Specificity of Gβ and γ subunits to SNARE complex both at rest and after α_{2a} adrenergic receptor stimulation

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Summary

Specific Gβγ dimers interact with SNARE complex following presynaptic α_{2a}AR activation in both adrenergic and non-adrenergic neurons.

Abstract

G proteins are major transducers of signals from G-protein coupled receptors (GPCRs). They are made up of α, β, and γ subunits, with 16 Gα, 5 Gβ and 12 Gγ subunits. Though much is known about the specificity of Gα subunits, the specificity of Gβγs by a given GPCR and those that activate each effector *in vivo* is not clear. In a previous paper, we were able to identify Gβ and Gγ interacting specifically neuronal α_{2a}-adrenergic receptors (α_{2a}ARs). However, it still remains unclear how G protein specificity plays out in α_{2a}AR-mediated effector interactions. This receptor is the major autoreceptor that acts as a brake to synaptic transmission in adrenergic neurons and the sympathetic nervous system, and as heteroreceptors on other neurons throughout the brain. Here, we examined the *in vivo* specificity of Gβγ to the soluble NSF attachment proteins (SNARE) complex upon presynaptic α_{2a}AR activation in both adrenergic (auto-α_{2a}ARs) and non-adrenergic (heteroreceptor) neurons for the first time. To understand how this interaction underlies inhibition of synaptic transmission in diverse physiological functions such as spontaneous locomotor activity, anesthetic sparing, and working memory enhancement, we applied a quantitative MRM proteomic analysis of Gβ and Gγ subunits co-immunoprecipitation from transgenic FLAG-α_{2a}ARs and wildtype mice. We evaluated Gβ and Gγ subunit binding to the SNARE complex with and without activation of auto-α_{2a}AR and hetero-α_{2a}AR using epinephrine. Without epinephrine stimulation, $G\beta_1$ and $G\gamma_3$ interact with SNARE. When auto-α_{2a}ARs are activated, $G\beta_1$, $G\beta_2$, and $G\gamma_3$ interact with SNARE. Further understanding of *in vivo* Gβγ specificity to various effectors offers new insights into the multiplicity of genes for Gβ and Gγ, and the mechanisms underlying GPCR signaling through Gβγ subunits to this interaction as a potential therapeutic target.

Introduction

Gβγ dimers play essential roles in regulating cellular signaling cascades. Numerous studies have demonstrated that different Gβ and Gγ subunits may selectively interact to form unique Gβγ dimers (*1-3*) which in turn target specific G protein-coupled receptors (GPCRs) and effectors (*3-12*). Although many of these Gβγ-effector interactions and downstream signaling cascades have been *in vitro*, factors controlling the specificity of Gβγ dimers to associated with unique GPCRs or their effectors, as well as the *in vivo* expression levels of Gβ and Gγ subunits, or Gβγ dimers remains unclear. To understand the specificity of Gβγ interactions, we developed targeted multiple reaction monitoring (MRM) proteomic methods that allow us to evaluate the protein abundance and subcellular localization of neuronal Gβ and Gγ subunits(*13*).

Soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE), one of Gβγ effectors, is a key protein involved in synaptic transmission, an essential method of inter-neuronal communication in both the central and peripheral nervous systems. The arrival of an action potential activates presynaptic voltage-gated calcium channels and increases the intracellular calcium concentration at the presynaptic terminal. Synaptotagmin 1 (Syt1), a calcium sensor, senses the increase in Ca^{2+} concentration and interacts with SNARE, comprised of SNAP25, syntaxin 1A, and synaptobrevin (VAMP) (*14-18*). Mediated by the Syt1 interaction, the SNARE complexes zip up, bringing the vesicle membrane and plasma membrane into close proximity, and leading to vesicle fusion (*19*). As membrane fusion continues, neurotransmitter is released into the synaptic junction, permitting communication between neurons which conveys both external and internal sensory inputs, and promotes responsive behaviors in postsynaptic cells(*18*). Thus, it is crucial to understand how synaptic transmission is regulated in healthy

subjects, and mechanisms of dysfunction in neurological diseases.

Inhibitory GPCRs, which are $G_{i/o}$ coupled, play an important role in regulating synaptic transmission by acting as auto- and hetero-receptors in feedback mechanisms limiting neurotransmitter release (*20-24*). The α_{2a}ARs is one of the best studied inhibitory GPCRs, with excellent availability of knock out and transgenic mice with tagged receptors (*25-29*). The activation of α_{2a}ARs inhibits synaptic transmission by Gβγ in both adrenergic and non-adrenergic neurons such as glutamatergic, serotonergic and dopaminergic neurons (*30-33*). In particular, numerous studies have reported that Gβγ subunits released from activated α_{2a}ARs reduce presynaptic Ca^{2+} influx through the inhibition of VDCC (*34-36*) and inhibit exocytosis through direct interaction with the SNARE complex (*37-39*).

The inhibition of presynaptic norepinephrine release via direct interaction between Gβγ and SNARE has been shown to be a primary mechanism inhibiting exocytosis by α_{2a}ARs in the central amygdala (*39*). Delaney *et al*. showed that inhibition of norepinephrine release, where high levels of presynaptic α_{2a}ARs are present, is mediated by a $G_{i/o}$-coupled receptor, and that Gβγ dimers released from active α_{2a}ARs directly interact with the SNARE complex downstream of calcium entry to inhibit norepinephrine release (*39*). Although the Gβγ–SNARE interaction has been well-characterized in α_{2a}AR-mediated inhibition of synaptic transmission, the specificity of Gβγ dimer isoforms for the SNARE complex is not known. Here, we evaluated which Gβγ dimers released from epinephrine (epi)-activated α_{2a}ARs interact with SNARE to regulate synaptic transmission. Interestingly, we discovered that only a subset of Gβ and Gγ subunits released from activated α_{2a}ARs interact with SNARE. Further understanding of α_{2a}AR-mediated Gβγ specificity to SNARE may lead to the development of new therapeutic targets that complement or contrast

with currently available α_{2a}AR-targeted drugs.

Results

Gβγ and SNARE interaction. To study the Gβγ specificity to SNARE upon activation of α_{2a}ARs, we used synaptosomes from wildtype, α_{2a}ARs KO, and FLAG-α_{2a}ARs mice. Using SNAP25 antibody, Gβγ and the SNARE complex were co-immunoprecipitated (coIP) and detected by Western blot (Fig. 1A). To further validate that we were indeed IPing the ternary SNARE, we detected VAMP2 and Syntaxin1 in addition to SNAP25 from these coIP samples (Supplementary Fig. 1). Interestingly, the Gβγ and SNARE interaction was found in both unstimulated and stimulated coIP samples, but greater amounts were present in epi-stimulated coIP samples of wildtype and FLAG-α_{2a}-ARs mice (Fig.1B and C). We know from a number of studies (*40*, *41*) that the interaction between Gβγ and SNARE is low affinity. However, the interaction is specific, as little non-specific binding was observed in IgG controls. Quantitative western blot analysis estimated approximately 1μg Gβγ was pulled down with SNAP25 per half brain (Supplementary Fig. 2).

