Molecular Pharmacology Fast Forward. Published on September 27, 2017 as DOI: 10.1124/mol.117.109462
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Effective attenuation of adenosine A1R signaling by neurabin requires oligomerization of neurabin

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Molecular Pharmacology Fast Forward. Published on September 27, 2017 as DOI: 10.1124/mol.117.109462
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a) Running title: Regulation of A1R signaling by neurabin

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c) Number of text pages:

Number of tables: 0

Number of figures: 7

Number of references: 34

Number of words in the Abstract: 197

Number of words in the Introduction: 531

Number of words in the Discussion: 803

d) Abbreviations.

GPCR, G protein-coupled receptor; RGS, regulator of G protein signaling; A1R, adenosine A1 receptor; SAM, sterile alpha motif; GST, glutathione S-transferase; IP, immunoprecipitation; DPCPX, 8-Cyclopentyl-1,3-dipropylxanthine; TIRF, total internal reflection fluorescence

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Abstract

The adenosine A1 receptor (A1R) is a key mediator of the neuroprotective effect by endogenous adenosine. Yet, targeting this receptor for neuroprotection is challenging due to its broad expression throughout the body. A mechanistic understanding of the regulation of A1R signaling is necessary for the future design of therapeutic agents that can selectively enhance A1R-mediated responses in the nervous system. In the present study, we demonstrate that A1R activation leads to a sustained localization of RGS4 at the plasma membrane, a process that requires neurabin, a neural tissue-specific protein. A1R and RGS4 interact with the overlapping regions of neurabin. Additionally, neurabin domains required for oligomerization are essential for formation of the A1R/neurabin/RGS4 ternary complex, as well as for stable localization of RGS4 at the plasma membrane and attenuation of A1R signaling. Thus, A1R and RGS4 each likely interacts with one neurabin molecule in a neurabin homo-oligomer to form a ternary complex, representing a novel mode of regulation of G protein-coupled receptor signaling by scaffolding proteins. Our mechanistic analysis of neurabin-mediated regulation of A1R signaling in this study will be valuable for the future design of therapeutic agents that can selectively enhance A1R-mediated responses in the nervous system.

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Introduction

Endogenous adenosine is a potent anticonvulsant agent protective against various excitatory insults *via* activation of the adenosine A1 receptor (A1R) (Boison, 2008; Cunha, 2001; Dunwiddie and Masino, 2001; Haas and Selbach, 2000; Sebastiao and Ribeiro, 2009; Stone, 2002). The A1R belongs to the G protein-coupled receptor (GPCR) superfamily and couples to the Gi/o subfamily of heterotrimeric G proteins. Activation of A1R inhibits cAMP production and voltage-gated calcium channels, and activates G protein-operated inwardly rectifying potassium channels. Consequently, A1R activation reduces neurotransmitter release from presynaptic terminals and provokes hyperpolarization of postsynaptic membranes (Cunha, 2001; Dunwiddie and Masino, 2001; Haas and Selbach, 2000; Sebastiao and Ribeiro, 2009). Despite its potential therapeutic effectiveness, targeting A1R for neuroprotection has been particularly challenging due to its broad expression in multiple peripheral tissues including heart, lung, and kidney (Stone, 2002), which leads to undesired peripheral actions that result in dramatic side effects. A better understanding of the regulatory mechanism governing A1R signaling and activity is essential for development of novel strategies targeting this receptor for treatment of neurological diseases.

One important modulator of G protein signaling is the family of regulator of G protein signaling (RGS) proteins, which accelerate hydrolysis of GTP by the Gα subunit of the G protein, thus terminating GPCR signaling (Hollinger and Hepler, 2002; Kach et al., 2012; Neitzel and Hepler, 2006; Neubig and Siderovski, 2002; Ross and Wilkie, 2000; Sjogren and Neubig, 2010; Xie and Palmer, 2007). A role for endogenous RGS proteins in regulating A1R signaling has been indicated by enhanced A1R-mediated responses in cells expressing an RGS-insensitive mutant form of Gi or Go protein (Chen and Lambert, 2000; Fu et al., 2006). We have previously discovered that RGS4 attenuates A1R signaling, and this effect relies on a neural tissue-specific scaffolding protein, neurabin (Chen et al., 2012). Significantly, neurabin deficiency enhances A1R signaling and promotes the protective effect of adenosine against excitotoxic seizure, suggesting that the A1R-neurabin interaction as a valid therapeutic target for specifically enhancing the anticonvulsant effects of endogenous adenosine. Neurabin is a multi-domain protein containing (from N- to C-terminus) an actin-binding domain, a protein phosphatase 1 binding and regulatory sequence, a

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protein-interaction PDZ domain, three coiled-coil domains, and a putative protein-interaction sterile alpha motif (SAM) domain (Burnett et al., 1998; Nakanishi et al., 1997). Sequences that are responsible for its interaction with A1R and RGS4 remain to be determined. In addition, whether and how other functional domains may contribute to regulation of A1R signaling have not been investigated. This information is not only important for mechanistic understanding of neurabin-dependent regulation of GPCR signaling but is also necessary for the future design of therapeutic agents that can selectively enhance A1R-mediated responses in the nervous system.

In the present study, we elected to tease out the molecular mechanism by which neurabin fine tunes A1R signaling using combined genetic, biochemical, and live-cell imaging approaches. Our live-cell imaging studies reveal, for the first time, that A1R activation induces a long-lasting localization of RGS4 at the plasma membrane, which requires the presence of neurabin. Our structure-function analysis suggests that the A1R/neurabin/RGS4 ternary complex formation relies on neurabin oligomerization, which is also essential for stable membrane localization of RGS4 and attenuation of A1R signaling.

Materials and Methods

Heterologous cell culture. HEK293 and Cos7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals), 1% penicillin/streptomycin, and maintained in a humidified 5% CO2 incubator. CHO-K1 cells were cultured in DMEM/F-12 (Invitrogen) with 10% FBS, 1% penicillin/streptomycin and 2 mM glutamine. Cells were transfected with Lipofectamine 2000 (Invitrogen).

DNA constructs. cDNAs encoding A1R aa 202-326 and RGS4 were amplified by PCR and cloned into the pCMVTNT vector (Promega). cDNAs encoding different regions of neurabin sequences were amplified by PCR and cloned into the GST fusion vector pGEX4T (GE Healthcare) through *Eco*RI and *Xho*I sites to generate the GST-fused Nrb constructs described in Fig. 3. The GST-NrbΔ404-413 construct and GST-Nrb331-453 with mutations were constructed from the GST-Nrb331-453 construct using the Quickchange kit (Stratagene). For eukaryotic expression of GST-RGS4 fusion protein, RGS4 cDNA was

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cloned into a pCMV-GST vector (Sigma).

In vitro GST pull-down assays. [^{35}S]-labeled, *in vitro* translated, A1R202-326 and RGS4 probes were prepared using the TNT rabbit reticulocyte lysate kit (Promega). Preparation of GST fusion proteins and pull-down assays were described previously (Wang and Limbird, 2002). In brief, 2 μg of purified GST or GST-fusion protein were incubated with [^{35}S]-labeled probe (100,000cpm to 300,000cpm) in 0.25 ml binding buffer (50 mM Tris-HCl, pH 7.4, 0.05% Triton X-100, 10% glycerol, 0.01%BSA, 100 μM PMSF, 1 μg/ml soybean trypsin inhibitor, 1 μg/ml leupeptin, 10 units/ml aprotinin) for 2 hours. 25 μl GSH agarose was then added into this incubation and rotated for an additional 2 hrs. In all studies where GST fusion proteins were employed, the same SDS-PAGE gel for autoradiography was stained with Coomassie Blue to confirm the presence of the GST-fusion proteins at equivalent amounts.

