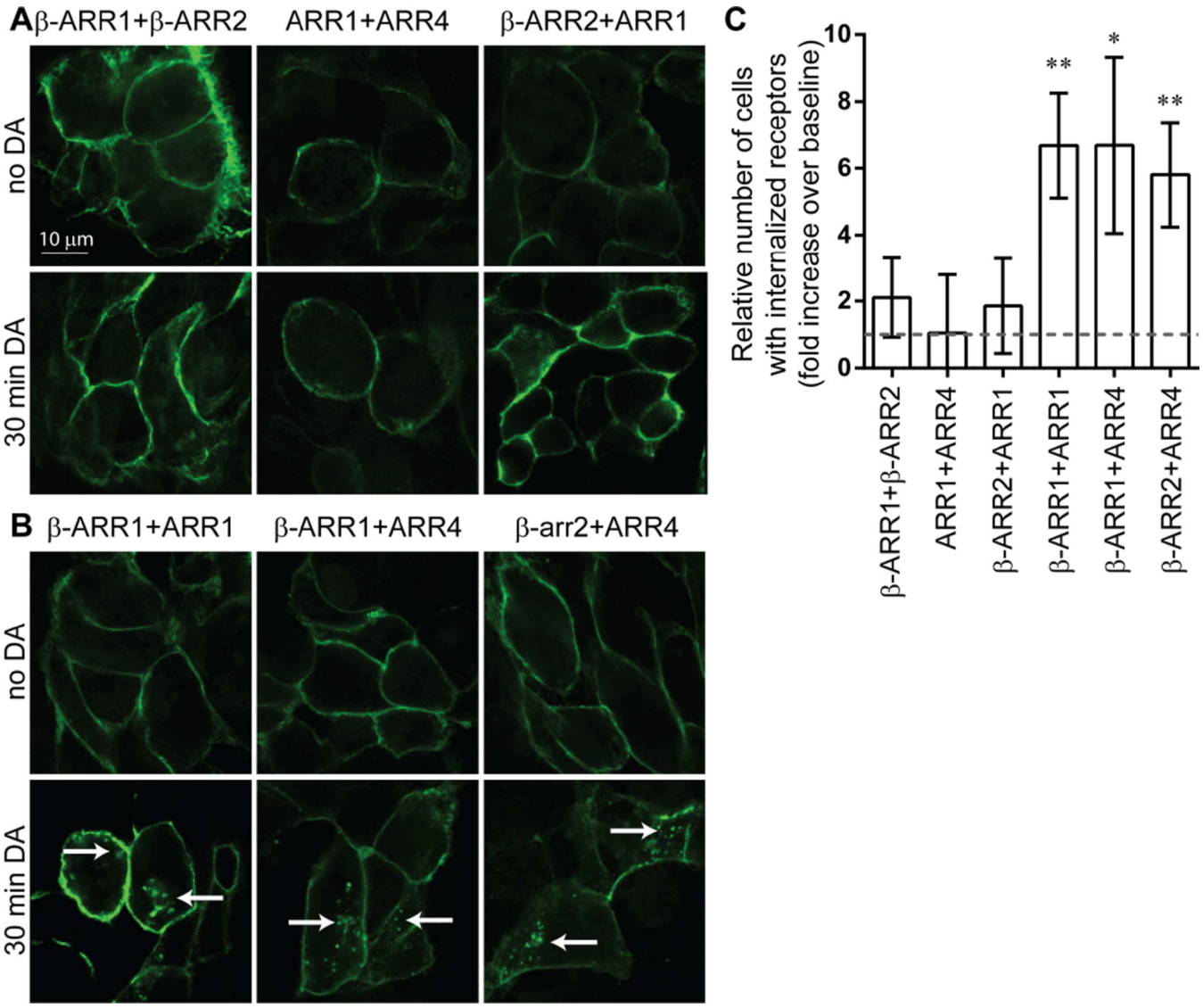

Fig 6.

