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

Yun Young Yim[1†], W. Hayes McDonald[2], Katherine M. Betke[1,5], Ali Kaya[1,6], Karren Hyde[1,7], Kevin Erreger[1], Ralf Gilsbach[3, 4], Lutz Hein[4], and Heidi E. Hamm[1*]

[1]Department of Pharmacology, Vanderbilt University, Nashville TN 37232-6600
†Currently at Nash Department of Neuroscience, Icahn School of Medicine at Mount Sinai, New York, NY 10029
[2]Department of Biochemistry and Mass Spectrometry Research Center, Vanderbilt University, Nashville TN 37232-6600
[3]Fachbereich Medizin, Institute for Cardiovascular Physiology, Goethe University, Theodor-Stern-Kai 7, 60590, Frankfurt, Germany
[4]Institute of Experimental and Clinical Pharmacology and Toxicology, Faculty of Medicine, University of Freiburg, 79104, Freiburg, Germany
[5]Currently at Department of Anesthesiology and Perioperative Medicine, Oregon Health & Science University, Portland, Oregon, 97239
[6]Currently at Department of Chemistry and Chemical Biology, Cornell University Address, APS 9700 S. Cass Ave. Bldg. 436E Lemont, IL 60439
[7]Currently at Department of Cell and Developmental Biology, Vanderbilt University, Nashville TN 37232-6600
*Correspondence to heidi.hamm@vanderbilt.edu

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Summary

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

Abstract

Though much is known about the various physiological functions of each GPCR and the specificity of Gα subunits, the specificity of G$\beta\gamma$ activated by a given GPCR and activating each effector in vivo is not known. Previously, we identified different Gβ and Gγ subunits interacting specifically with α_{2a}-adrenergic receptors (α_{2a}AR). In this study, we examined its in vivo specificity to the soluble NSF attachment proteins (SNARE) complex in adrenergic (auto-α_{2a}AR) and non-adrenergic (hetero-α_{2a}AR) neurons. We applied a quantitative targeted multiple reaction monitoring proteomic analysis of Gβ and Gγ subunits bound to the SNARE complex, and found only a subset of Gβ and Gγ bound. Without stimulation of auto-α_{2a}AR, Gβ_1 and Gγ_3 interacted with the SNARE complex. When auto-α_{2a}AR were activated, Gβ_1, Gβ_2, and Gγ_3 were found. Further understanding of in vivo G$\beta\gamma$ specificity to its effectors provides new insights into the multiplicity of genes for Gβ and Gγ.

Introduction

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 of Gβγ (*1, 3-11*). Although many of these Gβγ-effector interactions and downstream signaling cascades have been well studied in vitro, factors controlling the specificity of Gβγ dimers associated with unique GPCRs or their effectors, as well as the in vivo expression abundance of Gβ and Gγ subunits or Gβγ dimers remain unclear. Only a few in vivo genetic deletion studies and knout-out animal studies have attempted to identify the specific roles of different Gβ and Gγ subunits(*11, 12*). With the recent development of targeted multiple reaction monitoring (MRM) proteomic method to detect each neuronal Gβ and Gγ subunits, our lab has showen the protein abundance and subcellular localization of each neuronal Gβ and Gγ subunits (*13*) and their specificity to α_{2a}-adrenergic receptors in adrenergic neurons (auto-α_{2a}AR) and non-adrenergic neurons (hetero-α_{2a}AR)(*14*). Only a subset of neuronal Gβ and Gγ subunits interact with auto-α_{2a}AR and hetero-α_{2a}AR. However, it remains unclear how G protein specificity plays out in α_{2a}AR-mediated effector interactions.

α_{2a}-adrenergic receptors (α_{2a}AR) are one of the best studied $G_{i/o}$ coupled GPCRs, given the availability of both knock out (α_{2a}AR KO) mice and transgenic mice with tagged receptors (FLAG-α_{2a}AR) (*15-19*). Briefly, the activation of α_{2a}AR releases GTP-$G\alpha_{i/o}$ and Gβγ, and these interact with various effectors to initiate the downstream signaling cascades(*11*). FLAG-α_{2a}AR transgenic mice express only auto-α_{2a}AR under the control of the dopamine-β-hydroxylase (Dbh) promoter while α_{2a}AR KO mice are the endogenous knockout of α_{2a}AR(*16*). Using these mice, auto-α_{2a}AR are found to be important for bradycardia and hypotension while α_{2a}AR in hetero-α_{2a}AR are involved in anesthetic sparing, hypothermia, analgesia, bradycardia, and hypotension

(*16*). However, it is unclear how G protein specificity, especially Gβγ, plays out in α_{2a}AR-mediated downstream signaling cascades and underlies such diverse physiological functions. Given the physiological importance of α_{2a}AR, and the different roles of auto-and hetero-α_{2a}AR, the signaling mechanisms of α_{2a}AR in both adrenergic and non-adrenergic neurons, especially in relation to the specificity of Gβγ, need to be further elucidated.

Soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE complex), Gβγ effector, is a key protein involved in synaptic transmission, essential for inter-neuronal communication in both the nervous and endocrine systems. The neuronal SNARE complex, comprised of SNAP25, syntaxin 1A, and synaptobrevin (VAMP) (*20-24*), brings the vesicle membrane and plasma membrane into close proximity, resulting in vesicle fusion at the plasma membrane (*25*). After membrane fusion, neurotransmitter is released into the synaptic junction, permitting communication between neurons encoding both external and internal sensory inputs, and promoting responsive behaviors in postsynaptic cells (*24*). Thus, it is crucial to understand how synaptic transmission is regulated in healthy subjects, and mechanisms of dysfunction in neurological diseases (*26-28*).

The activation of α_{2a}AR results in the inhibition of synaptic transmission via the Gβγ subunit in both adrenergic (auto-α_{2a}AR) and non-adrenergic neurons (hetero-α_{2a}AR) such as glutamatergic, GABAergic, serotonergic and dopaminergic neurons (*29-32*). In particular, numerous studies have reported that Gβγ subunits released from activated α_{2a}AR reduce presynaptic Ca^{2+} influx through the inhibition of voltage-gated calcium channels (VDCC) (*33-35*) and inhibit exocytosis through direct interaction with the SNARE complex (*36-38*). Furthermore, the direct interaction between Gβy and the SNARE complex upon α_{2a}AR activation is a primary mechanism to inhibit exocytosis of norepinephrine-containing vesicles in the central amygdala

(*38*). Delaney *et al*. show that inhibition of norepinephrine release, where high abundance of presynaptic α_{2a}AR are present, is mediated by a $G_{i/o}$-coupled receptor, and that Gβγ dimers released from active α_{2a}AR directly interact with the SNARE complex downstream of calcium entry to inhibit norepinephrine release (*38*). In addition, our knout-out study with SNAP25Δ3 mice no longer shows the inhibition of adrenergic signaling in the BNST, a key area for the integration of stress signals (*39*). The loss of 3 C-terminal residues in SNAP25 causes a loss of Gβγ`s ability to regulate α_{2a}AR mediated inhibition of synaptic transmission. Although the Gβγ–SNARE complex interaction has been well-characterized during α_{2a}AR-mediated inhibition of synaptic transmission, the specificity of Gβγ dimer isoforms for the SNARE complex is not known.

Together, we use various biochemical techniques and tools such as co-immunoprecipitation (coIP), quantitative MRM proteomic method, and transgenic animals to understand the specificities of Gβ and Gγ subunits to the SNARE complex. Here, we evaluated which Gβγ dimers released from epinephrine (epi)-activated α_{2a}AR interact with SNARE complex to regulate synaptic transmission. We discovered that only a subset of Gβ and Gγ subunits released from activated α_{2a}AR interact with the SNARE complex. Further understanding of α_{2a}AR-mediated Gβγ subunit specific interaction with the SNARE complex may lead to the development of new therapeutic targets that complement or contrast with currently available α_{2a}AR-targeted drugs.