Coimmunoprecipitation (co-IP) assays. Co-IP assays were performed as described previously (Chen et al., 2012). Briefly, HEK293 cells (plated at 4x10^6/100mm dish) were transfected with the desired plasmids. 48 hrs post transfection, cells were stimulated with a selective A1R agonist, R-PIA (Sigma), for 5 min. Cells were then lysed in 350 μl IP buffer (10 mM Tris, 1% NP-40, 10% glycerol, 5 mM EDTA, 5 mM EGTA, pH7.6, plus protease inhibitors) and incubated with an antibody against HA (rat anti-HA, Roche, 1:100), Myc (Cell Signaling Technology, 1:100) or RGS4 (Santa Cruz, 1:50) overnight at 4ºC, followed by an additional 2 hr incubation with 30 μl protein G beads (Pierce) slurry at 4ºC. Proteins in the IP complex or total lysates were detected by Western blot. HA-A1R was detected by mouse HA.11 antibody followed by a cross-adsorbed anti-mouse secondary antibody.

For sequential GST pull-down-co-IP experiments, HEK293 cells were plated on 100mm dishes at 4x10^6/dish, and 16 hrs later transfected with HA-A1R, myc-Nrb, and GST or GST-RGS4 (at 1 μg, 7 μg and 2 μg, respectively). 48 hrs post-transfection, cells were stimulated and lysed in 350 μl IP buffer. Cell lysates were first incubated with 30 μl glutathione (GSH) agarose slurry (GE Healthcare Life Science) for 2 hrs at 4ºC. The bound GST-fusion proteins were then eluted with 200 μl free GSH (Sigma) at 25mM dissolved in IP buffer. The GSH eluates were then incubated with rat anti-HA antibody (1:100 dilution) overnight at

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4ºC followed by an additional 2 hr incubation with 20 µl protein G beads (Pierce) slurry at 4ºC. Quantitation of Western blots was performed using the LI-COR Odyssey Imaging System.

Measurement of cAMP levels. cAMP assays were performed using AlphaScreen® Assay Kit (PerkinElmer). In brief, CHO-K1 cells were cotransfected with cDNAs encoding HA-A1R together with Myc-Nrb or Myc-Nrb1-597. 48 hrs post-transfection, cells were collected in PBS. The pellet was then resuspended with the stimulation buffer (1x HBSS, 0.1% BSA, 0.5 mM IBMX, 5 mM HEPES, pH 7.4) and mixed with anti-cAMP acceptor beads. The mix was divided into 3 groups for the following treatments: (1) vehicle; (2) 10 µM forskolin (Sigma); (3) 10 µM forskolin and 5 µM R-PIA. 20min post-stimulation at 37ºC, biotinylated cAMP/streptavidin donor beads in lysis buffer (0.1% BSA, 0.3% Tween-20, 5 mM HEPES, pH 7.4) were added to the cells/acceptor beads mix. After 30 min incubation at room temperature, fluorescence intensity was analyzed on a Biotek Synergy2 plate reader using standard α-screen settings.

Live imaging of RGS4 translocation. Cos7 cells were transfected with cDNA encoding A1R-CFP and RGS4-YFP with either full length neurabin or Nrb1-597 for 48 hours. Cells were serum starved for 24 hours before imaging. Cells were also pretreated with adenosine deaminase (5 U/mL, Sigma) or 1 µM 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX, Sigma) overnight to eliminate adenosine in the medium before imaging. Live cell CFP and YFP images were taken at 30 second intervals on a Zeiss Observer Z1 epifluorescent microscope (Carl Zeiss) equipped with an Evolve EMCCD camera (Photometrics). Cells were stimulated with 1 µM R-PIA. Translocation of RGS4 for each cell was measured using a ratio of different plasma membrane ROIs to a single cytoplasmic reference ROI (ROI Ratio). The translocation responses were plotted as individual traces for each plasma membrane ROI. The responses were normalized to the frame prior to drug addition, t=0. Translocation measurements were plotted using Prism (Graphpad Software, Inc.). Quantification was performed by comparing the slopes of the responses after the initial maximum response is reached. The slope of the response was measured by determining the initial maximum response and calculating the slope of the response from this point through the end of the acquisition. The slopes for each experimental group were averaged and graphed as a box-and-whisker plot.

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Live-cell total internal reflection fluorescence (TIRF) imaging of RGS4 translocation. Cos7 or HEK293T cells were cotransfected with cDNA encoding A1R-CFP, RGS4-YFP, and neurabin (WT or mutant) for 24-48 hrs with Polyjet (Signagen). Control cells were only transfected with A1R-CFP and RGS4-YFP. Cells were serum starved and pretreated with 1 μM DPCPX overnight before imaging. Live cell TIRF images were taken for YFP at 30 second intervals on an Olympus IX83 microscope with the CellTIRF4-Line system and an Andor iXon Ultra EMCCD camera. For YFP excitation, a 491 nm cell* laser (Olympus) was utilized and all images were taken with a 150x TIRF objective (Olympus). CFP imaging was only performed during sample focusing to confirm presence of A1R in the cells being imaged for the time-lapse. For CFP excitation a 405 nm OBIS laser was used (Coherent). To stimulate the translocation, 1 μM R-PIA was added to the cells. Translocation for each cell was determined by taking an ROI of the cell and measuring intensity averages over time. The translocation responses for each cell in each experimental group were plotted as an average trace with SEM bars. The responses were normalized to the frame prior to drug addition, t=0. Quantification was performed by comparing the slopes of the responses after the initial maximum response is reached. The slope of the response was measured by determining the initial maximum response and calculating the slope of the response from this point through the end of the acquisition. The slopes for each experimental group were averaged and graphed as a box-and-whisker plot. The slopes were also statistically compared using an unpaired t-test with a 99% confidence level. Translocation measurements were plotted and statistically analyzed using Prism (Graphpad Software, Inc).

Statistical Analysis. All statistical analyses were performed using GraphPad Prism software (GraphPad Software Inc). Data were analyzed with two-tailed Student's *t*-tests. $p<0.05$ was considered statistically significant.

Results

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Neurabin is required for prolonged localization of RGS4 on the plasma membrane following A1R stimulation. RGS4 is mainly localized in cytosol. In order to turn off the long-lasting A1R signaling (Palmer et al., 1996; Ramkumar et al., 1991; Wetherington and Lambert, 2002), RGS4 needs to be localized at the plasma membrane in a prolonged fashion following A1R stimulation. We examined RGS4 localization in live cells before and after A1R stimulation by a selective agonist, R-PIA, using total internal reflection fluorescence (TIRF) imaging. In cells co-expressing neurabin with A1R and RGS4, a rapid and stable translocation of RGS4 from cytosol to plasma membrane was observed following R-PIA stimulation (Fig. 1A), which lasted the entire recording period (30 min, Fig. 1, B and E). However, in cells without neurabin expression, although R-PIA was still able to induce a rapid translocation of RGS4 to the plasma membrane, the membrane localization of RGS4 declined over time and returned to basal or near-basal levels by 30 min (Fig. 1, C and F). The recruitment of RGS4 to the plasma membrane in these cells was much more transient compared to that in cells with neurabin expression (Fig. 1, D and G). These data suggest that although not required for the initial recruitment of RGS4 to the plasma membrane by A1R activation, neurabin is crucial for stabilizing a prolonged localization of RGS4 at the plasma membrane.

A1R and RGS4 interact with the overlapping regions of neurabin. Neurabin has multiple functional domains (illustrated in Fig. 2A). We have previously defined aa146-453 as the A1R binding region (Chen et al., 2012). Consistently, A1R was readily co-immunoisolated with neurabin aa 1-597 (Nrb1-597, Fig. 2, B and F) but not with neurabin aa 454-593 (Nrb454-593, Fig. 2C) in cells stimulated with R-PIA. Intriguingly, RGS4 was also found to strongly interact with Nrb1-597 (Fig. 2, D and F) but showed no interaction with Nrb454-593 (Fig. 2E) following R-PIA stimulation. These data suggest that the interactions of neurabin with A1R and RGS4 both occur within neurabin aa 146-453.