$G\beta_1$, $G\beta_2$, $G\beta_4$, $G\gamma_2$, $G\gamma_3$, and $G\gamma_4$ interact specifically with SNARE upon epinephrine stimulation. To examine which neuronal Gβ and Gγ subunits interact with SNARE upon the activation of α_{2a}-ARs, we applied a quantitative MRM method (*42*) to TCA precipitated and trypsin digested SNAP25 coIP samples (approximately 11 coIPs per condition per genotype) of wildtype (WT), α_{2a}AR KO, and FLAG-α_{2a}AR mouse synaptosomes (see Materials and Methods for details). Previously, only a subset of neuronal Gβ and Gγ subunits were identified to be selectively associated with α_{2a}AR(*42*). WT samples were used to detect Gβγ–SNARE interactions mediated by epinephrine stimulation, including both auto- and hetero-α_{2a}ARs, while α_{2a}AR KO samples were used to detect non α_{2a}AR-mediated interactions. Lastly, FLAG-α_{2a}AR samples were

used to detect presynaptic auto-α_{2a}AR-mediated Gβγ-SNARE interactions. We used unstimulated WT (WT no epi), α_{2a}AR KO (α_{2a}AR KO no epi), and FLAG-α_{2a}AR (FLAG-α_{2a}ARs no epi) samples as controls to detect basal Gβγ–SNARE interactions. By comparing the amount of Gβ and Gγ subunits detected per mg of lysate between stimulated WT (WT + epi), α_{2a}AR KO (α_{2a}AR KO +epi), and FLAG-α_{2a}AR (FLAG-α_{2a}AR +epi), we can detect not only auto-receptor mediated Gβγ-SNARE interactions, but also quantify those mediated by heteroreceptor interactions.

We used IgG alone controls (without primary antibody) to identify nonspecific interactions of Gβ and Gγ subunits. We compared the amount of Gβ subunit detected per mg of lysate between WT IgG +epi and WT +epi samples and we found $G\beta_1$, $G\beta_2$, and $G\beta_4$ significantly interacted with SNARE upon α_{2a}AR stimulation (Fig. 2). $G\beta_1$ was detected significantly higher than $G\beta_2$ and $G\beta_4$ subunits. As expected, we did not identify interaction between $G\beta_5$ and SNARE following α_{2a}AR activation.

Next, we identified the Gγ subunits that interact with SNARE following receptor activation. We found enrichment of the $G\gamma_2$, $G\gamma_3$, and $G\gamma_4$ subunits with SNARE upon epi stimulation (Fig.3). $G\gamma_3$ is the most abundant followed by $G\gamma_2$ and$G\gamma_4$, respectively. We detected a similar level of $G\gamma_2$ and $G\gamma_4$ associated with SNARE. Interestingly, we were not able to detect an interaction between $G\gamma_{12}$ and SNARE (Fig. 3). Although $G\gamma_{12}$ is specifically bound to α_{2a}AR (*42*), it may not interact with SNARE but rather with other Gβγ effectors such as VDCCs (see Discussion). Even though we are not detecting specific Gβγ dimers in this study, according to our data, we can postulate 9 different combinations of Gβγ dimers ($G\beta_1\gamma_2$, $G\beta_1\gamma_3$, $G\beta_1\gamma_4$, $G\beta_2\gamma_2$, $G\beta_2\gamma_3$, $G\beta_2\gamma_4$, $G\beta_4\gamma_2$, $G\beta_4\gamma_3$, and $G\beta_4\gamma_4$) which might interact with SNARE upon α_{2a}ARs activation. These dimers may occur in the brain and play roles in α_{2a}AR mediated Gβγ-SNARE interactions. Based on the detected

abundances of $G\beta_1$ and $G\gamma_3$, $G\beta_1\gamma_3$ may be the most likely dimer to interact with SNARE following receptor stimulation. Further biochemical analysis will be needed to validate the presence of these $G\beta\gamma$ dimers and their specificities for SNARE upon α_{2a}AR activation in both adrenergic and non-adrenergic neurons.

Basal interaction of $G\beta\gamma$ and SNARE. We showed in Fig 1B and C that $G\beta\gamma$ and SNARE interact in unstimulated coIPed samples of wildtype and FLAG-α_{2a}-ARs mice. To understand the basal $G\beta\gamma$–SNARE interaction without epi stimulation, we compared IgG control to no epi coIPed samples from α_{2a}AR KO and FLAG-α_{2a}AR synaptosomes using the quantitative mass spectrometry via multiple reaction monitoring (MRM). α_{2a}AR KO samples were used to identify non-α_{2a}AR-mediated basal interactions while FLAG-α_{2a}AR samples were used to detect auto-α_{2a}AR-mediated $G\beta\gamma$-SNARE interactions. Here, we detected all neuronal $G\beta$ subunits but found only $G\beta_1$ significantly interacting with SNARE in both α_{2a}AR KO and FLAG-α_{2a}AR no epi conditions compared to that of the IgG no epi condition (Figure 4). Interestingly, the level of $G\beta_1$ between these two conditions was not different. This suggests that the interaction of $G\beta_1$ with SNARE may be basal and not mediated through α_{2a}AR. Moreover, we detected $G\gamma_3$ interacting with SNARE in the unstimulated conditions of both genotypes compared to IgG control (Figure 5). Similar to the $G\beta$ subunit, we did not see any difference in the level of $G\gamma_3$ interacting with SNARE between α_{2a}AR KO and FLAG-α_{2a}AR no epi samples suggesting that this is, once again, basal, non-α_{2a}AR mediated interaction. Various GPCRs have been implicated to mediate presynaptic inhibition through this mechanism(*43*). This may be caused by one of these.

$G\beta_1$, $G\beta_2$, and $G\gamma_3$ interact specifically with SNARE upon auto-α_{2a}ARs activation. As previously described, the adrenergic auto-α_{2a}AR inhibits presynaptic neurotransmitter release

through the interaction between Gβγ and SNARE(*39*). To test whether auto-α_{2a}ARs and presynaptic hetero-α_{2a}ARs utilize different Gβγ dimers upon activation, we coIPed samples from α_{2a}AR KO and FLAG-α_{2a}AR synaptosomes and again applied the quantitative MRM method to understand auto-α_{2a}ARs mediated Gβγ-SNARE interaction. α_{2a}AR KO (no epi) and FLAG-α_{2a}AR (no epi) samples were used as controls to identify non-auto-α_{2a}ARs mediated interactions, and α_{2a}AR KO +epi samples were used to detect non-α_{2a}AR-mediated Gβγ-SNARE interactions. By comparing the result from α_{2a}AR KO and FLAG-α_{2a}AR to wildtype, we can speculate the Gβ and Gγ specificity to SNARE mediated by hetero-α_{2a}ARs.

Here, we were able to detect a subset of Gβ and Gγ subunit interactions with SNARE upon auto-α_{2a}AR activation. When we compared the results from α_{2a}AR KO +epi and FLAG-α_{2a}AR +epi synaptosomes, we identified that $G\beta_1$ and $G\beta_2$, but not $G\beta_4$, enrich with SNARE following auto-α_{2a}AR activation (Fig.6). We also detected significantly more $G\beta_1$ than $G\beta_2$ (Fig.6).

In contrast to the 3 Gγ subunits enriched with SNARE upon stimulation of WT containing both auto- and hetero-α_{2a}ARs, we only detect the enrichment of $G\gamma_3$ with SNARE following auto-α_{2a}AR activation (Fig. 7). Basally, $G\gamma_3$ was found to interact with SNARE (Fig 5). These findings suggest that $G\beta_1\gamma_3$ may be basally interacting with SNARE, as well as after epi stimulation of auto-α_{2a}AR, whereas $G\beta_2\gamma_3$ is present only after epi stimulation.