Results

Gβγ - SNARE complex interaction. To study Gβγ specificity for the SNARE complex upon activation of α_{2a}AR, we used synaptosomes collected from the brains of wildtype, α_{2a}AR KO,

and FLAG-α_{2a}AR mice. Using a SNAP25 antibody (S9684) and DSP, a lipid-soluble thiol cleavable crosslinker, Gβγ and the SNARE complex were co-immunoprecipitated (coIP) and detected by Western blot (Fig. 1A). We showed that the SNAP25 (S9684) antibody selectively recognizes SNAP25 (fig.S1). To further validate that we were indeed coIPing the ternary SNARE complex, we detected Syntaxin1 in addition to SNAP25 from these coIP samples (fig. S2). The Gβγ and SNARE complex 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}-AR mice (Fig.1B to E). We know from a number of studies (*40, 41*) that there is a low affinity for interaction between Gβγ and the SNARE complex. 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 (fig. S3). Together, these results suggest that this method is suitable to determine which Gβγ subunits associate with the SNARE complex in vivo.

$G\beta_1$, $G\beta_2$, $G\beta_4$, $G\gamma_2$, $G\gamma_3$, and $G\gamma_4$ interact specifically with the SNARE complex upon epinephrine stimulation. To examine which neuronal Gβ and Gγ subunits interact with the SNARE complex upon activation of α_{2a}-AR, we applied a quantitative MRM method (*14*) to TCA precipitated and trypsin digested SNAP25 coIP samples of wildtype (WT), α_{2a}AR KO, and FLAG-α_{2a}AR mouse synaptosomes. Previously, only a subset of neuronal Gβ and Gγ subunits are selectively associated with α_{2a}AR(*14*). WT samples were used to detect the Gβγ–SNARE complex interactions mediated by epi stimulation, including both auto- and hetero-α_{2a}AR, while α_{2a}AR KO samples were used to detect non α_{2a}AR-mediated interactions only. Lastly, FLAG-α_{2a}AR samples were used to detect presynaptic auto-α_{2a}AR-mediated Gβγ-SNARE complex interactions. We used unstimulated WT samples (WT no epi), α_{2a}AR KO (α_{2a}AR KO no epi),

and FLAG-α_{2a}AR (FLAG-α_{2a}ARs no epi) as controls to detect basal Gβγ–SNARE complex 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 detected not only auto-receptor mediated Gβγ-SNARE complex interactions, but also quantify those mediated by non-α_{2a}AR and estimated heteroreceptor interactions.

To identify nonspecific interactions of Gβ and Gγ subunits, we used IgG alone controls (without primary antibody). We compared the amount of Gβ subunit detected per mg of lysate between WT IgG +epi and WT +epi samples and found $G\beta_1$, $G\beta_2$, and $G\beta_4$ significantly interacted with the SNARE complex upon α_{2a}AR stimulation (Fig. 2). As expected from previous studies (*13, 14*), $G\beta_1$ was detected at a significantly higher concentration than $G\beta_2$ and $G\beta_4$ subunits. Moreover, we did not identify interaction between $G\beta_5$ and the SNARE complex following α_{2a}AR activation.

Next, we identified the Gγ subunits that interact with the SNARE complex following receptor activation. We found enrichment of the $G\gamma_2$, $G\gamma_3$, and $G\gamma_4$ subunits with the SNARE complex upon epi stimulation (Fig.3). $G\gamma_3$ was the most abundant followed by $G\gamma_2$ and $G\gamma_4$, respectively. We detected a similar abundance of $G\gamma_2$ and $G\gamma_4$ associated with the SNARE complex. We were not able to detect an interaction between $G\gamma_{12}$ and the SNARE complex (Fig. 3). Although $G\gamma_{12}$ is specifically bound to α_{2a}AR (*14*), it may not interact with the SNARE complex 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 (Fig. 2 and 3), we detect only a subset of possible Gβγ dimers and 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 the SNARE complex upon epi stimulation. These dimers may occur in the brain and play roles in the α_{2a}AR mediated Gβγ-SNARE complex interactions. Based on the detected abundances of $G\beta_1$ and $G\gamma_3$, we suggest that $G\beta_1\gamma_3$ is the most likely dimer to interact with the SNARE complex following receptor stimulation. Further biochemical analysis will be needed to validate the presence of these Gβγ dimers and their specificities for the SNARE complex upon α_{2a}AR activation in both adrenergic and non-adrenergic neurons.

Basal interaction of Gβγ and the SNARE complex. We showed in Fig 1B and C that Gβγ and the SNARE complex interact basally in unstimulated coIPed samples of wildtype and FLAG-α_{2a}-AR mice. To understand the basal Gβγ–SNARE complex interaction in the absence of epi stimulation, we compared IgG control to no epi coIPed samples from α_{2a}AR KO and FLAG-α_{2a}AR synaptosomes using MRM mass spectrometry. 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 Gβγ-SNARE complex interactions. Here, we detected all neuronal Gβ subunits but found only $G\beta_1$ significantly interacting with the SNARE complex in both α_{2a}AR KO and FLAG-α_{2a}AR no epi conditions compared to that of the IgG no epi condition (Fig.4). This suggests that there is a basal interaction of $G\beta_1$ with the SNARE complex. The abundance of $G\beta_1$ between these two conditions was not different, suggesting that the basal interaction of $G\beta_1$ and the SNARE complex may not be α_{2a}AR mediated. Similarly, we detected $G\gamma_3$ interaction with the SNARE complex in the unstimulated conditions of both genotypes compared to IgG control (Figure 5). Thus we suggest that the $G\beta_1\gamma_3$ dimer is a basal, non-α_{2a}AR mediated interaction. Various GPCRs have been implicated to mediate presynaptic inhibition through this mechanism; therefore, other endogenous GPCRs present in the synaptosomes could be causing

this effect, or alternatively, since no agonists of other GPCRs were present in the experiments, it may be a basal interaction with SNARE complex

$G\beta_1$, $G\beta_2$, and $G\gamma_3$ interact specifically with the SNARE complex upon auto-α_{2a}ARs activation. As previously described, adrenergic auto-α_{2a}AR inhibits presynaptic neurotransmitter release through the interaction between $G\beta\gamma$ and the SNARE complex (*38*). To test whether auto-α_{2a}AR~~s~~ and presynaptic hetero-α_{2a}AR utilize different $G\beta\gamma$ 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}AR~~s~~ mediated $G\beta\gamma$-SNARE complex interactions. α_{2a}AR KO (no epi) and FLAG-α_{2a}AR (no epi) samples were used as controls to identify non-auto-α_{2a}AR mediated interactions, and α_{2a}AR KO +epi samples were used to detect non-α_{2a}AR-mediated $G\beta\gamma$-SNARE complex interactions. By comparing the result from α_{2a}AR KO, FLAG-α_{2a}AR, and wildtype, we can propose the specificity of $G\beta$ and $G\gamma$ interactions with the SNARE complex mediated by hetero-α_{2a}AR.

Here, we were able to detect a subset of $G\beta$ and $G\gamma$ subunit interactions with the SNARE complex 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 the SNARE complex following auto-α_{2a}AR activation (Fig.6). We also detected significantly more $G\beta_1$ than $G\beta_2$ (Fig.6). Moreover, we detected a ~ 10-fold increased interaction of $G\beta_1$ and the SNARE complex upon epi stimulation compared to the basal state (compare Fig. 4 and 6).