We further mapped sequences within neurabin aa 146-453 (Nrb146-453) for direct interaction with A1R and RGS4 by generating and analyzing a series of deletion mutations within this region (illustrated in Fig. 3A). In *in vitro* GST pull-down assays, neurabin aa 331-453 (Nrb331-453) directly interacted with both A1R (aa 202-326, 3iloop plus C-terminal tail, regions on A1R that are responsible for neurabin interaction) (Fig. 3B) and RGS4 (Fig. 3C) whereas aa 146-330 failed to interact with either of these two

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proteins. Neurabin sequences that interact with A1R were further narrowed down to aa 383-413 (Fig. 3B). In particular, neurabin aa 404-413 is essential as deletion of these 10 amino acids abolished interaction of Nrb331-453 with A1R (Fig. 3B). Similarly, we also detected direct interaction of Nrb383-413 with RGS4 (Fig. 3C). However, deletion of aa 404-413 only slightly reduced Nrb331-453 interaction with RGS4. In addition, Nrb331-403 could still bind to RGS4, albeit to a lower extent compared with Nrb383-413 (Fig. 3C). On the other hand, Nrb331-403 failed to interact with A1R (Fig. 3B). Together, these data suggest that A1R and RGS4 bind to overlapping regions of neurabin (i.e., aa 383-413). While A1R binding requires a linear stretch of neurabin sequence (i.e. aa 404-413), RGS4 binding likely involves multiple non-contiguous amino acids within neurabin region aa 383-413.

Since sequence 404-413 is required for neurabin interaction with A1R, we further analyzed this region. Within this 10 aa region, there are two valines (V404 and V407), which are highly hydrophobic, and three arginines (R406, R408 and R410), which are positively charged. We generated mutant Nrb331-453 constructs with valines and arginines at these positions mutated to alanines, respectively, and tested their interactions with A1R (aa 202-326). Mutations at the two valine sites markedly decreased the binding of Nrb331-453 to A1R, whereas mutations at the three arginine sites only had a slight effect (Fig. 3D). These data suggest that the interaction interface between A1R and neurabin is likely hydrophobic and not influenced much by charges.

Domains of neurabin that mediate oligomerization are required for assembly of the A1R/neurabin/RGS4 ternary complex. Our data shown above suggest that A1R and RGS4 interact with overlapping regions of neurabin. The question then became whether neurabin interacts with A1R and RGS4 sequentially or the three proteins form a triple, or ternary, complex in cells. To address this, we performed sequential GST pull-down and coimmunoprecipitation (co-IP) experiments (procedure illustrated in Fig. 4A). As shown in Fig. 4B, Myc-tagged neurabin (Myc-Nrb), HA-A1R, and GST-RGS4 (but not GST alone) were co-isolated in the same complex from the lysates of cells stimulated with R-PIA. These data establish the formation of an A1R/neurabin/RGS4 ternary complex in cells upon agonist stimulation.

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Since A1R and RGS4 interact with overlapping regions of neurabin and the three protein form a ternary complex upon agonist stimulation, we speculate that A1R and RGS4 may not bind to the same neurabin molecule simultaneously. Instead, since neurabin forms homo-oligomers in cells through the coiled-coil and SAM domains (Oliver et al., 2002), A1R and RGS4 may each bind one monomer of neurabin to form the A1R/neurabin/RGS4 ternary complex (illustrated in Fig. 5A). To test this hypothesis, we examined whether neurabin lacking the coiled-coil and SAM domains can still scaffold the A1R-RGS4 interaction. Consistent with formation of neurabin homo-oligomers (MacMillan et al., 1999; Nakanishi et al., 1997; Oliver et al., 2002), we readily detected interaction between Myc and GFP-tagged full-length neurabin (Fig. 5B). Myc-Nrb1-597, in which the coiled-coil and SAM domains are deleted, failed to interact with GFP-tagged full-length neurabin (Fig. 5B). In cells expressing the full-length Myc-Nrb, we detected stable interaction between A1R and RGS4 (Fig. 5C). However, in cells expressing Nrb1-597, we were unable to co-immunoisolate RGS4 in the same complex with A1R, despite the fact that this neurabin truncation itself can be co-immunoisolated with A1R (Fig. 5C). Together with the fact that Nrb1-597 is able to interact with A1R and RGS4 individually (Fig. 2, B and D), these data suggest that domains mediating neurabin oligomerization are required for assembly of the A1R/neurabin/RGS4 ternary complex, and support the interaction model illustrated in Fig. 5A.

The neurabin oligomerization domains are required for stabilizing RGS4 on the cell surface and attenuating A1R signaling. Next we examined the importance of neurabin oligomerization domains in regulating RGS4 translocation in live cells. Consistent with our data shown in Fig. 1, in cells expressing the full-length neurabin, A1R activation with R-PIA led to a rapid and long-lasting redistribution of RGS4 to the plasma membrane (Fig. 6, A and B). RGS4 stayed associated with the plasma membrane for the entire recording period up to 60 min (Fig. 6D). On the other hand, in cells expressing Nrb1-597, RGS4 translocation to the cell surface was rather transient (Fig. 6C); plasma membrane association of RGS4 started to decline within 20 min after stimulation in most cells (Fig. 6, C and E). These data suggest that the neurabin oligomerization domains are crucial for the sustained plasma membrane localization of RGS4 induced by A1R stimulation.

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Since Nrb1-597 cannot stabilize RGS4 at the plasma membrane (Fig. 6, C and E), nor can it scaffold the interaction between A1R and RGS4 (Fig. 5C), we predict that expression of this mutant form of neurabin would not be able to attenuate A1R signaling in cells. We measured A1R-mediated inhibition of cAMP production stimulated by *R*-PIA in CHO-K1 cells, in which endogenous RGS4 is required for neurabin-mediated attenuation of A1R signaling (Chen et al., 2014). In cells expressing the full-length neurabin, A1R-mediated inhibition of cAMP production was abolished (Fig. 7A). However, expression of Nrb1-597 showed a negligible effect on A1R-mediated cAMP inhibition (Fig. 7A). Expression of either the full-length neurabin or Nrb1-597 had no effect on the A1R expression level (Fig. 7B). These data suggest that domains mediating neurabin oligomerization are required for attenuation of A1R signaling.

Discussion

In the present study, we demonstrate that in the presence of neurabin, A1R activation is able to induce prolonged localization of RGS4 on the plasma membrane (Fig. 1 and Fig. 6B). Both A1R and RGS4 interact with neurabin aa 146-453 in cells (Fig. 2). Further characterization of the interaction between neurabin and A1R and between neurabin and RGS4 reveal that A1R and RGS4 interact with overlapping sequences of neurabin (Fig. 3). Thus, in order to form a ternary complex (as shown in Fig. 4B), A1R and RGS4 likely each interacts with one neurabin molecule in a neurabin oligomer. Indeed, neurabin domains that are required for oligomerization are essential for formation of the A1R/neurabin/RGS4 ternary complex (Fig. 5C). Despite its ability to interact with A1R and RGS4 individually, the truncated neurabin mutant that cannot oligomerize (Nrb1-597) fails to stabilize plasma membrane localization of RGS4 (Fig. 6C) and is unable to attenuate A1R signaling (Fig. 7A). Taken together, these data reveal a novel mode of regulation of GPCR signaling by scaffolding proteins, specifically the formation of a ternary complex with the receptor and a regulator through homo-oligomerization (Fig. 5A).

Scaffolding proteins play crucial roles in regulation of GPCR trafficking and signaling (Romero et al., 2011; Walther and Ferguson, 2015). They can target receptors to specific subcellular compartments, determine the endocytic route of the receptor, and link the receptor to specific effectors and regulators.

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Although a number of scaffolding proteins are predicted to have oligomerization domains (Hu et al., 2015), the significance of oligomerization of these proteins in GPCR regulation remains largely elusive, except for homer proteins. The long forms of homer proteins form dimers and tetramers through the C-terminal coiled-coil domains (Hayashi et al., 2009). The resulting hub structure connects synaptic proteins and signaling components, and targets the group I mGluRs to postsynaptic compartments and their effectors (Bockaert et al., 2010). Our current study provides the first example of oligomerization being required for a scaffolding protein to link the receptor to a RGS protein to modulate receptor signaling strength. In addition to forming homo-oligomers, neurabin can form hetero-oligomers with its homolog, spinophilin (MacMillan et al., 1999; Nakanishi et al., 1997; Oliver et al., 2002). Since spinophilin does not interact with A1R, neurabin-spinophilin oligomerization in the postsynaptic compartments may reduce the ability of neurabin to attenuate A1R responses, adding another level of complexity in A1R regulation.