Discussion

Numerous *in vitro* studies have been performed to determine the effectors of Gβγ and their downstream signaling cascades; however, only a few studies have attempted to identify the specificity of Gβγ isoforms to their effectors. Here, we addressed the *in vivo* specificity of Gβγs to the SNARE complex, as a part of the Gβγ-SNARE interaction that modulates synaptic

transmission. Gβγs can interact with the SNARE complex regardless of receptor activation, and only a subset of neuronal Gβγ dimers, from $G\beta_1$, $G\beta_2$, $G\beta_4$, $G\gamma_2$, $G\gamma_3$, and $G\gamma_4$, appear to interact with SNARE (Fig. 2 and 3) following epinephrine stimulation. Upon the activation of auto-α_{2a}ARs, only $G\beta_1$, $G\beta_2$, and $G\gamma_3$ interact with SNARE (Fig. 6 and 7). From 9 possible Gβγ dimers, auto-α_{2a}ARs utilize only two Gβγ dimers, $G\beta_1\gamma_3$ and $G\beta_2\gamma_3$, to inhibit neurotransmitter release through the Gβγ-SNARE interaction. We postulate $G\beta_1\gamma_3$ to be associated with SNARE basally while $G\beta_2\gamma_3$ only associated upon auto-α_{2a}ARs activation.

From biochemical studies, we know which Gβγ dimers can form (*1-3, 44, 45*), but there is nothing known about which Gβγ dimers are present *in vivo* or underlie particular physiologically relevant functions *in vivo*. Some Gβ and Gγ subunits were found to be ubiquitously expressed, while others are localized in specific brain regions and cell types (*46-51*). Numerous genetic deletion studies and knockout animal studies have suggested specific roles for different Gβ and Gγ subunits in intact cells and mice (*12, 52*). However, it is not clear how particular Gβγ dimers form or how they localize to particular sites in cells. Researchers suspect that the different affinities between Gβ and Gγ subunits, in combination with the expression and localization of individual Gβ and Gγ subunits, may determine which Gβγ dimers are active in a given cell (*46*). Here, we find a surprising specificity in which Gβ and γ subunits associate with the SNARE complex. From our results, $G\beta_1\gamma_3$ and $G\beta_2\gamma_3$ may be specific for auto-α_{2a}AR-mediated Gβγ–SNARE interactions (Fig. 6 and 7), while $G\beta_4\gamma_2$ and $G\beta_4\gamma_4$ may be specifically utilized by presynaptic hetero-α_{2a}ARs. Thus, this study provides a window into the specificity of Gβγ dimer function *in vivo.*

Interestingly, $G\beta_1$ was determined to specifically interact with SNARE (Fig.2), although it

was not found associated with auto-α_{2a}ARs in co-IPs (*42*). This suggests that $G\beta_1$ may have a unique role in the basal association with the SNARE complex. So far, the role of this basal Gβγ association with the SNARE complex is not known, but it is well-known that basal exocytotic events (measured electrophysiologically as mini EPSCs) can be regulated by $G_{i/o}$ GPCR agonists(*53*). Receptor-independent regulation of minis by Gβγ is currently under active investigation.

Our studies of the specificity of Gβγ subunits for α_{2a}ARs and the SNARE complex suggest that the specificity may be encoded by an association of a particular GPCR with a subset of Gβγs. For example, we've shown that $G\gamma_2$ and $G\gamma_4$ are selectively associated with presynaptic hetero-α_{2a}ARs as well as auto-α_{2a}ARs (*42*). We hypothesize that the abundance and affinity of each neuronal Gβ and Gγ subunit, and their localization at the presynaptic terminal, may affect Gβγ subunit specificity to SNARE complexes. In our previous receptor study comparing the protein abundance detected with receptors, we speculated that $G\gamma_2$, $G\gamma_3$, and $G\gamma_4$ associated with auto-α_{2a}ARs while $G\gamma_{12}$ is specific for hetero-α_{2a}AR (*42*). However, in the current study, we found $G\gamma_2$, and $G\gamma_4$ may also be hetero-α_{2a}AR-specific. This may be due to the quantification of abundance. As previously stated, we can accurately determine the expression patterns and selectivity of neuronal Gβ and Gγ subunits in any given sample(*13*), however, the absolute quantification is difficult due to technical challenges associated with sample preparations (*54*).

In both Western blot and quantitative MRM studies, we detected Gβγ-SNARE interactions in the basal state, without receptor stimulation. Because the 'no epi' condition was identical to that of the previous receptor study, and it was clear that the α_{2a}AR did not basally interact with Gβ (*42*), it is surprising to see this basal interaction of Gβγ and SNARE (Fig.4 and 5). The detection of Gβ

and Gγ subunits with SNARE without receptor activation in FLAG-α_{2a}ARs suggests that Gβγ may be pre-coupled to SNARE before receptor activation (Fig. 4 and 5). The fast rate of Gβγ-SNARE-mediated inhibition (*55*) also supports the hypothesis that Gβγ, SNARE, and other synaptic proteins may be scaffolded together prior to receptor activation to facilitate Gβγ-SNARE interaction.

Previously, we identified Gβγ binding sites on both amino- and carboxy-terminal regions of SNAP25 (*41, 56, 57*), far enough apart to not both be occupied by a single Gβγ. Using SNAP25Δ3 mice with a truncated c-terminus SNAP25, we see a two-fold reduction in Gβγ-SNARE interaction, as well as an increased in stress-induced hyperthermia, defective spatial learning, impaired gait, supraspinal nociception(*58*), and enhancement of long-term potentiation at Schaffer collateral-CA1 synapse (*59*). However, the physiological and biochemical importance of these interaction sites still remains unclear. We might speculate that the two sites have different functions; the amino terminal region may be a Gβγ binding (or "holding") site that can hand Gβγ off to the physiologically important carboxy-terminal interaction site (*57*). Activation of α_{2a}ARs could produce a conformational change to promote Gβγ translocation from the amino terminus to the carboxy terminus, to compete with Syt1 to inhibit exocytosis. It will be interesting to determine the stoichiometry of Gβγ binding to SNARE complexes, and whether identical Gβγ dimers bind to both amino- and carboxy-termini of SNARE. Detailed quantitative analysis in combination with SNAP25 mutagenesis studies will be necessary to answer these questions.

The specificity of Gβγ dimers to the SNARE complex is not fully understood, but its importance and the relationship of GPCRs to this interaction have been well-studied. *In vitro* binding studies of calcium-triggered exocytosis demonstrated that different Gβγ dimers may exhibit functional differences in their ability to modulate SNARE activity (*56*). Hamid et al

demonstrated that $5HT_{1b}$ and $GABA_B$ receptors inhibit exocytotic release at the same synaptic terminal through different mechanisms (*53*), with $5HT_{1b}$ receptor-mediated inhibition of exocytosis using the Gβγ-SNARE interaction, while $GABA_B$ receptor-mediated inhibition working through Gβγ-VDCC interaction and decreased calcium entry. The authors suggested that this difference may depend on the N-terminal SNAP25 residues. The association of specific Gβγ subunits to GPCRs, such as $5HT_{1b}$ and $GABA_{B,}$ and to SNARE may add a further layer of regulation of Gβγ-effector selectivity. Future studies to understand this specificity will clarify Gβγ isoforms important for both receptor and effector binding.

Materials and Methods

Animals. Adult, male FLAG-tagged alpha2a adrenergic receptors (α_{2a}-ARs), α_{2a}-AR knockout (KO), and wildtype mice (*60*) were decapitated and brain tissues were immediately homogenized to produce crude synaptosomes as described below. To minimize post-mortem differences, all tissues were processed in parallel. All animal handling and procedures were conducted in accordance with the Care and Use of Laboratory Animals of the National Institutes of Health and approved by the Vanderbilt Institutional Animal Care and Use Committee.

Drugs. Epinephrine (catalog E4642), prazosin (catalog P7791), and propranolol (catalog P0884) were purchased from Sigma-Aldrich.