Among the 3 $G\gamma$ subunits enriched with the SNARE complex upon stimulation of WT containing auto- and hetero-α_{2a}AR and non-α_{2a}AR (Fig. 3), the enrichment of $G\gamma_3$ with the SNARE complex was detected following auto-α_{2a}AR activation (Fig. 7). Basally, without epi

stimulation, $G\gamma_3$ was found to interact with the SNARE complex (Fig 5). These findings suggest that $G\beta_1\gamma_3$ may be basally interacting with the SNARE complex and not α_{2a}ARs mediated (Fig. 4 and 5), whereas $G\beta_2\gamma_3$ may interact with the SNARE complex only after epi stimulation of auto-α_{2a}AR.

Discussion

Numerous in vitro studies have studied the effectors of Gβγ and their downstream signaling cascades(*1-4, 42*); however, only a few in vivo genetic deletion studies and knout-out animals studies have examined the specific roles of different Gβ and Gγ subunits (*11, 12*). It remains unclear how particular Gβγ dimers form or how they localize to particular sites in vivo. In this paper, we addressed the in vivo specificity of Gβγ subunit interaction with the SNARE complex. We determined a surprising specificity in which Gβ and γ subunits associate with the SNARE complex, including the interesting finding that some Gβγs interact with the SNARE complex regardless of receptor activation. Only a subset of neuronal Gβγ dimers—$G\beta_1$, $G\beta_2$, $G\beta_4$, $G\gamma_2$, $G\gamma_3$, and $G\gamma_4$—interact with the SNARE complex (Fig. 2 and 3) following epi stimulation in WT mice. Upon the activation of auto-α_{2a}AR, only $G\beta_1$, $G\beta_2$, and $G\gamma_3$ interacted with the SNARE complex (Fig. 6 and 7). From 9 possible Gβγ dimers, auto-α_{2a}AR may utilize only two Gβγ dimers, $G\beta_1\gamma_3$ and $G\beta_2\gamma_3$, to inhibit neurotransmitter release through the Gβγ-SNARE complex interaction, In addition, comparing WT and auto-α_{2a}AR samples (Fig.2 and 6), we found that $G\beta_4\gamma_2$ and $G\beta_4\gamma_4$ may be specifically utilized by presynaptic hetero-α_{2a}AR. Thus, this study provides a window into the specificity of Gβγ dimer function in vivo.

In both Western blot and quantitative MRM study in FLAG-α_{2a}AR animals, we detected Gβγ-SNARE complex interactions in the basal state, without α_{2a}AR stimulation (Fig.1C, 4, and

5). Because we showed previously that the α_{2a}AR does not basally interact with G$\beta\gamma$ (*14*) and no basal G$\beta\gamma$ interaction to the SNARE complex was detected in the quantitative MRM study of wildtype animals (Fig. 2), we were surprised to see a basal interaction of G$\beta_1\gamma_3$ with the SNARE complex (Fig.4 and 5). With the limited tools, we cannot evaluate and understand the observed difference of basal interaction in wildtype and FLAG-α_{2a}AR animals However, the ~4-fold increase of abundance in Gβ and ~20-fold increase of abundance in Gγ observed in FLAG-α_{2a}AR animals raise an interacting hypothesis. The change in G$\beta\gamma$ associated to the SNARE complex may occur upon the activation of auto-α_{2a}AR. Further studies are necessary to understand the basal interaction of G$\beta_1\gamma_3$ with the SNARE complex, especially in the wildtype animal G$\beta_2\gamma_3$ appears only associated with the SNARE complex upon auto-α_{2a}AR activation.

Gβ_1 specifically interacts with the SNARE complex not only in the basal state and but also upon epi stimulation in FLAG-α_{2a}AR mice (Fig.4 and 6). The abundance of Gβ_1 interacting with the SNARE complex in WT samples (Fig. 2) was expected as Gβ_1 is the most abundant Gβ subunit in whole synaptosome as well as at both pre-and post-synaptic fractions(*13*). However, an increase of abundance in Gβ_1 (~10 fold) interacting with the SNARE complex upon auto-α_{2a}AR activation was not expected (Fig.6). This suggests that the basally bound Gβ_1 may have a unique role in basal modulation of exocytosis. So far, the role of this basal G$\beta\gamma$ 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(*43, 44*).

Combining this study of the G$\beta\gamma$ specificity to the SNARE complex with our previous studies of the presynaptic abundance specificity of G$\beta\gamma$ subunits for α_{2a}AR(*13, 14*), we hypothesize that the G$\beta\gamma$ subunit specificity may be encoded by an association of a particular

GPCR with a subset of Gβγs, –the abundance of each neuronal Gβ and Gγ subunit, and the distance to their effectors at the presynaptic terminal. In our previous receptor study comparing the protein abundance detected with α_{2a} receptors, we speculated that $G\gamma_{2,}$ $G\gamma_{3,}$ and $G\gamma_4$ were associated with auto-α_{2a}AR while $G\gamma_{12}$ is specific for hetero-α_{2a}AR (*14*). Given our current understanding with this study on the Gβ and Gγ specificity to the SNARE complex, we can more accurately show that $G\gamma_2$ and $G\gamma_4$ are involved in hetero-α_{2a}AR-mediated Gβγ-SNARE complex interaction, with no evidence for $G\gamma_{12}$ association to the SNARE complex. $G\gamma_{12}$ associated with hetero-α_{2a}AR may be associated with another Gβγ effector.

The Gγ subunits, more than Gβ, play key roles in Gβγ specificity. Previously, knock-down and knock-out studies showed that particular Gγ subunits have distinct physiological importance in biological systems (*11, 12*). To date, only 4 out of 12 Gγ subunits have been studied using knockdown or knock-out models. Mainly expressed in retina, $G\gamma_1$ is involved in photoreceptor degeneration. $G\gamma_2$ is involved in nociception and angiogenesis. $G\gamma_3$ is involved in increased susceptibility to seizures and resistance to diet induced obesity while $G\gamma_7$ affects the startle response(*4, 7*). Unique physiological phenotypes of each Gγ subunit suggest a great deal of specificity in Gβγ dimerization and signaling(*45-47*). The main molecular mechanisms behind binding selectivity between various Gβγs and their effectors are still unknown. However, ongoing investigations of interactions between each Gγ and t-SNARE are suggesting the importance of charged residues during this process(*48*).

Moreover, the fast rate of Gβγ-SNARE complex-mediated inhibition(*44*) suggests that Gβγ, the SNARE complex, and other synaptic proteins may be associated by scaffolding interactions prior to receptor activation, to facilitate Gβγ-SNARE complex interaction and

mediate Gβγ specificity. Previously, we identified two Gβγ binding sites on vesicle proximal and distal regions of SNAP25 (*41, 49, 50*), far enough apart to not both be occupied by a single Gβγ. However, the physiological and biochemical importance of these interaction sites still remains unclear. The two sites could underlie interaction via the SNARE complex with two different effectors (*51, 52*) or 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 (*50*). Using SNAP25Δ3 mice with a truncated C-terminus of SNAP25, we see a two-fold reduction in Gβγ-SNARE complex interaction, as well as an increase in stress-induced hyperthermia, defective spatial learning, impaired gait, supraspinal nociception (*39*), enhancement of long-term potentiation at Schaffer collateral-CA1 synapse (*53*), and resistance to diet-induced obesity(*54*). It will be interesting to determine the stoichiometry of Gβγ binding to the SNARE complexes, and whether identical Gβγ dimers bind to both amino- and carboxy-terminal sites of the SNARE complexes. 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. Hamid *et al*. demonstrated that $5HT_{1b}$ and $GABA_B$ receptors inhibit exocytotic release at the same synaptic terminal through different mechanisms (*43*), with $5HT_{1b}$ receptor-mediated inhibition of exocytosis using the Gβγ-SNARE complex interaction, while $GABA_B$ receptor-mediated inhibition work~~s~~ 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 the SNARE complex may

add a further layer of regulation of Gβγ-effector selectivity. Future studies to understand this specificity will clarify Gβγ isoform specificity important for both receptor and effector binding.