We have previously demonstrated that neurabin specifically links RGS4, but not its close homolog, RGS2, to A1R signaling (Chen et al., 2012). RGS4 is highly expressed in the brain (Larminie et al., 2004), and regulates multiple Gq or Gi-coupled receptors to modulate synaptic signaling and plasticity (Gerber et al., 2016). Plasma membrane localization of RGS proteins is critical for their ability to terminate G protein signaling (Hollinger and Hepler, 2002; Kach et al., 2012). RGS4 is mainly localized in the cytosol, and could be recruited to plasma membrane through interactions with GPCRs or Gα proteins (Roy et al., 2003). Using live-cell imaging, we demonstrate that RGS4 rapidly translocates to the plasma membrane upon A1R stimulation (Figs. 2 and 7), likely through direct interaction with the receptor or G proteins. In cells lacking neurabin expression or expressing the mutant neurabin, Nrb1-597, plasma membrane localization of RGS4 is transient (Figs. 2 and 7), and A1R-mediated cAMP inhibition is not affected (Fig. 7). On the other hand, when the full-length neurabin is present, plasma membrane localization of RGS4 is prolonged (Figs. 2 and 7) and A1R signaling is attenuated (Fig. 7). These results suggest that although not required for the initial membrane recruitment of RGS4, neurabin is essential for the sustained localization of RGS4 at the plasma membrane and subsequent attenuation of A1R signaling.

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We and others have shown that A1R does not undergo rapid phosphorylation following agonist stimulation (Chen et al., 2012; Palmer et al., 1996), which is often a prerequisite for arrestin binding to and desensitization of many GPCRs. Consistently, desensitization of A1R signaling takes place at a much slower pace than many GPCRs, including its homologs, adenosine A2R and A3R (Palmer et al., 1996; Ramkumar et al., 1991; Wetherington and Lambert, 2002). Collectively, our data suggest that the prolonged membrane recruitment of RGS4, which is scaffolded by neurabin, represents a critical mechanism for fine-tuning A1R signaling. In addition, it has been reported that A1R activation reduces M2 muscarinic acetylcholine receptor responsiveness in neurons (Shakirzyanova et al., 2006). The A1R-induced, sustained RGS4 membrane localization revealed in our current study may contribute to the cross-desensitization of M2 muscarinic receptor signaling following A1R agonist stimulation.

In summary, the current study reveals that homo-oligomerization of neurabin is required for stabilizing RGS4 on the plasma membrane to attenuate A1R signaling. Our mechanistic analysis of neurabin-mediated regulation of A1R signaling provides valuable information for the future development of therapeutic interventions targeting A1R in the nervous system.

Acknowledgements

The authors thank Dr. Christopher Cottingham (Morehead State University) for critical reading and helpful editing of the manuscript.

Authorship Contributions

Participated in research design: Chen, Booth, Zachariou, Jiao, Zhang, and Q. Wang.

Conducted experiments: Chen, Booth, H. Wang, R.X. Wang, Terzi.

Contributed new reagents or analytic tools: Booth, Zhang.

Performed data analysis: Chen, Booth, Jiao, Zhang and Q. Wang.

Wrote or contributed to the writing of the manuscript: Chen, Booth, Zachariou, Jiao, Zhang, and Q. Wang.

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Footnotes

This work was supported by National Institutes of Health grant MH081917.

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Figure legends

Fig. 1. A1R activation led to prolonged RGS4 localization at the plasma membrane of live cells with expression of neurabin and failed to do so when neurabin was absent. (A-D) Cos7 cells were transfected with A1R-CFP, RGS4-YFP, with or without neurabin in addition. (A) Representative TIRF images of RGS4 localization in neurabin-expressing cells before and after R-PIA stimulation. (B) 1μM R-PIA stimulated a sustained translocation of RGS4 to the plasma membrane in the presence of neurabin. Multiple translocation traces from different cells demonstrate uniform responses across the cells. n=13. Data are presented as mean ± SEM. 1μM R-PIA was added at t=0. (C) 1μM R-PIA stimulates a transient translocation of RGS4 to the plasma membrane in the absence of neurabin. Data are mean ± SEM. n=20. (D) The stability of RGS4 at the plasma membrane in Cos7 cells was analyzed by comparing the slope of the trace after the maximal response in each cell. ****, $p<0.0001$ between the slopes of cells with or without neurabin. (E-G) HEK293T cells were transfected with A1R-CFP, RGS4-YFP, with or without neurabin in addition. (E) A sustained translocation of RGS4 to the plasma membrane following R-PIA stimulation in the presence of neurabin. Data are mean ± SEM. n=14. R-PIA was added at t=0. (F) A transient translocation of RGS4 to the plasma membrane in the absence of neurabin. Data are mean ± SEM. n=16. (G) The stability of RGS4 at the plasma membrane in HEK293T cells was analyzed by comparing the slope of the trace after the maximal response in each cell. ****, $p<0.0001$ between the slopes of cells with or without neurabin.

Fig. 2. Neurabin a.a.146-453 is involved in interaction with both the A1R and RGS4 in cells. (A) Scheme of neurabin structural domains. (B) HEK293 cells co-transfected with cDNAs encoding HA-A1R and Myc-Nrb1-597 were stimulated with vehicle or R-PIA (1 μM, 5 min), then lysed and subjected to the IP assay using an HA antibody. (C) HEK293 cells co-expressing HA-A1R with GFP-Nrb454-593 were stimulated and subjected to the IP assay using an HA antibody. (D) HEK293 cells co-transfected with

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cDNAs encoding HA-A1R, Myc-Nrb1-597 and RGS4 were stimulated with vehicle or R-PIA (1 μM, 5 min), then lysed and subjected to the IP assay using an anti-RGS4 antibody. (E) HEK293 cells co-expressing HA-A1R, GFP-Nrb454-593 and RGS4 were stimulated and subjected to the IP assay using an anti-RGS4 antibody. Blots representative of at least three independent experiments are shown. (F) Quantitation of Nrb1-597 interactions with A1R or RGS4. n=4 for each condition. Data are expressed as fold change of Nrb1-597 in complex with the A1R or RGS4 over no-stimulation control (defined as 1.0 fold). Values shown are mean ± SEM. **, $p<0.01$, *R*-PIA stimulated *vs.* control.

Fig. 3. Binding to the A1R and RGS4 involves overlapping sequences within neurabin aa 146-453. (A) Schematic diagram of GST-fused WT and mutant neurabin a.a. 146-453 with deletions or truncations. Estimated binding strength of each structure to the A1R 3iloop or RGS4, as revealed by in vitro GST pull-down assays, is indicated. (B) *In vitro* GST pull-down assays with ^{35}S-labeled A1R 3iloop and C-tail (a.a. 202-326) as a probe. Bound [^{35}S]A1R202-326 was detected by autoradiograph. GST and GST-fused neurabin fragments were detected by Coomassie staining. Lanes are numbered as corresponding to structures shown in (A). Input probe represents 1/10 of the total input in each reaction. (C) *In vitro* GST pull-down assays with ^{35}S-labeled RGS4 as a probe. Bound [^{35}S]RGS4 was detected by autoradiograph. GST and GST-fused neurabin fragments (numbered corresponding to structures shown in A) were detected by Coomassie staining. Input probe in *left* and *right* panels represents 1/5 of the total input in each reaction whereas input probe in the *middle* panels represents 1/30 of the total input in the reaction. (D) *In vitro* GST pull-down assays testing interaction between ^{35}S-labeled A1R202-326 and GST-Nrb331-453 with indicated mutations within neurabin aa 404-413. Bound [^{35}S]A1R202-326 was detected by autoradiograph. Input probe represents 1/20 of the total input in each reaction. Images are representative of at least three experiments for each pull down assay.

Fig. 4. Neurabin forms a triple complex with the A1R and RGS4 through oligomerization. (A) Flowchart of the sequential pull-down and IP procedure. (B) Formation of a neurabin/A1R/RGS4 ternary complex.

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HEK293 cells co-expressing Myc-Nrb and HA-A1R, together with GST or GST-RGS4, were stimulated with vehicle or R-PIA (1 μM) for 5 min. Cell lysates were subjected to sequential GST pull-down and co-IP. Blots representative of three independent experiments showing the A1R, neurabin and RGS4 in total lysates and final eluate after sequential pull-down and co-IP.