Antibodies. For immunoprecipitation, rabbit anti-SNAP25 (Sigma, S9684) and rabbit ChromePure IgG (Jaskson Immuno Research, 011-030-003) were used. For the western blot analysis, mouse anti-SNAP25 (Santa Cruz, sc-376713, 1:500) and rabbit anti-Gβ (Santa Cruz, sc-378, 1:10,000 and 1:5000) were used. HRP-conjugated secondary antibodies were obtained from Perkin-Elmer and Jackson Immunoresearch and used at the following dilutions: goat anti-mouse (1:10,000), and mouse anti-rabbit light chain specific (1:7,500).

Synaptosome preparation, stimulation, and lysate protocol. Crude synaptosomes were isolated from mouse brain tissue, as described previously (*61-63*) in the presence of 1mM ethylenediaminetetraacetic acid and stimulated with 100µM epinephrine (epi). This mimics the local synaptic concentration of epinephrine and it is a commonly used concentration in α_{2a}ARs studies(*64-66*). Samples were frozen in liquid nitrogen and stored at -80°C.

Co-immunoprecipitation. Similar to the co-immunoprecipitation (coIP) of α_{2a}ARs and Gβγ (*42*), we coIPed SNARE and Gβγ from the precleared lysates (Fig.1A). Precleared lysates were aliquoted 400ul per tube. Instead of anti-HA or FLAG antibodies, they were incubated with an anti-SNAP25 coIP antibody. Because homozygous SNAP25 knockout mice exhibit neonatal lethally (*67*), we used rabbit IgG as a control.

Elution and trichloroacetic acid (TCA) precipitation of coIPs. Samples were eluted using 100mM glycine (pH 2.5). 40µL of glycine was added to coIP beads and placed on a room temperature vortex shaker at medium speed for 8 mins and centrifuged at 5,000 x g for 2mins to pellet beads. Elutants were transferred to clean 1.5mL Eppendorf tubes. The elution was repeated a second time, and the elutants were pooled together. For western blot analysis, one tube per condition per genotype was saved separately Remaining elutants were pooled per genotype per condition and TCA precipitated to concentrate the Gβ and Gγ subunits for MRM (*42*). All samples were stored at -80°C freezer for the western blot or MRM analysis.

Immunoblot Analysis. The analysis was done as described previously (*42*). We used 15%, instead of 10% SDS-PAGE gel.

Heavy labeled peptide cocktail. A heavy labeled peptide cocktail was made as described previously (*13*).

Quantitative MRM of Gβ and Gγ subunits. As described previously (*42*), coIP samples

containing Gβ and Gγ subunits were separated by SDS-PAGE, in-gel trypsin digested, and analyzed by scheduled multiple reaction monitoring (MRM) using a TSQ vantage triple quadrupole mass spectrometer (Thermo Scientific) coupled directly to a nanoAcquity liquid chromatography system (Waters).

Statistical analysis. One-way analysis of variance (ANOVA) with a Tukey post hoc test and student T tests were used to account for differences in protein abundance of Gβ and Gγ subunits (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). All statistical tests were performed using GraphPad Prism v.7.0 for Windows, (GraphPad Software, La Jolla, California, USA, www.graphpad.com).

Supplementary Materials

Figure 1. Western blot analysis of SNARE complex.

Figure 2. Quantification of Gβ precipitated with SNAP25.

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Authors Contribution

Y.Y., W.H.M., K.M.B. and H.E.H. participated in research design and preliminary study. Y.Y. conducted experiments and data analysis. W.H.M. ran mass spectrometry and helped with data analysis. K.H., A.K., R.G., and L.H. contributed in mouse breeding and sampling. Y.Y. A.K., and H.E.H. wrote or contributed to the writing of the manuscript. All authors reviewed the results and approved the final version of the manuscript.

Competing Interests Statement

There are no conflicts of interest on the part of any author and no financial and/or non-financial competing interests in relation to the work described.

Figure legends

Figure 1. Co-immunoprecipitation of SNARE and Gβγ. Co-immunoprecipitation (coIP) experimental protocol (**A**), and representative western blot of coimmunoprecipitation of SNAP25 and Gβγ in wildtype (**B**) or FLAG-α_{2a}AR (**C**) mice following the stimulation of synaptosomes with (+, 100μM epi) or without epi (-). We detected Gβγ in the pulldown regardless of receptor stimulation (**B-C**). coIP: co-immunoprecipitated fraction; Sup: depleted supernatant

Figure 2. Gβ specificity to SNARE upon α_{2a}AR activation. Quantification of Gβ subunits interacting with SNARE in wildtype synaptosomes (N=8, unless otherwise noted). $G\beta_1$, $G\beta_2$, and $G\beta_4$ interact specifically with SNARE upon α_{2a}AR activation with epi. Data are presented as mean ± SEM. One-way ANOVA* $p<0.05$, **$p<0.01$, *Post hoc* analysis by Tukey's multiple comparison.

Figure 3. Gγ specificity to SNARE upon α_{2a}AR activation. Quantification of Gγ subunits interacting with SNARE in wildtype synaptosomes (N=8,unless otherwise noted). $G\gamma_2$, $G\gamma_3$, and $G\gamma_4$ may specifically interact with SNARE upon α_{2a}AR activation. Data are presented as mean ± SEM. One-way ANOVA* $p<0.05$, **$p<0.01$, *Post hoc* analysis by Tukey's multiple comparison.

Figure 4. Gβ subunits interacting basally with SNARE. Quantification of Gβ subunits interacting with SNARE basally (N=8, unless otherwise noted). $G\beta_1$, but not $G\beta_2$, specifically interacting with SNARE in both α_{2a}AR KO and FLAG-α_{2a}AR no epi conditions compared to that of IgG no epi. Data are presented as mean ± SEM. One-way ANOVA* $p<0.05$, **$p<0.01$, *Post hoc* analysis by Tukey's multiple comparison.

Figure 5. Gγ subunits interacting basally with SNARE. Quantification of Gγ subunits interacting with SNARE basally (N=8, unless otherwise noted). $G\gamma_3$ specifically interacted with SNARE in both α_{2a}AR KO and FLAG-α_{2a}AR no epi conditions compared to that of IgG no epi. Data are presented as mean ± SEM. One-way ANOVA* $p<0.05$, **$p<0.01$, *Post hoc* analysis by Tukey's multiple comparison

Figure 6. Gβ specificity to SNARE upon auto-α_{2a}ARs activation. Quantification of Gβ subunits interacting with SNARE in α_{2a}AR KO and FLAG-α_{2a}AR synaptosomes (N=8, unless otherwise noted). $G\beta_1$ and $G\beta_2$ specifically interact with SNARE upon auto-α_{2a}AR activation. Data are presented as mean ± SEM. One-way ANOVA* $p<0.05$, **$p<0.01$, *Post hoc* analysis by Tukey's multiple comparison.

Figure 7. Gγ subunit specificity to auto-α_{2a} adrenergic receptors. Quantification of Gγ subunits interacting with SNARE in α_{2a}ARs KO and FLAG-α_{2a}AR synaptosomes (N=8, unless otherwise noted). $G\gamma_3$ specifically interacts with SNARE upon auto-α_{2a}AR activation. Data are presented as

mean ± SEM. One-way ANOVA* $p<0.05$,, **$p<0.01$, *Post hoc* analysis by Tukey's multiple comparison.

Figure 8. **Summary Figure**. While the Gβγ-SNARE interaction may exist basally (ex. $G\beta_1\gamma_3$), only a subset of Gβγ dimers made with $G\beta_1$, $G\beta_2$, $G\beta_4$, $G\gamma_2$, $G\gamma_3$, and $G\gamma_4$ (ex. $G\beta_2\gamma_3$) specifically interact with SNARE upon presynaptic α_{2a}AR activation in both adrenergic and non-adrenergic neurons.