Materials and Methods

Animals. Adult, male FLAG-tagged alpha2a adrenergic receptors (α_{2a}-AR), α_{2a}-AR knock-out (KO), and wildtype mice (*55*) 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 (*14, 56-58*) in the presence of 1mM ethylenediaminetetraacetic acid, 1uM propranolol, 1uM prazosin, and 2mM 3,3'-dithiobis [sulfosuccinimydlypropinate], the lipid soluble, thiol cleavable crosslinker (Pierce, 22585) and stimulated with 100μM epinephrine (epi). This mimics the local synaptic concentration of epi and it is a commonly used concentration in α_{2a}AR studies(*59-61*). As epi is not an α2aAR

selective agonist, we also used prazosin and propranolol to prevent off-target effects from non-α2aAR adrenergic receptors. Samples were frozen in liquid nitrogen and stored at -80°C.

Co-immunoprecipitation. Similar to the co-immunoprecipitation (coIP) of α_{2a}AR and Gβγ (*14*), we coIPed the SNARE complex 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 knock-out mice exhibit neonatal lethally(*62*) , we used rabbit IgG as a control.

Elution and trichloroacetic acid (TCA) precipitation of coIPs. Samples (approximately 11 coIPs per condition per genotype) 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 min~~s~~ and centrifuged at 5,000 x g for 2min~~s~~ 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 (*14*). All samples were stored at -80°C freezer for the western blot or MRM analysis.

Immunoblot Analysis. The analysis was done as described previously (*14*). 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 (*14*), 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 was used to account for differences in protein abundance of Gβ and Gγ subunits and for the quantification of western blots (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). All statistical tests were performed using GraphPad Prism v.9.0 for Windows, (GraphPad Software, La Jolla, California, USA, www.graphpad.com).

Supplementary Materials

Figure 1. Selectivity of SNAP25 IP Antibody

Figure 2. Western blot analysis of SNAP25 coIP.

Figure 3. Quantification of Gβ precipitated with SNAP25.

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Authors Contributions

Y.Y., W.H.M., K.M.B. , and H.E.H. participated in research design and preliminary study. Y.Y. and K.E. 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., K.M.B. 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

All authors declare that they have no competing interests.

Data Availability

All data supporting the findings of this study are available from the lead author Heidi E. Hamm upon reasonable request.

Figure legends

Figure 1. Co-immunoprecipitation of the SNARE complex 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 (-). DSP, a lipid-soluble thiol cleavable crosslinker, was added to ensure the receptor and Gβγ remained intact during co-IP experiments. We detected Gβγ in the SNAP25 pulldown but not the IgG control pulldown regardless of receptor stimulation (**B** and **C**, red box). Quantified Gβ intensities were normalized to that of input and presented as relative fold change in wildtype (**D**) or FLAG-α_{2a}AR (**E**) mice (N=3). Data are presented as mean ± SEM. One-way ANOVA* $p<0.05$, **$p<0.01$, *Post hoc* analysis by Tukey's multiple comparison. coIP: co-immunoprecipitated fraction; Sup: depleted supernatant

Figure 2. Gβ specificity to the SNARE complex upon α_{2a}AR activation. Quantification of Gβ subunits interacting with the SNARE complex in wildtype synaptosomes (N=8, unless otherwise noted). $G\beta_1$, $G\beta_2$, and $G\beta_4$ interact specifically with the SNARE complex 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 the SNARE complex upon α_{2a}AR activation. Quantification of Gγ subunits interacting with the SNARE complex in wildtype synaptosomes (N=8,unless otherwise noted). $G\gamma_2$, $G\gamma_3$, and $G\gamma_4$ may specifically interact with the SNARE complex 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 the SNARE complex. Quantification of Gβ subunits interacting with the SNARE complex basally (N=8, unless otherwise noted). $G\beta_1$, but not $G\beta_2$, specifically interacting with the SNARE complex 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 the SNARE complex. Quantification of Gγ subunits interacting with the SNARE complex basally (N=8, unless otherwise noted). $G\gamma_3$ specifically interacted with the SNARE complex 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 the SNARE complex upon auto-α_{2a}ARs activation. Quantification of Gβ subunits interacting with the SNARE complex 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 the SNARE

complex 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 the SNARE complex 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 complex 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 the SNARE complex upon presynaptic α_{2a}AR activation in both adrenergic and non-adrenergic neurons.

Figure 1.