Fig. 5. Truncated neurabin lacking coiled-coil and SAM domains failed to scaffold formation of the A1R-RGS4 complex. (A) A working model for A1R/neurabin/RGS4 complex formation. (B) Truncated neurabin lacking coiled-coil and SAM domains did not form oligomers. HEK293 cells co-expressing GFP-Nrb with Myc-Nrb or GFP-Nrb1-597 with Myc-Nrb1-597 were lysed and subjected to the IP assay using an anti-Myc antibody. Blots representative of at least three independent experiments are shown. (C) No complex formation between A1R and RGS4 was detected upon *R*-PIA stimulation in cells expressing Nrb1-597. HEK293 cells co-expressing HA-A1R, Myc-Nrb1-597 and RGS4 were stimulated with *R*-PIA (1 μM) for 5 min. Cell lysates were subjected to IP assay using an anti-Myc antibody and analyzed by Western blot. Blots representative of four independent experiments are shown.

Fig. 6. A1R activation led to sustained localization of RGS4 at the plasma membrane of live cells with expression of full-length neurabin, but failed to do so when Nrb1-597 was expressed. (A) Representative images of RGS4 localization before and after 1 μM R-PIA stimulation in Cos7 cells co-expressing A1R-CFP and RGS4-YFP with the full length neurabin. (B) Representative traces of RGS4 showing sustained RGS4 translocation within different plasma membrane ROIs in a single Cos7 cell with full length neurabin co-expressed with A1R-CFP and RGS4-YFP; n=6. 1 μM R-PIA was added at t=0. (C) Representative traces showing transient RGS4 translocation within different plasma membrane ROIs in a single Cos7 cell with Nrb1-597 co-expressed with A1R-CFP and RGS4-YFP; n=4. 1 μM R-PIA was added at t=0. (D) Multiple translocation traces from different cells expressing the full length neurabin demonstrate uniform responses of sustained RGS4 localization at the plasma membrane across the cells;

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n=8. (E) Multiple traces from different cells expressing Nrb1-597 demonstrate non-uniform responses of RGS4 translocation across the cells with a general transient nature; n=6. (F) The sustained plasma membrane localization of RGS4 was analyzed by comparing the slope of the trace after the maximal response in each cell. ***, $p<0.001$ between the slopes of cells in the presence of full length neurabin and Nrb1-597A.

Fig. 7. Expression of Nrb1-597 failed to cause attenuation of A1R signaling. (A) Expression of Nrb1-597 had no effect on A1R-mediated inhibition of cAMP production. CHO-K1 cells expressing HA-A1R alone (plus empty vector) or in combination with either neurabin WT or Nrb1-597 were treated with 10 μM forskolin alone or forskolin plus 1 μM *R*-PIA. The average cAMP concentration at the basal level in cells was $3.45x10^{-9}$M, and became $3.89x10^{-8}$M with forskolin stimulation (i.e. forskolin-alone). Data are expressed as fold change in cAMP production over forskolin-alone control (defined as 1.0 fold). n=6 for cells expressing the A1R alone; n=8 for cells expressing the A1R and neurabin; n=7 for cells expressing the A1R and Nrb1-597. *, $p<0.05$, *R*-PIA stimulated *vs.* control by Student's *t* test. (B) Representative Western blots showing expression of HA-A1R, Nrb, and Nrb1-597 in the same preparation of cells analyzed for cAMP production. Note that a degradation band of full-length Nrb was detected (by Myc antibody) following the assay sample preparation.

Fig. 1

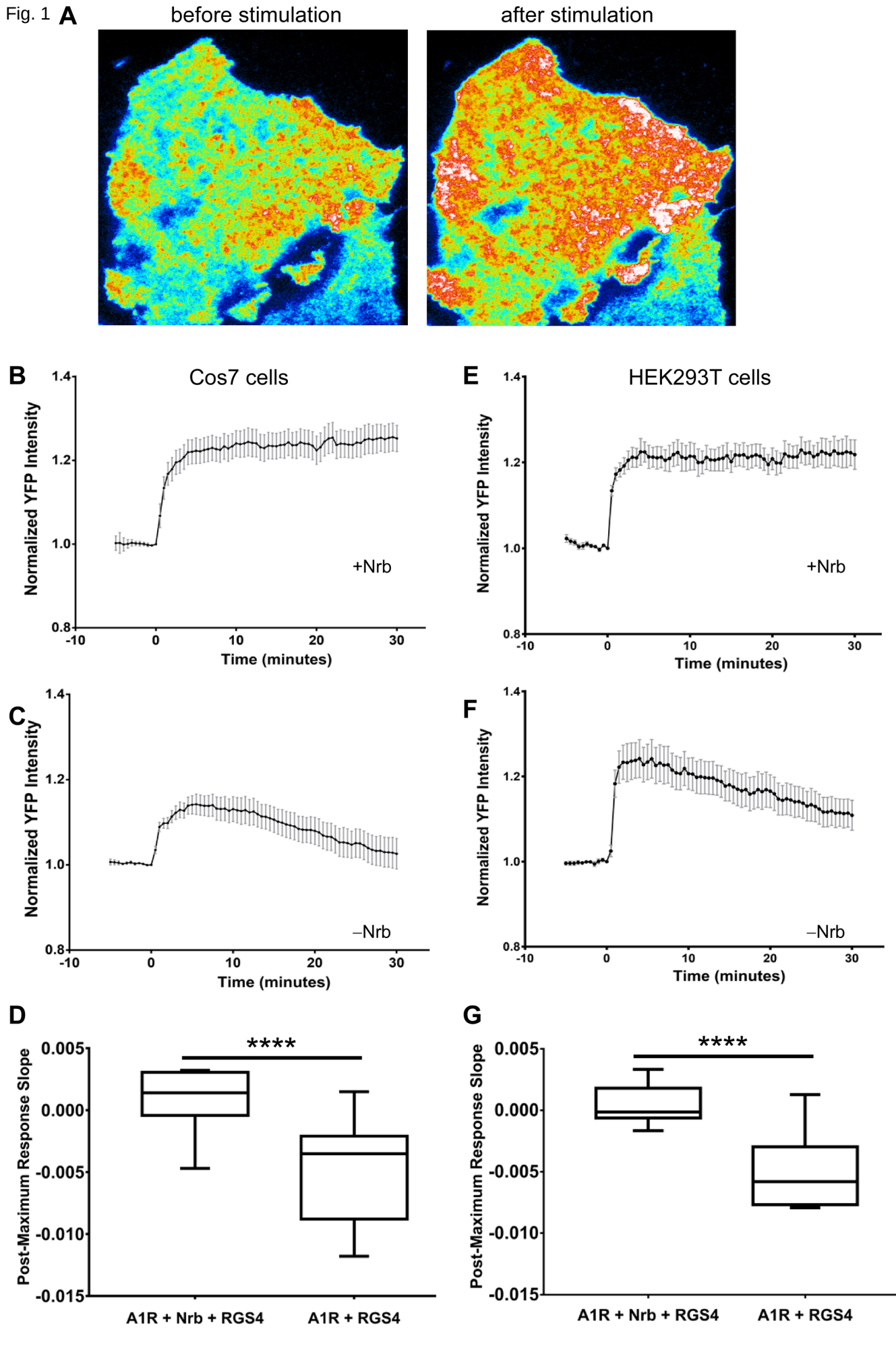
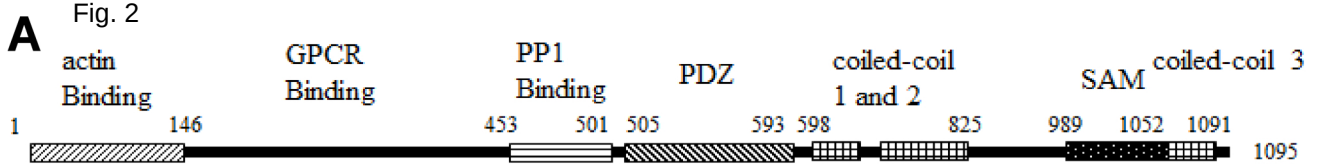
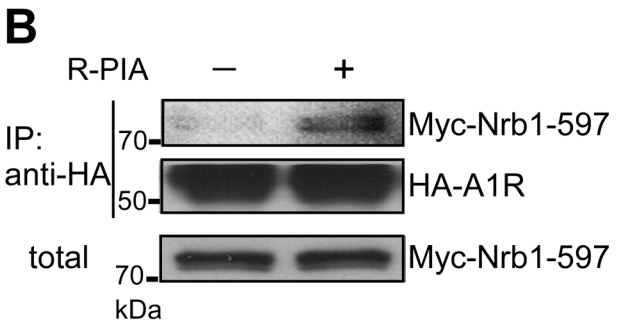
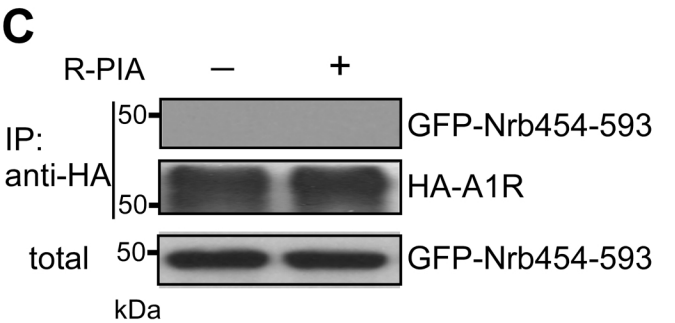
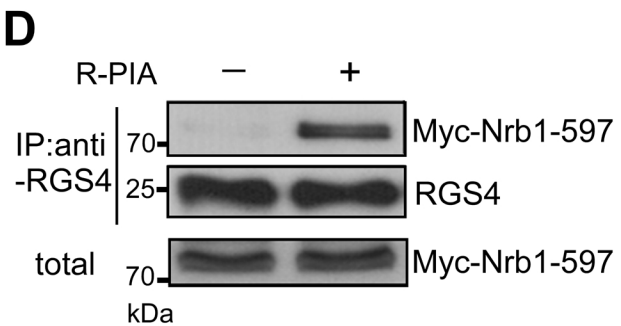