Figure 1.

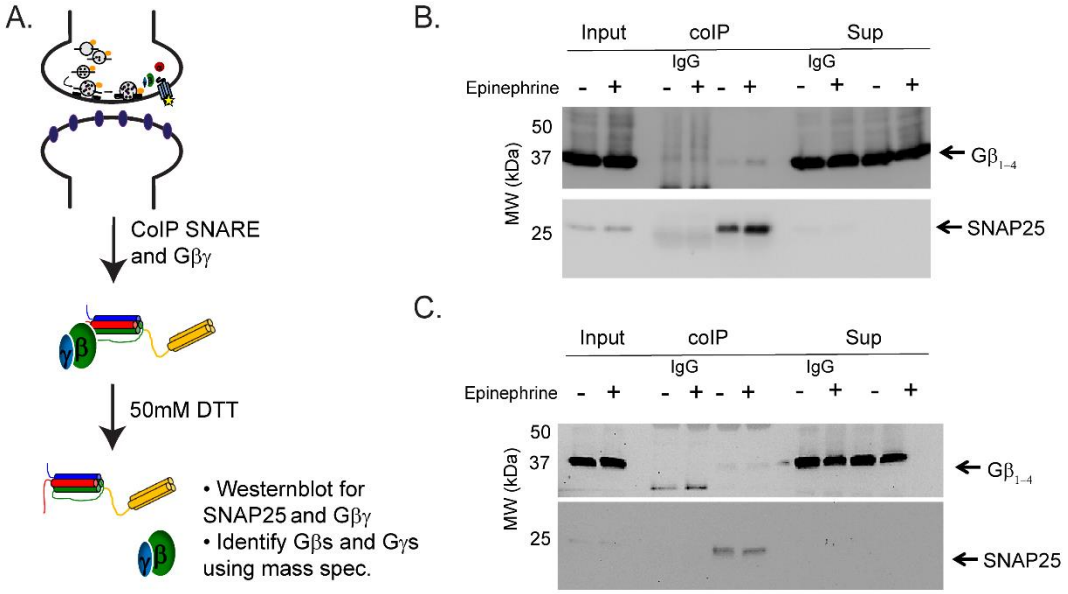

A.
CoIP SNARE
and Gβγ
50mM DTT
• Westernblot for
SNAP25 and Gβγ
• Identify Gβs and Gγs
using mass spec.
B.
Input
coIP
Sup
IgG
IgG
Epinephrine
- + - + - + - + - +
50
37
25
MW (kDa)
Gβ1-4
SNAP25
C.
Input
coIP
Sup
IgG
IgG
Epinephrine
- + - + - + - + - +
50
37
25
MW (kDa)
Gβ1-4
SNAP25

Figure 2.

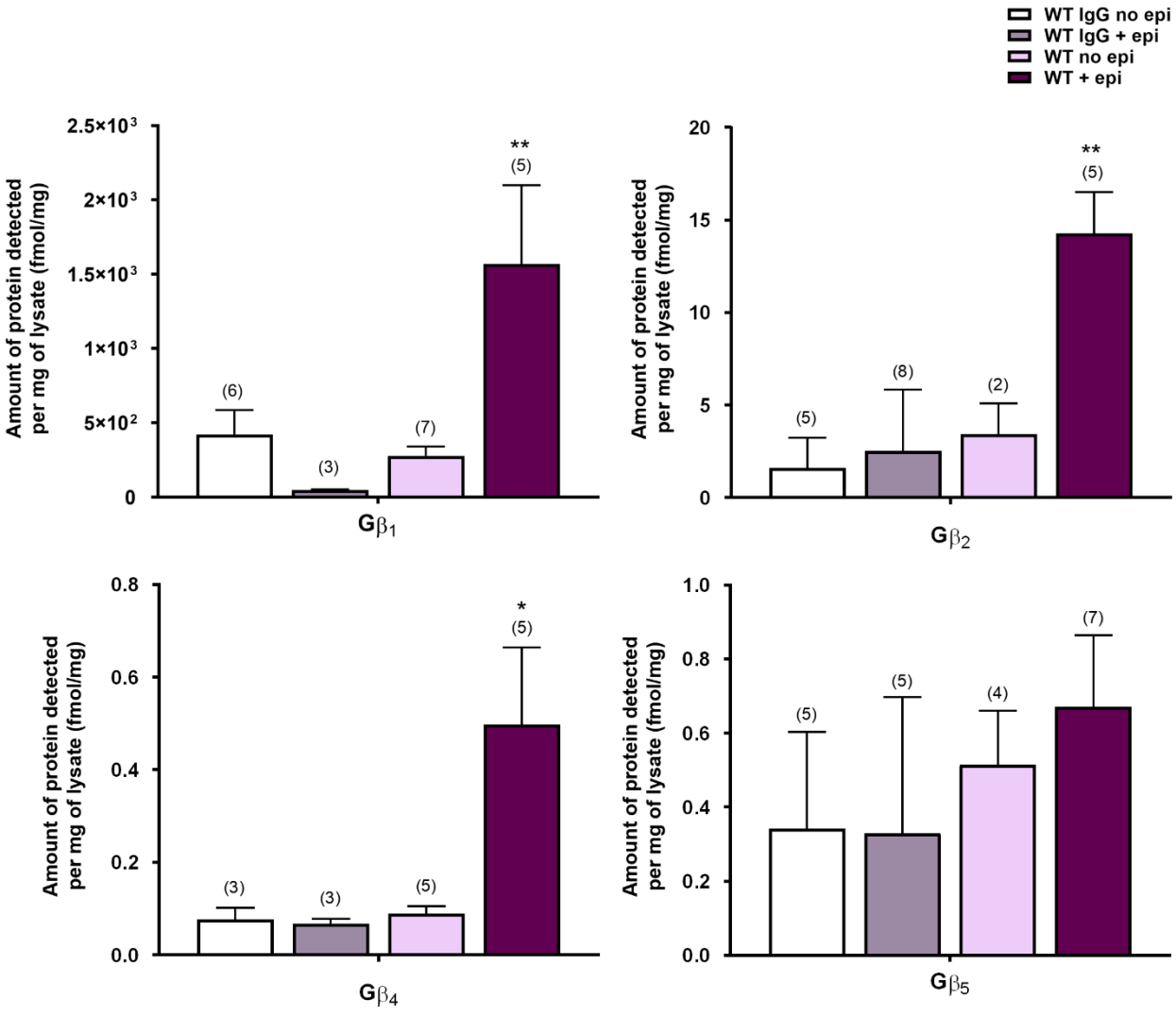

WT IgG no epi
WT IgG + epi
WT no epi
WT + epi
Amount of protein detected per mg of lysate (fmol/mg)
2.5×10³
2×10³
1.5×10³
1×10³
5×10²
0
(6)
(3)
(7)
**
(5)
Gβ1
20
15
10
5
0
(5)
(8)
(2)
**
(5)
Gβ2
0.8
0.6
0.4
0.2
0.0
(3)
(3)
(5)
*
(5)
Gβ4
1.0
0.8
0.6
0.4
0.2
0.0
(5)
(5)
(4)
(7)
Gβ5

Figure 3.