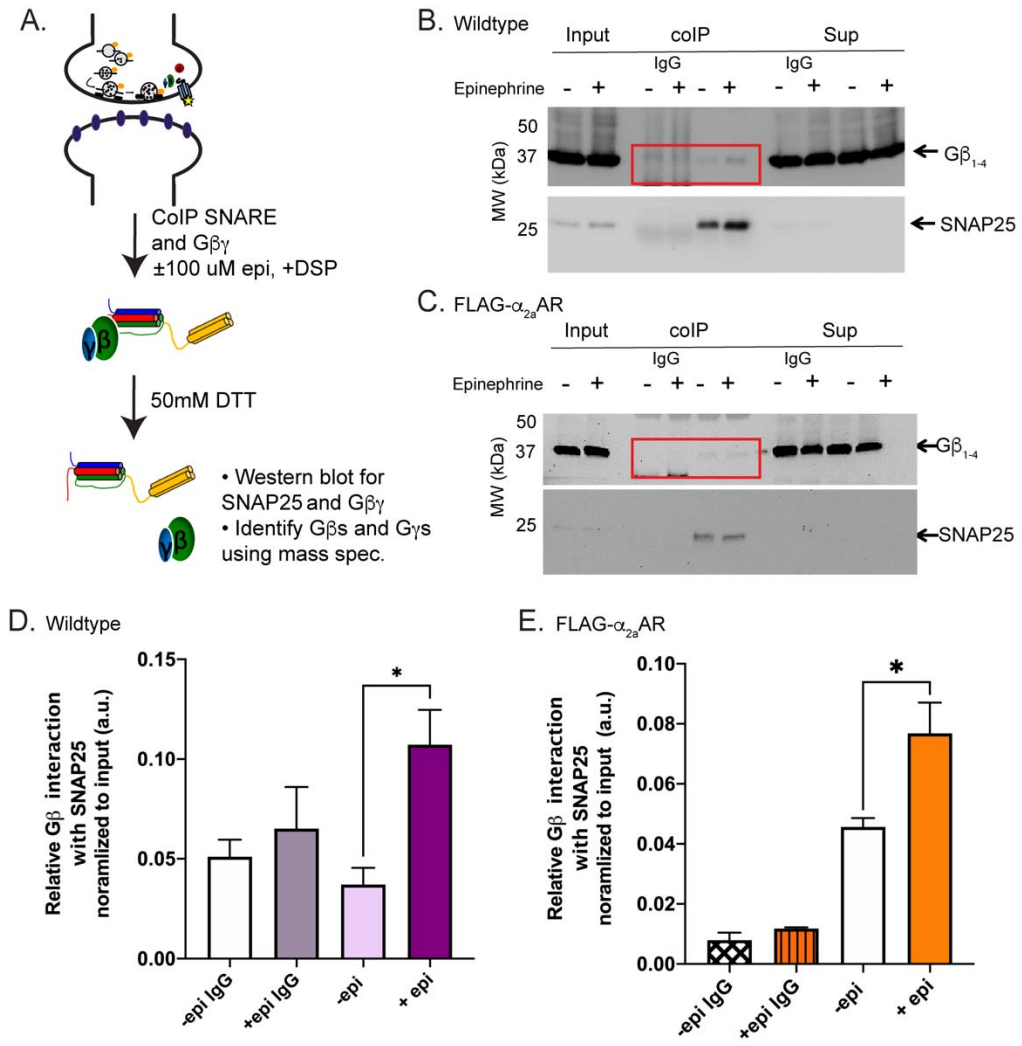

A.
CoIP SNARE
and Gβγ
±100 uM epi, +DSP
50mM DTT
• Western blot for
SNAP25 and Gβγ
• Identify Gβs and Gγs
using mass spec.
B. Wildtype
Input
coIP
Sup
IgG
IgG
Epinephrine
MW (kDa)
50
37
25
Gβ1-4
SNAP25
C. FLAG-α2aAR
Input
coIP
Sup
IgG
IgG
Epinephrine
MW (kDa)
50
37
25
Gβ1-4
SNAP25
D. Wildtype
Relative Gβ interaction
with SNAP25
noramlized to input (a.u.)
0.15
0.10
0.05
0.00
*
-epi IgG
+epi IgG
-epi
+ epi
E. FLAG-α2aAR
Relative Gβ interaction
with SNAP25
noramlized to input (a.u.)
0.10
0.08
0.06
0.04
0.02
0.00
*
-epi IgG
+epi IgG
-epi
+ epi

Figure 2.

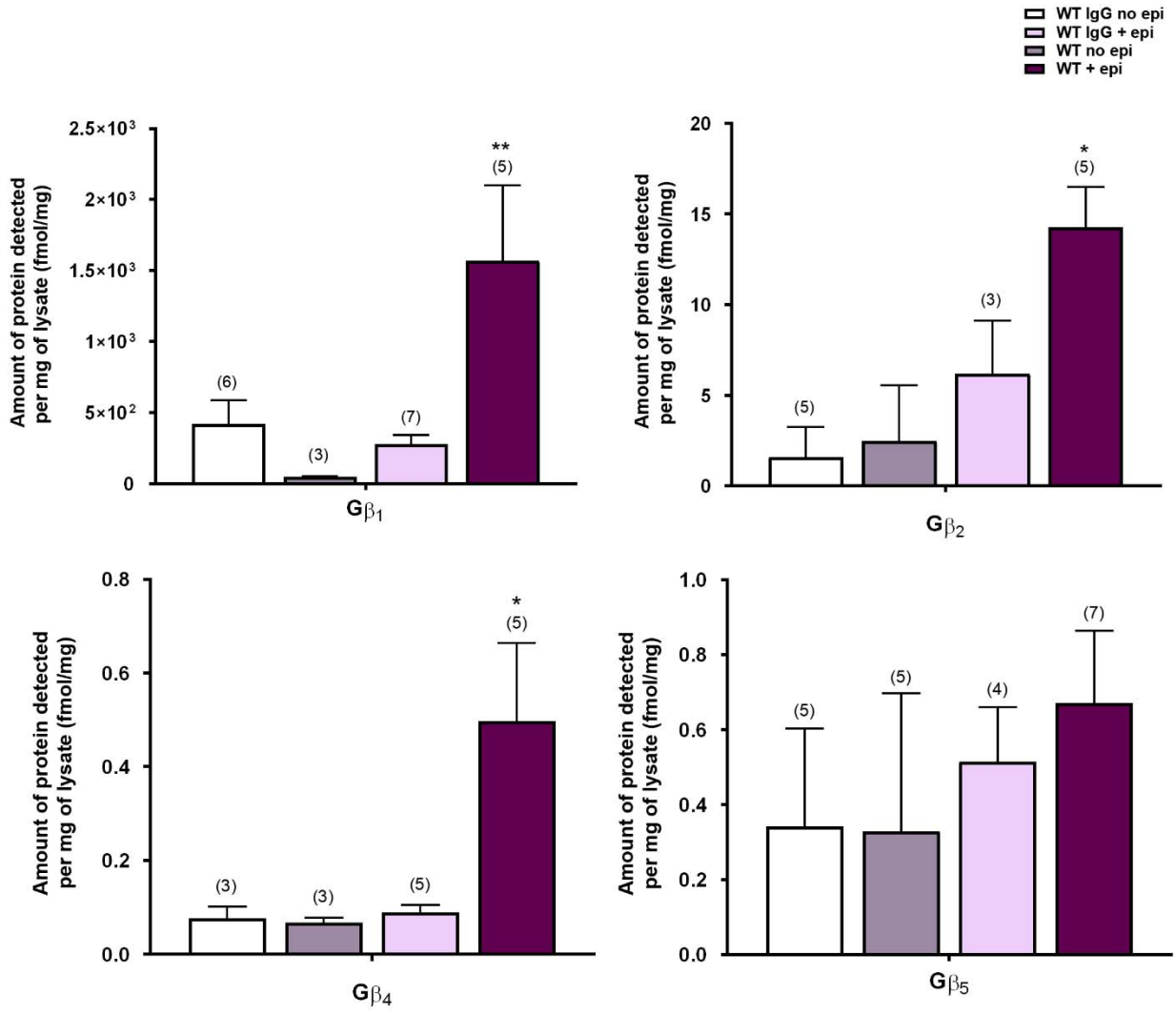

WT IgG no epi
WT IgG + epi
WT no epi
WT + epi
Amount of protein detected per mg of lysate (fmol/mg)
2.5×10³
2×10³
1.5×10³
1×10³
5×10²
0
(6)
(3)
(7)
**
(5)
Gβ1
20
15
10
5
0
(5)
(3)
*
(5)
Gβ2
0.8
0.6
0.4
0.2
0.0
(3)
(3)
(5)
*
(5)
Gβ4
1.0
0.8
0.6
0.4
0.2
0.0
(5)
(5)
(4)
(7)
Gβ5

Figure 3.