A
before stimulation
after stimulation
B
Cos7 cells
Normalized YFP Intensity
1.4
1.2
1.0
0.8
-10
0
10
20
30
Time (minutes)
+Nrb
C
Normalized YFP Intensity
Time (minutes)
–Nrb
D
Post-Maximum Response Slope
0.005
0.000
-0.005
-0.010
-0.015
****
A1R + Nrb + RGS4
A1R + RGS4
E
HEK293T cells
Normalized YFP Intensity
Time (minutes)
+Nrb
F
Normalized YFP Intensity
Time (minutes)
–Nrb
G
Post-Maximum Response Slope
****
A1R + Nrb + RGS4
A1R + RGS4

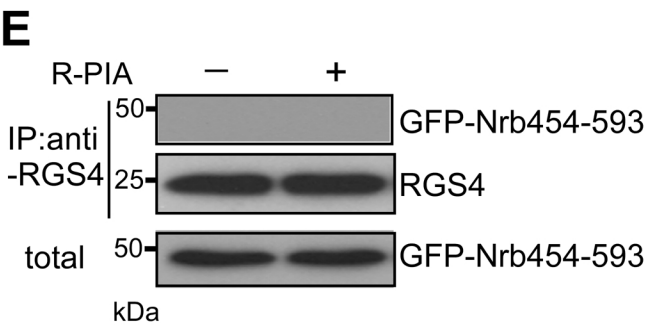
Fig. 2


A
actin Binding
GPCR Binding
PP1 Binding
PDZ
coiled-coil 1 and 2
SAM
coiled-coil 3
1
146
453
501
505
593
598
825
989
1052
1091
1095


B
R-PIA – +
IP: anti-HA
70
50
Myc-Nrb1-597
HA-A1R
total
70
Myc-Nrb1-597
kDa


C
R-PIA – +
IP: anti-HA
50
50
GFP-Nrb454-593
HA-A1R
total
50
GFP-Nrb454-593
kDa


D
R-PIA – +
IP:anti -RGS4
70
25
Myc-Nrb1-597
RGS4
total
70
Myc-Nrb1-597
kDa


E
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IP:anti -RGS4
50
25
GFP-Nrb454-593
RGS4
total
50
GFP-Nrb454-593
kDa

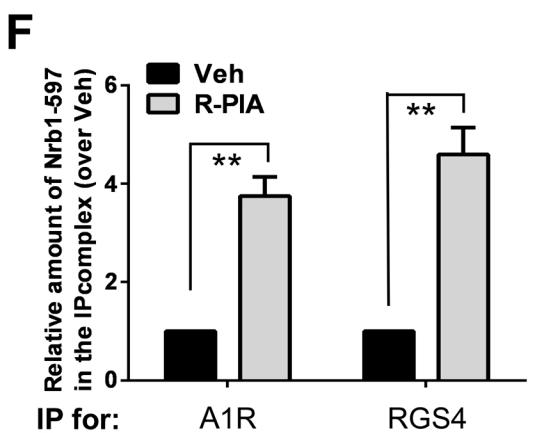

F
Veh
R-PIA
Relative amount of Nrb1-597 in the IPcomplex (over Veh)
0
2
4
6
**
**
IP for:
A1R
RGS4