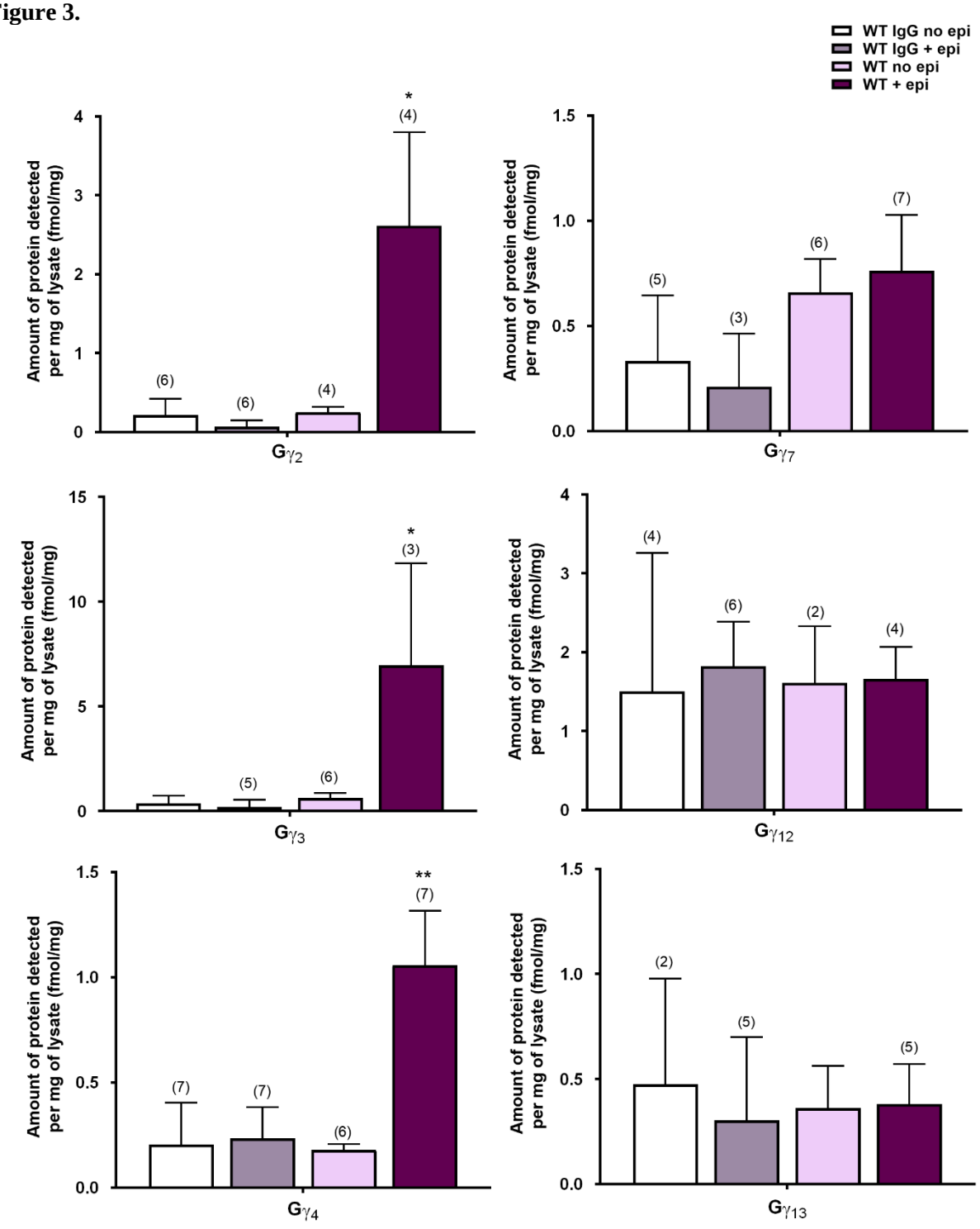

WT IgG no epi
WT IgG + epi
WT no epi
WT + epi
Amount of protein detected per mg of lysate (fmol/mg)
Gγ2
Gγ7
Gγ3
Gγ12
Gγ4
Gγ13
*
**
(6)
(4)
(5)
(3)
(7)
(2)

Figure 4.