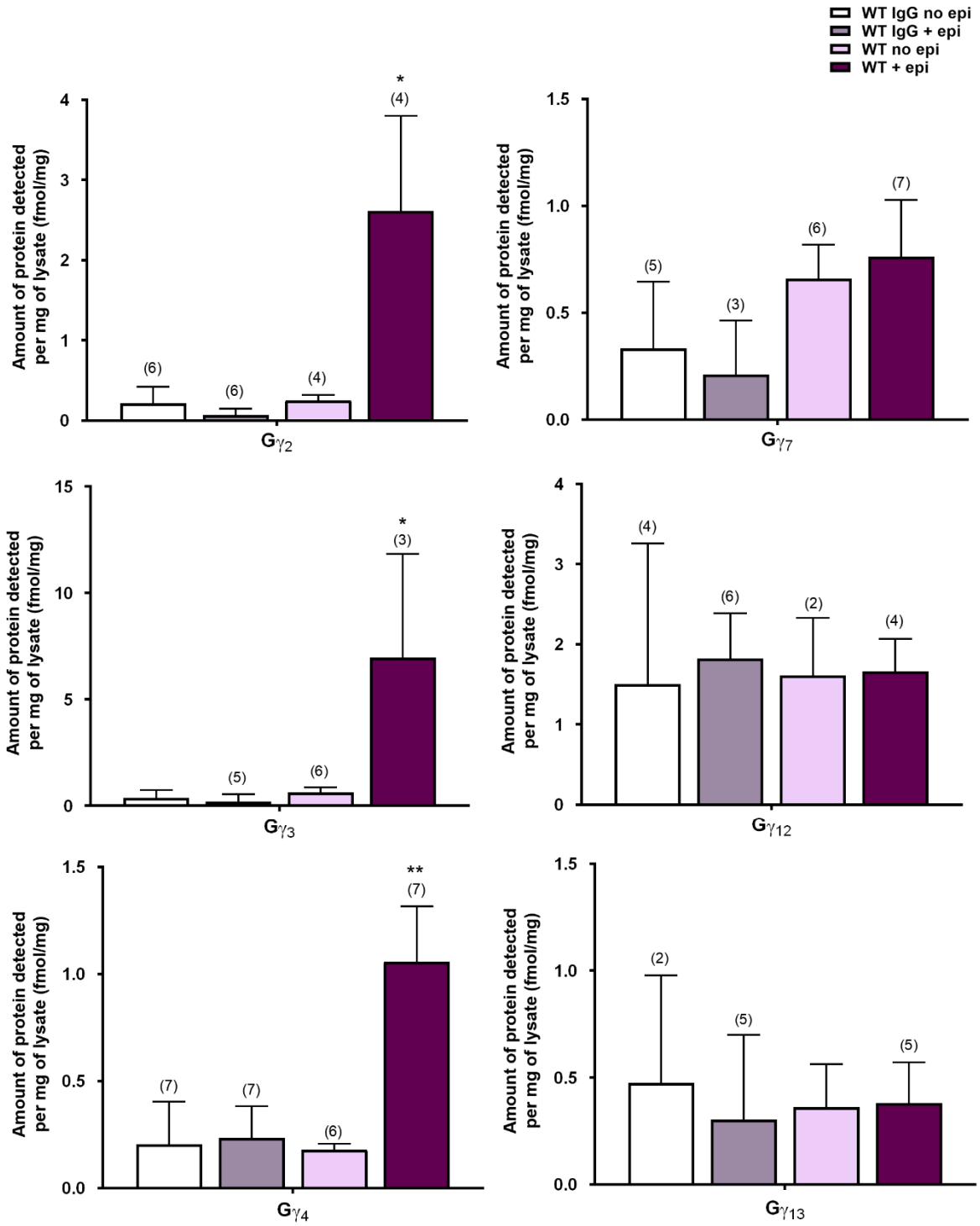

WT IgG no epi
WT IgG + epi
WT no epi
WT + epi
Amount of protein detected per mg of lysate (fmol/mg)
Gγ2
Gγ7
Gγ3
Gγ12
Gγ4
Gγ13
*
**
(6)
(4)
(5)
(3)
(7)
(2)

Figure 4.

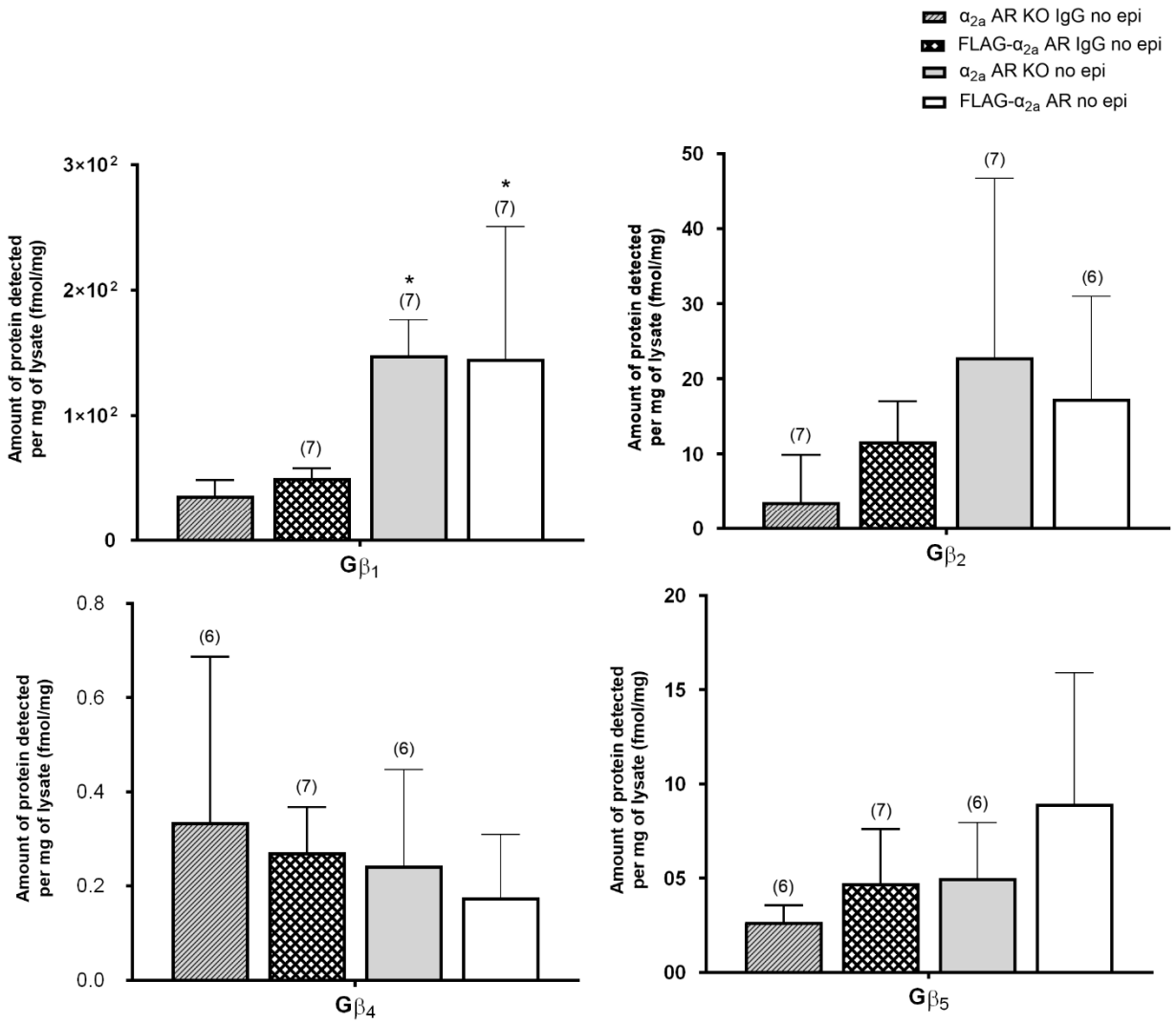

α2a AR KO IgG no epi
FLAG-α2a AR IgG no epi
α2a AR KO no epi
FLAG-α2a AR no epi
Amount of protein detected per mg of lysate (fmol/mg)
3×10²
2×10²
1×10²
0
(7)
*
(7)
*
(7)
Gβ1
50
40
30
20
10
0
(7)
(7)
(6)
Gβ2
0.8
0.6
0.4
0.2
0.0
(6)
(7)
(6)
Gβ4
20
15
10
05
00
(6)
(7)
(6)
Gβ5

Figure 5.