Fig. 3

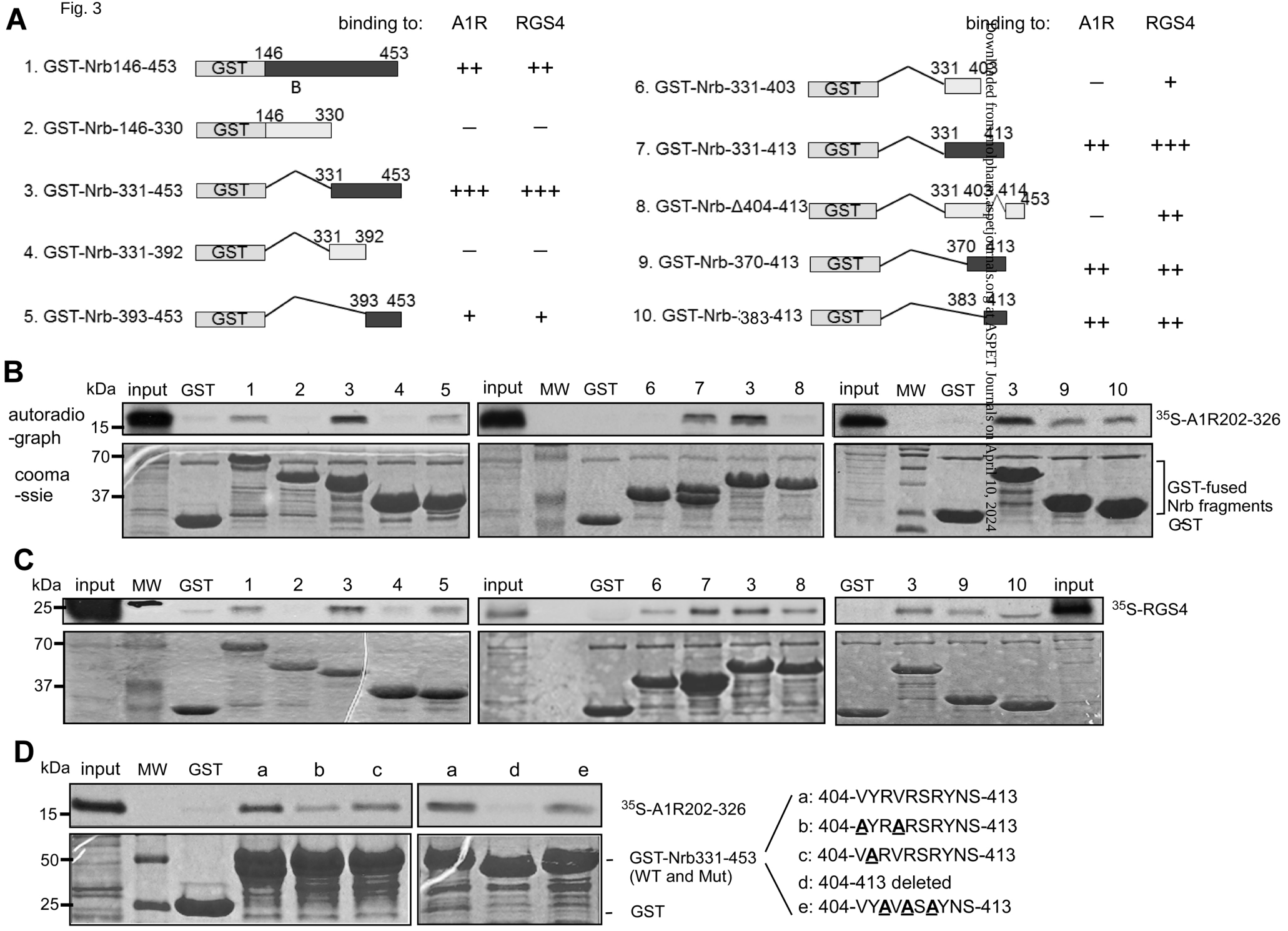
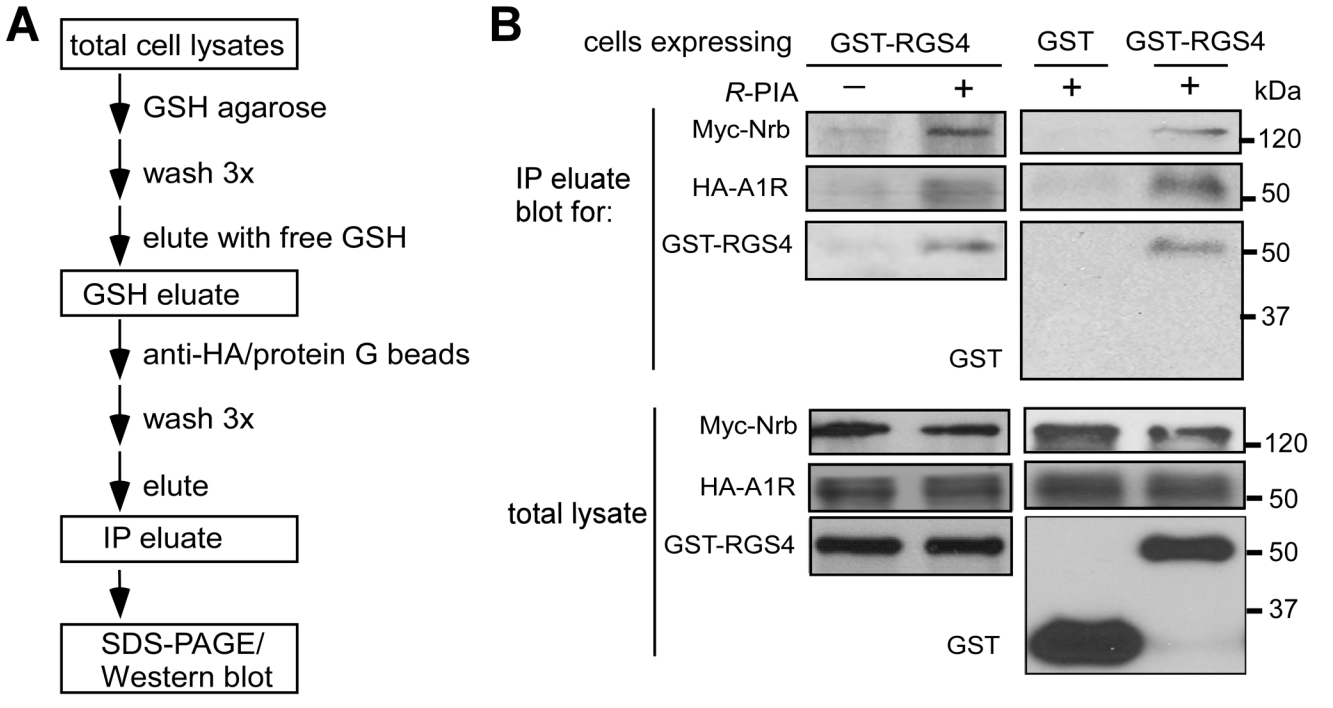


Fig. 4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total cell lysates
GSH agarose
wash 3x
elute with free GSH
GSH eluate
anti-HA/protein G beads
wash 3x
elute
IP eluate
SDS-PAGE/
Western blot
B
cells expressing
GST-RGS4
GST
GST-RGS4
R-PIA
–
+
+
+
kDa
IP eluate
blot for:
Myc-Nrb
HA-A1R
GST-RGS4
GST
120
50
50
37
total lysate
Myc-Nrb
HA-A1R
GST-RGS4
GST
120
50
50
37

Fig. 5

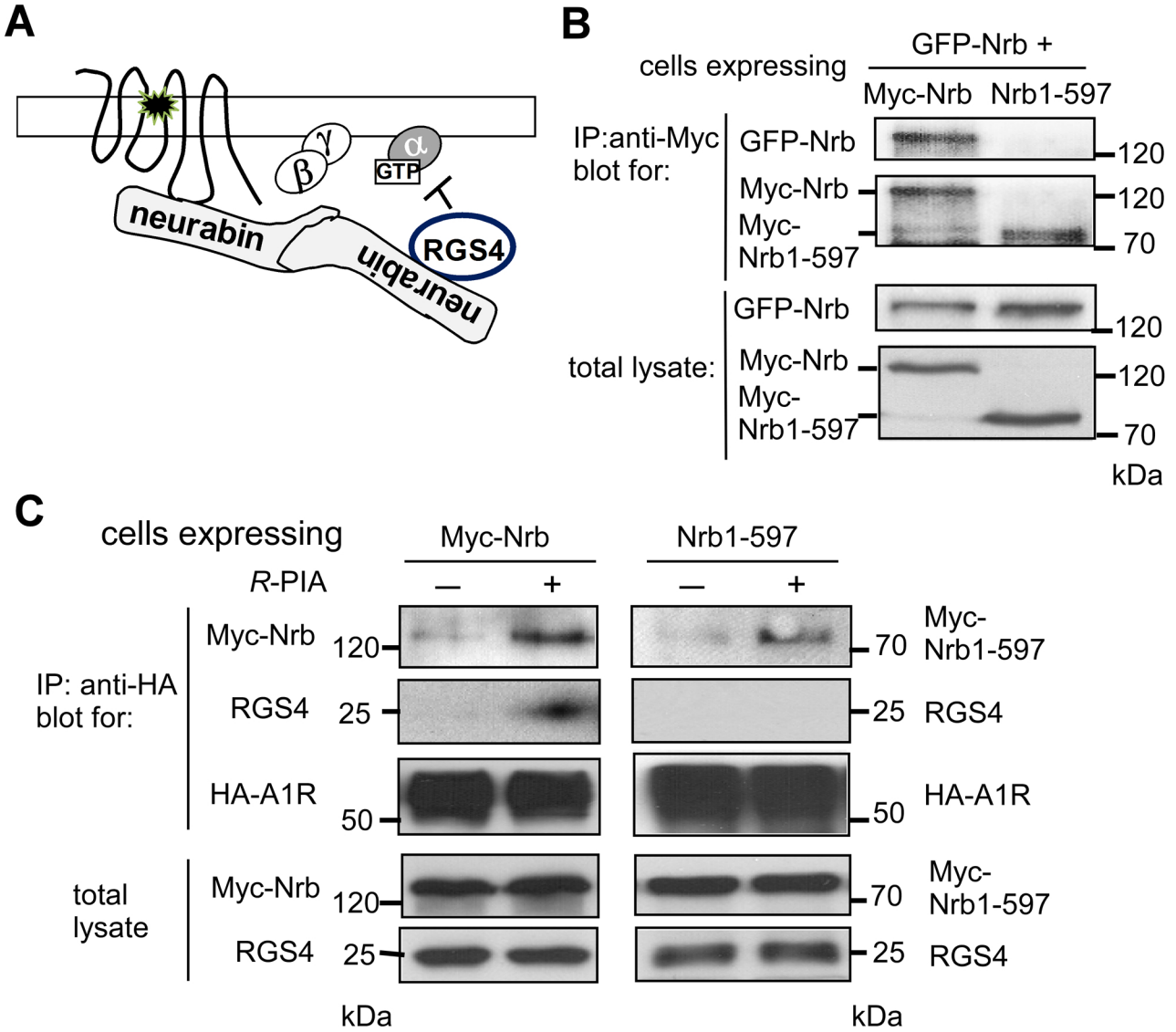

A
γ
β
α
GTP
neurabin
neurabin
RGS4
B
cells expressing
GFP-Nrb +
Myc-Nrb
Nrb1-597
IP:anti-Myc blot for:
GFP-Nrb
Myc-Nrb
Myc-Nrb1-597
120
120
70
total lysate:
GFP-Nrb
Myc-Nrb
Myc-Nrb1-597
120
120
70
kDa
C
cells expressing
Myc-Nrb
Nrb1-597
R-PIA
—
+
—
+
IP: anti-HA blot for:
Myc-Nrb
RGS4
HA-A1R
120
25
50
70
25
50
Myc-Nrb1-597
RGS4
HA-A1R
total lysate
Myc-Nrb
RGS4
120
25
70
25
Myc-Nrb1-597
RGS4
kDa
kDa