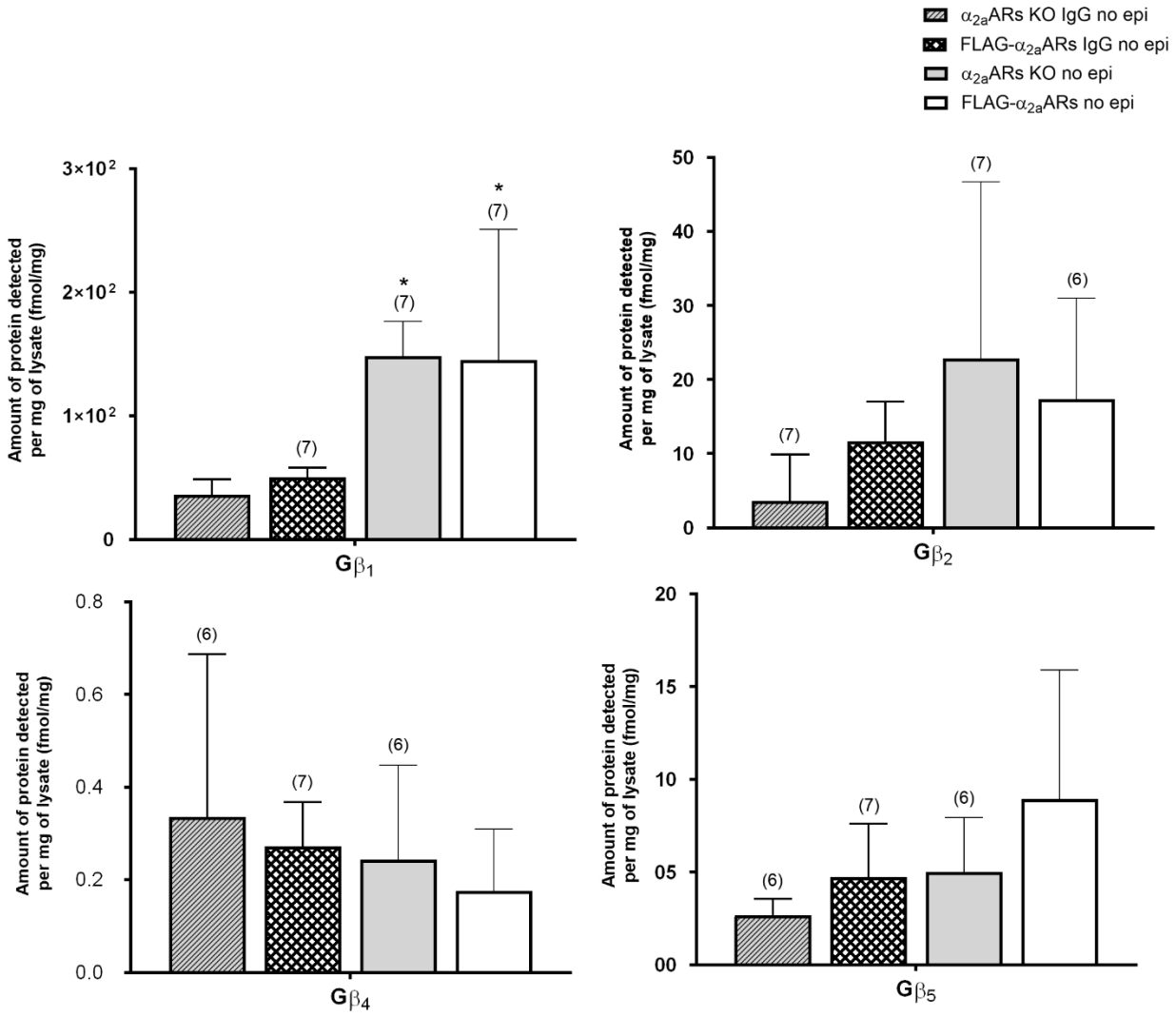
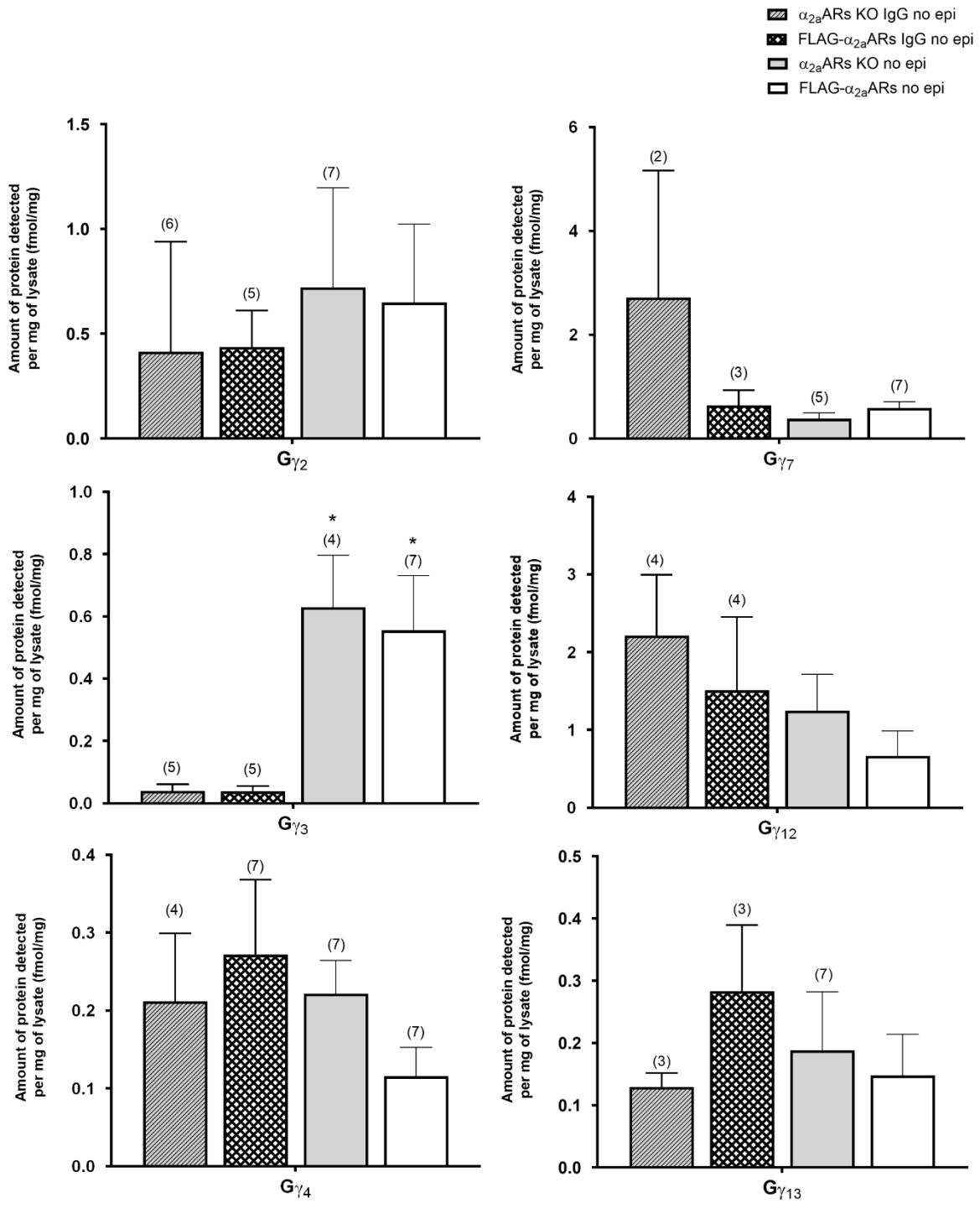

α2aARs KO IgG no epi
FLAG-α2aARs IgG no epi
α2aARs KO no epi
FLAG-α2aARs no epi
Amount of protein detected per mg of lysate (fmol/mg)
3×10²
2×10²
1×10²
0
(7)
*
(7)
*
(7)
Gβ1
50
40
30
20
10
0
(7)
(7)
(6)
Gβ2
0.8
0.6
0.4
0.2
0.0
(6)
(7)
(6)
Gβ4
20
15
10
05
00
(6)
(7)
(6)
Gβ5

Figure 5.


α2aARs KO IgG no epi
FLAG-α2aARs IgG no epi
α2aARs KO no epi
FLAG-α2aARs no epi
Amount of protein detected per mg of lysate (fmol/mg)
Gγ2
Gγ7
Gγ3
Gγ12
Gγ4
Gγ13
(6)
(5)
(7)
(2)
(3)
(5)
(7)
(5)
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(7)

Figure 6.