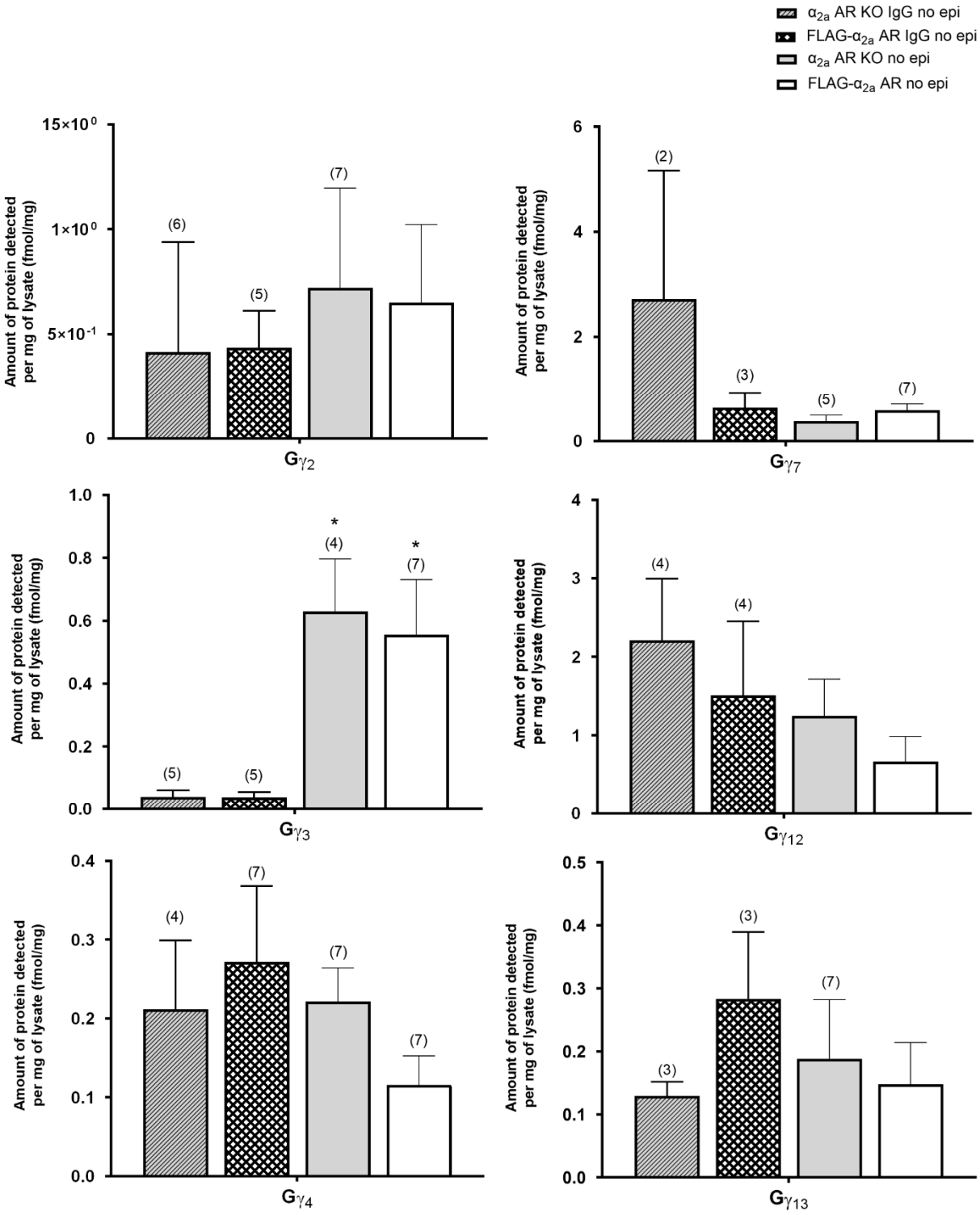

α2a AR KO IgG no epi
FLAG-α2a AR IgG no epi
α2a AR KO no epi
FLAG-α2a AR no epi
Amount of protein detected per mg of lysate (fmol/mg)
15×10⁰
1×10⁰
5×10⁻¹
0
(6)
(5)
(7)
Gγ2
6
4
2
0
(2)
(3)
(5)
(7)
Gγ7
1.0
0.8
0.6
0.4
0.2
0.0
(5)
(5)
*
(4)
*
(7)
Gγ3
4
3
2
1
0
(4)
(4)
Gγ12
0.4
0.3
0.2
0.1
0.0
(4)
(7)
(7)
(7)
Gγ4
0.5
0.4
0.3
0.2
0.1
0.0
(3)
(3)
(7)
Gγ13

Figure 6.

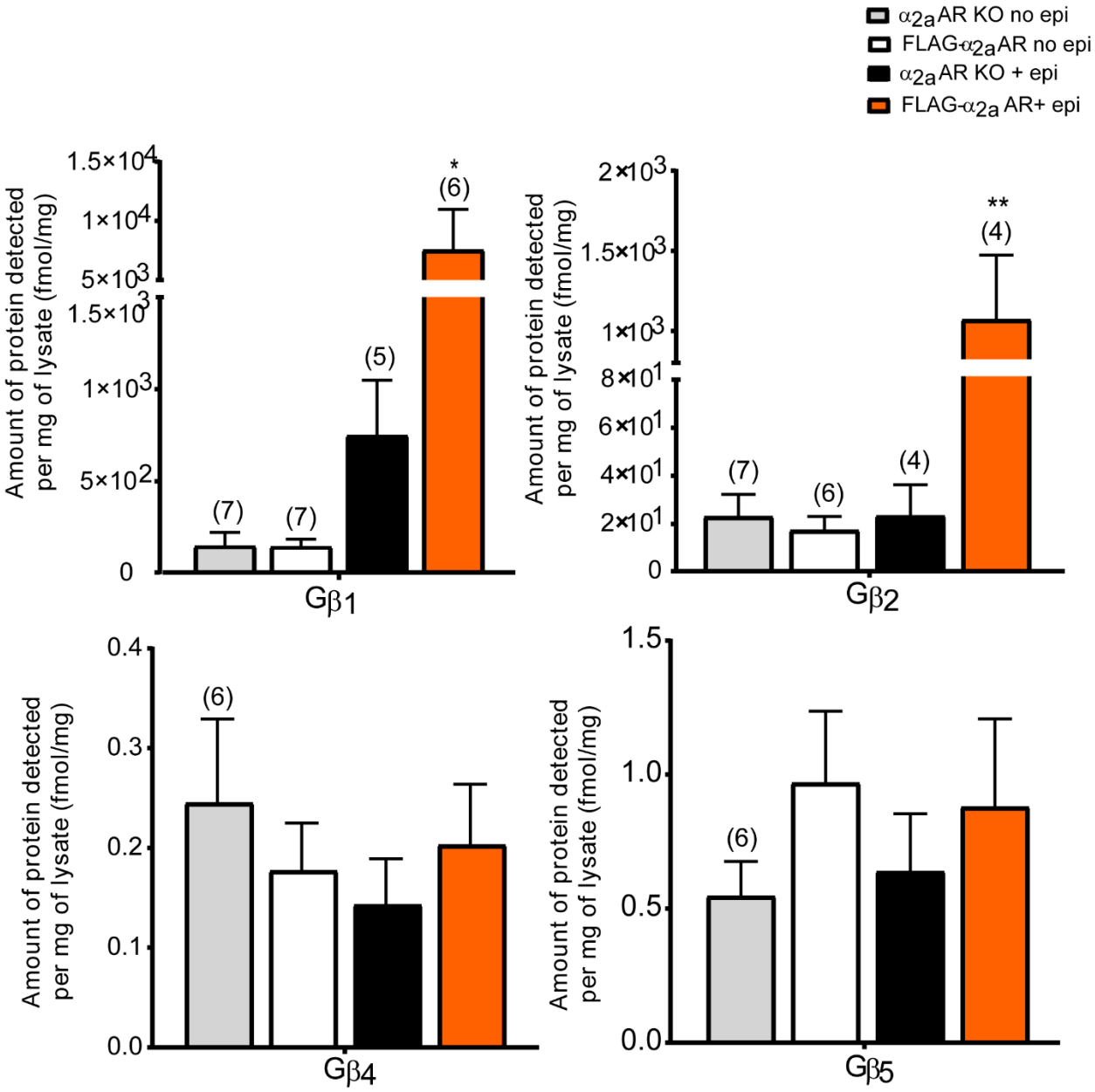

α2aAR KO no epi
FLAG-α2aAR no epi
α2aAR KO + epi
FLAG-α2a AR+ epi
Amount of protein detected per mg of lysate (fmol/mg)
1.5×10^4
1×10^4
5×10^3
1.5×10^3
1×10^3
5×10^2
0
(7)
(7)
(5)
*
(6)
Gβ1
2×10^3
1.5×10^3
1×10^3
8×10^1
6×10^1
4×10^1
2×10^1
0
(7)
(6)
(4)
**
(4)
Gβ2
0.4
0.3
0.2
0.1
0.0
(6)
Gβ4
1.5
1.0
0.5
0.0
(6)
Gβ5

Figure 7.