Fig. 6

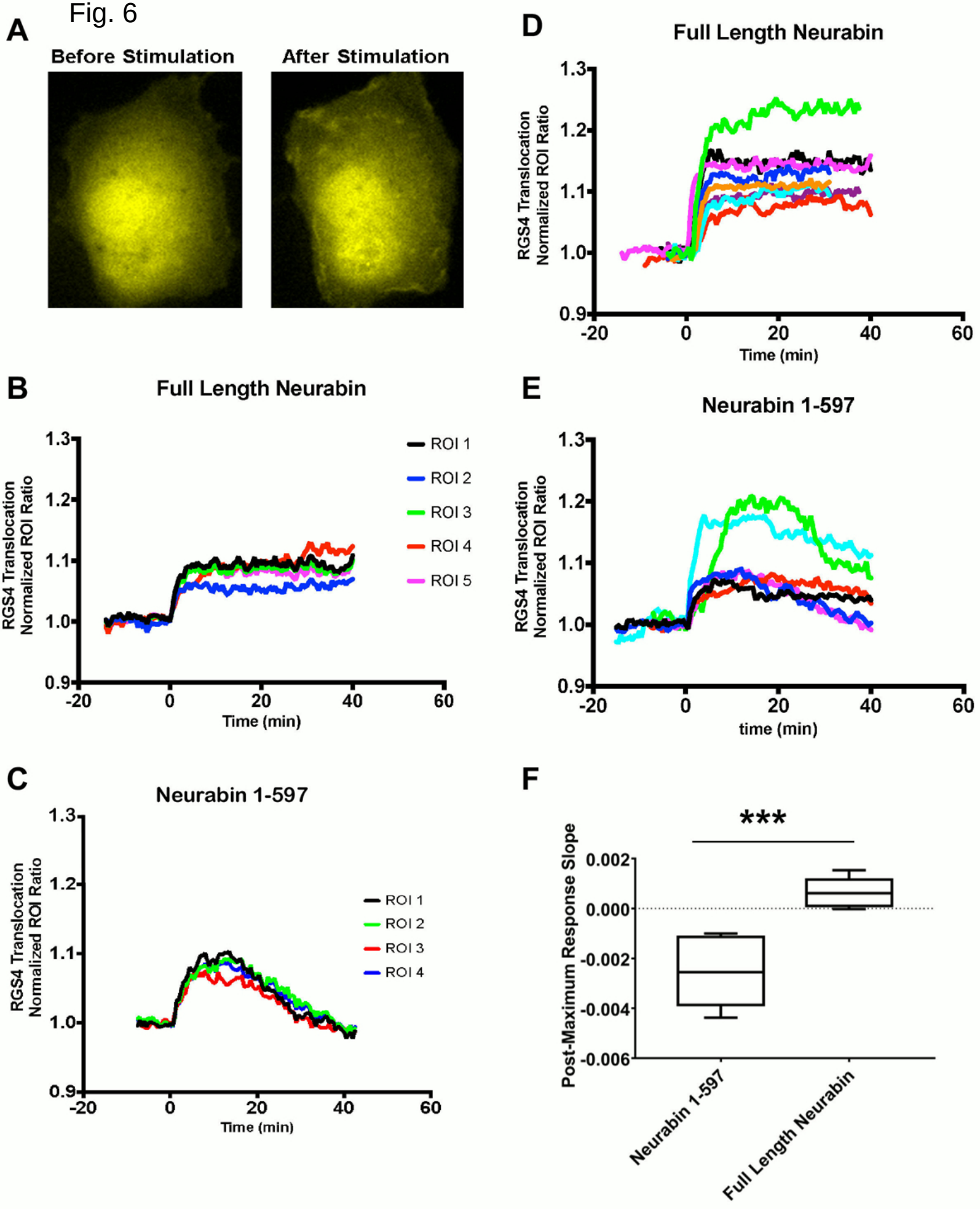
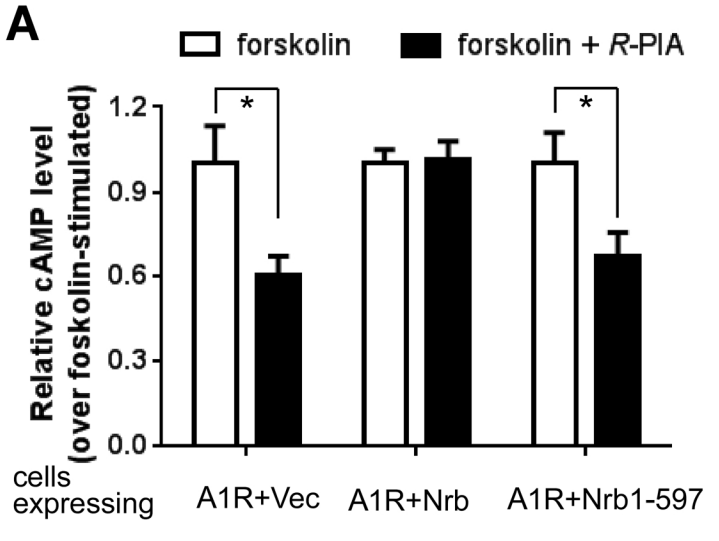
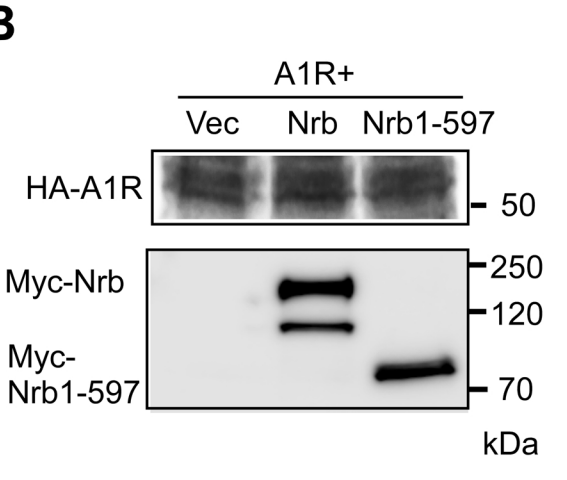

A
Before Stimulation
After Stimulation
B
Full Length Neurabin
RGS4 Translocation Normalized ROI Ratio
Time (min)
ROI 1
ROI 2
ROI 3
ROI 4
ROI 5
C
Neurabin 1-597
RGS4 Translocation Normalized ROI Ratio
Time (min)
ROI 1
ROI 2
ROI 3
ROI 4
D
Full Length Neurabin
RGS4 Translocation Normalized ROI Ratio
Time (min)
E
Neurabin 1-597
RGS4 Translocation Normalized ROI Ratio
time (min)
F
***
Post-Maximum Response Slope
Neurabin 1-597
Full Length Neurabin

Fig. 7


A
forskolin
forskolin + R-PIA
Relative cAMP level (over foskolin-stimulated)
1.2
0.9
0.6
0.3
0.0
*
*
cells expressing
A1R+Vec
A1R+Nrb
A1R+Nrb1-597


B
A1R+
Vec
Nrb
Nrb1-597
HA-A1R
50
Myc-Nrb
250
120
Myc-Nrb1-597
70
kDa