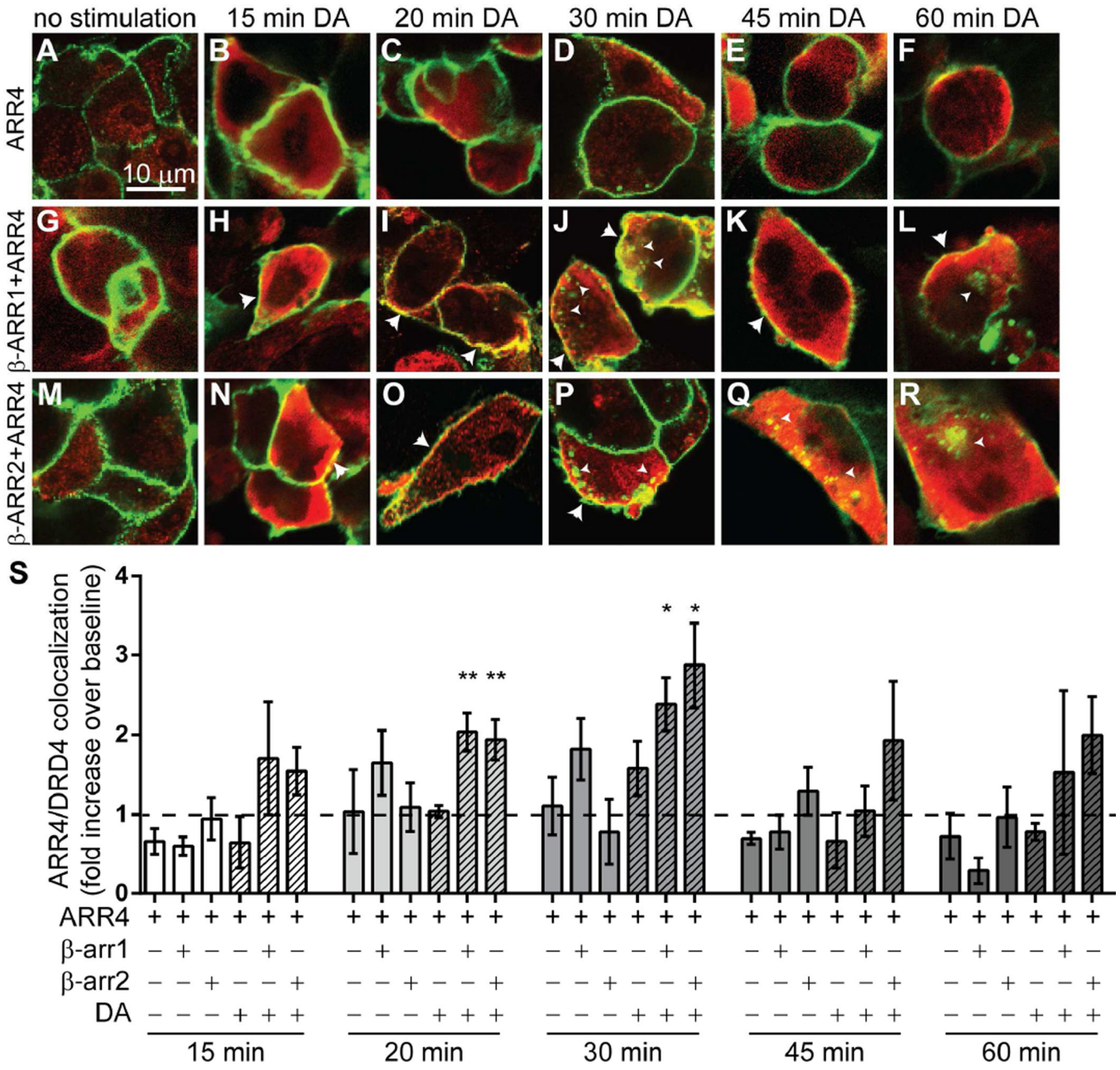

Fig 7.

Table 1

Primary antibodies used in the current study

Name	Source	Specificity	Species	Application	Dilution	Publication
M1 anti-FLAG	Sigma Aldrich (#F3040)	FLAG tag	mouse	IHC	1:500	
anti-HA (clone 6E2)	Cell Signaling (#2367)	HA-tag	mouse	IHC IB	1:500 1:500	
LUMIf-hCAR	Craft	human ARR4	rabbit	IHC IB co-IP	1:1000 1:10,000 1:200	(Zhang *et al.*, 2003)
LUMIj-mCAR	Craft	mouse ARR4	rabbit	IHC	1:1000	(Zhu *et al.*, 2002)
D9F2	Donoso	bovine S-antigen/ ARR1	mouse	IHC	1:10,000	(Donoso *et al.*, 1990)
A2CT	Lefkowitz	β-ARR1 and β-ARR2	rabbit	IHC IB	1:500 1:10,000	(Wei *et al.*, 2003)
N-20	Santa Cruz (#31480)	human DRD4	goat	IHC IB	1:100 1:200	(Deming *et al.*, 2015a)
β-arrestin 2	Santa Cruz (#13140)	human β-ARR2	mouse	IHC	1:500	

Abbreviations: IHC: immunohistochemistry; IB: immunoblot; co-IP: co-immunoprecipitation