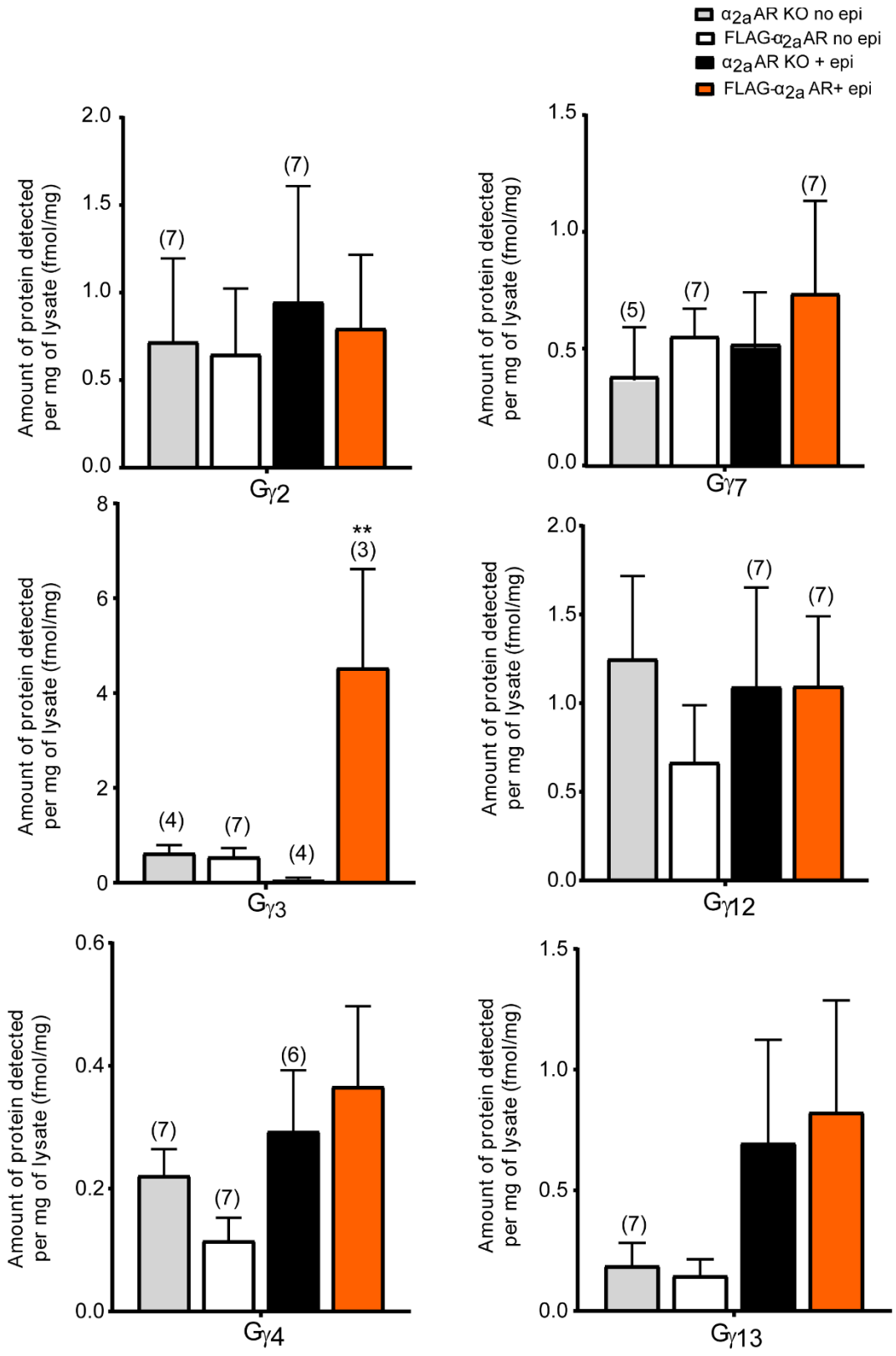
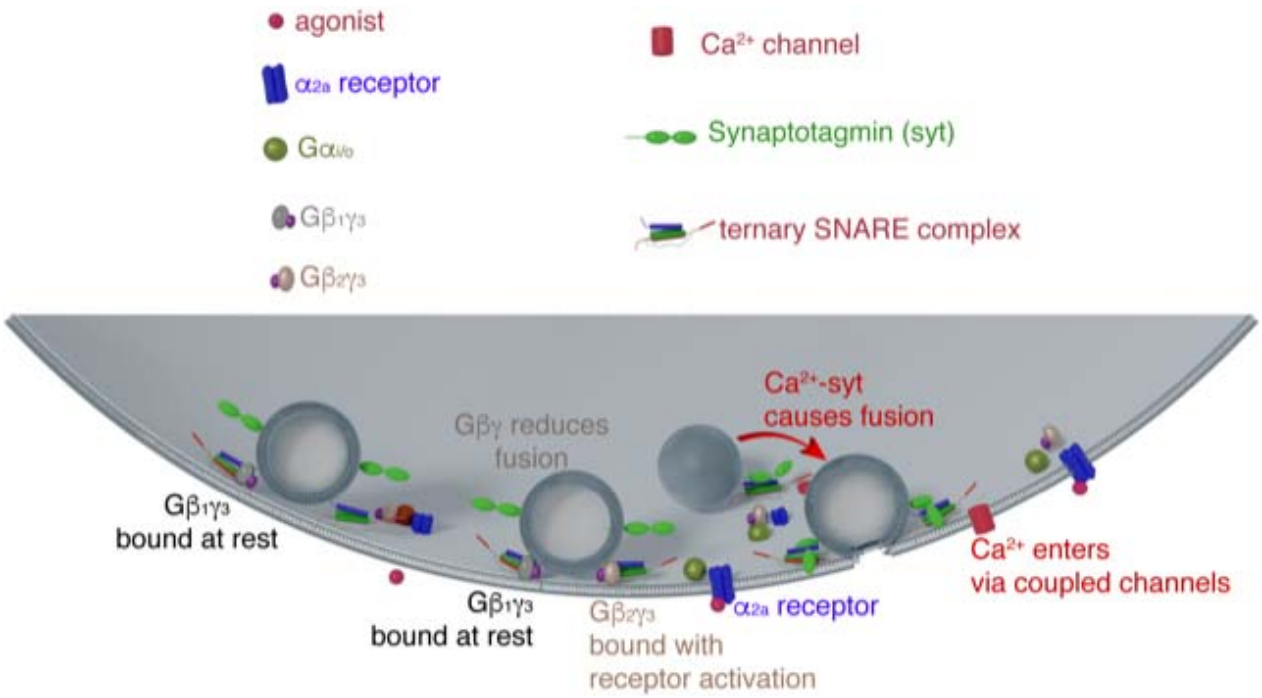

α2aAR KO no epi
FLAG-α2aAR no epi
α2aAR KO + epi
FLAG-α2a AR+ epi
Amount of protein detected per mg of lysate (fmol/mg)
Gγ2
Gγ7
Gγ3
Gγ12
Gγ4
Gγ13
**
(3)
(4)
(5)
(6)
(7)

Figure 8.


agonist
α2a receptor
Gαi/o
Gβ1γ3
Gβ2γ3
Ca2+ channel
Synaptotagmin (syt)
ternary SNARE complex
Ca2+-syt
causes fusion
Gβγ reduces
fusion
Gβ1γ3
bound at rest
Gβ1γ3
bound at rest
Gβ2γ3
bound with
receptor activation
α2a receptor
Ca2+ enters
via coupled channels