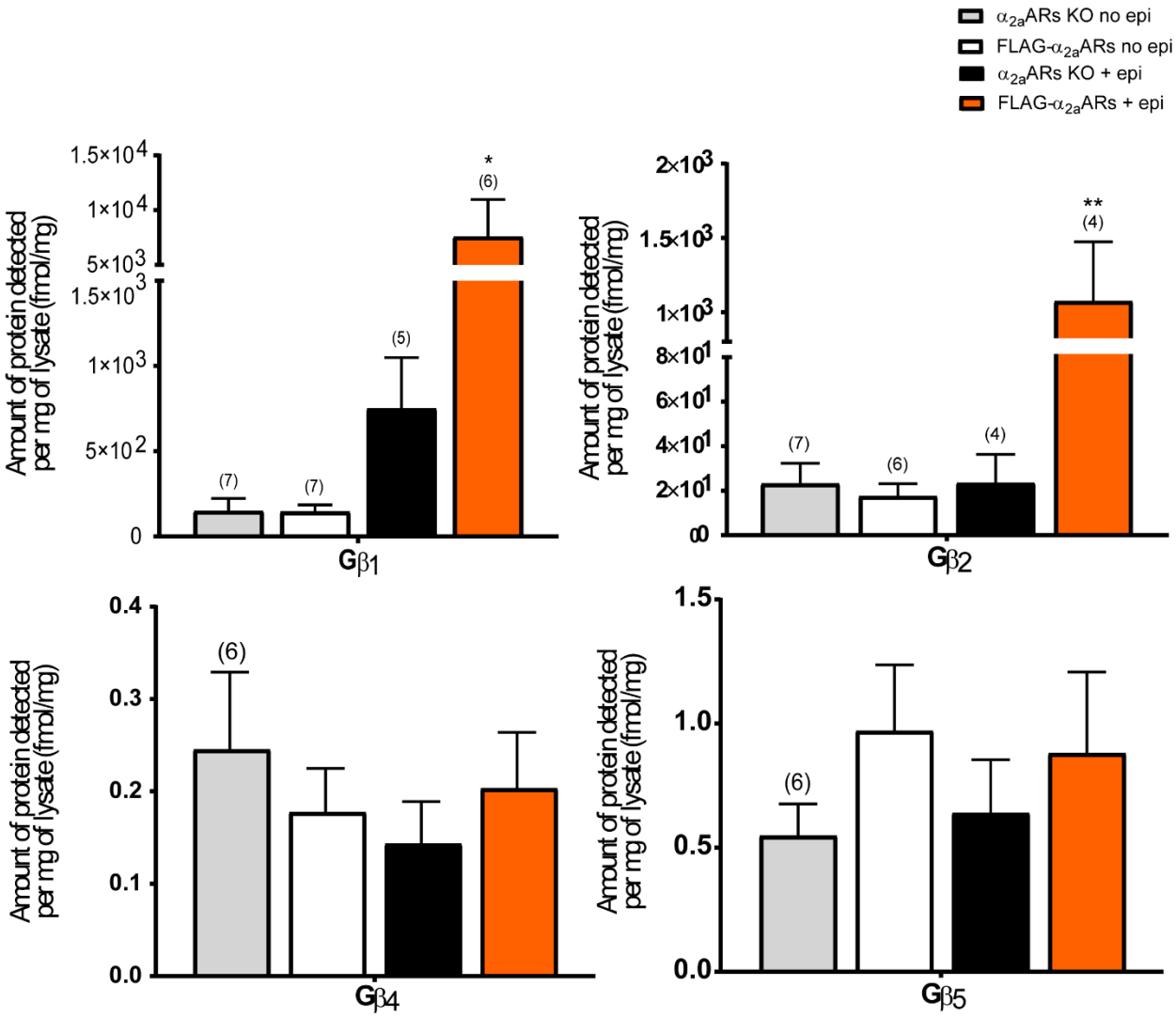

α2aARs KO no epi
FLAG-α2aARs no epi
α2aARs KO + epi
FLAG-α2aARs + epi
Amount of protein detected per mg of lysate (fmol/mg)
1.5×10^4
1×10^4
5×10^3
1.5×10^3
1×10^3
5×10^2
0
(7)
(7)
(5)
*
(6)
Gβ1
2×10^3
1.5×10^3
1×10^3
8×10^1
6×10^1
4×10^1
2×10^1
0
(7)
(6)
(4)
**
(4)
Gβ2
0.4
0.3
0.2
0.1
0.0
(6)
Gβ4
1.5
1.0
0.5
0.0
(6)
Gβ5

Figure 7.

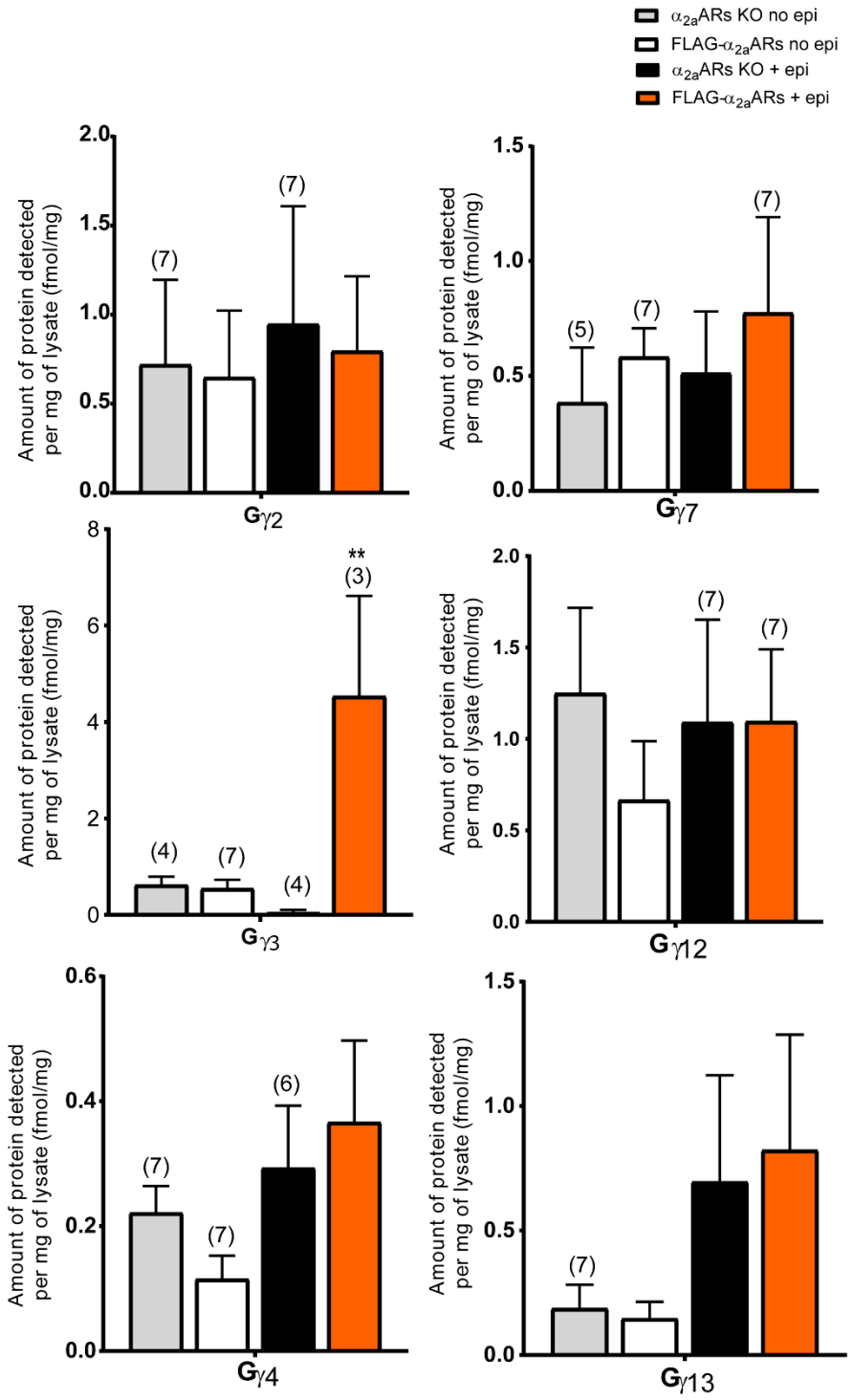
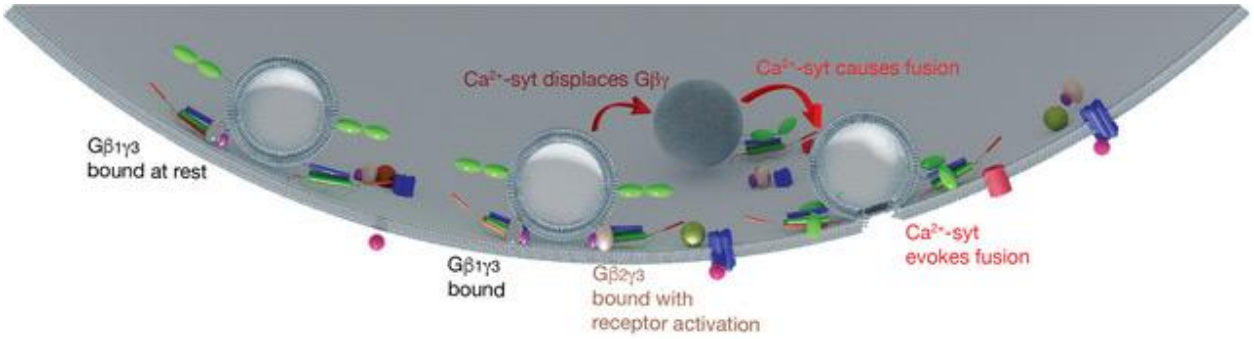

α2aARs KO no epi
FLAG-α2aARs no epi
α2aARs KO + epi
FLAG-α2aARs + epi
Amount of protein detected per mg of lysate (fmol/mg)
Gγ2
Gγ7
Gγ3
Gγ12
Gγ4
Gγ13
(7)
(5)
(4)
(3)
(6)
**

Figure 8.


Ca2+-syt displaces Gβγ
Ca2+-syt causes fusion
Gβ1γ3
bound at rest
Gβ1γ3
bound
Gβ2γ3
bound with
receptor activation
Ca2+-syt
evokes fusion