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The *in vivo* specificity of synaptic Gβ and Gγ subunits to the α_{2a} adrenergic receptor at CNS synapses

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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 activated by a given GPCR and that activate each effector *in vivo* is not known. Here, we examined the *in vivo* Gβγ specificity of presynaptic α_{2a}-adrenergic receptors (α_{2a}ARs) in both adrenergic (auto-α_{2a}ARs) and non-adrenergic neurons (hetero-α_{2a}ARs) for the first time. With a quantitative MRM proteomic analysis of neuronal Gβ and Gγ subunits, and co-immunoprecipitation of tagged α_{2a}ARs from mouse models including transgenic FLAG-α_{2a}ARs and knock-in HA-α_{2a}ARs, we investigated the *in vivo* specificity of Gβ and Gγ subunits to auto-α_{2a}ARs and hetero-α_{2a}ARs activated with epinephrine to understand the role of Gβγ specificity in diverse physiological functions such as anesthetic sparing, and working memory enhancement. We detected $G\beta_2$, $G\gamma_2$, $G\gamma_3$, and $G\gamma_4$ with activated auto α_{2a}ARs, whereas we found $G\beta_4$ and $G\gamma_{12}$ preferentially interacted with activated hetero-α_{2a}ARs. Further understanding of *in vivo* Gβγ specificity to various GPCRs offers new insights into the multiplicity of genes for Gβ and Gγ, and the mechanisms underlying GPCR signaling through Gβγ subunits.

G-protein coupled receptors (GPCRs) are the largest and most diverse superfamily of transmembrane receptors that convey signal transduction across cell membranes, and mediate a vast array of cellular responses necessary for human physiology[1–3]. Upon their activation, GTP-Gα and Gβγ subunits are released from the GPCR and interact with various effectors to initiate downstream signaling cascades. Theoretically, 60 different combinations of Gβγ dimers are possible (5 Gβ × 12 Gγ subunits)[4–8]. However, not all theoretical Gβγ dimers exist, are equally expressed, or interact with Gα subunits, receptors, effectors, and downstream signaling factors[5,9–17]. For example, $G\beta_1$ and $G\beta_4$ dimerize with all Gγ subunits, while $G\beta_2$ and $G\beta_3$ are unable to dimerize with $G\gamma_1$ and $G\gamma_{11}$[8]. In addition, $G\beta_5$ has low-affinity interaction with Gγ subunits[18,19] and preferentially forms a stable dimer with the RGS R7 subfamily[20–24]. Similarly, $G\beta_2\gamma_1$ shows a stronger association than $G\beta_2\gamma_4$[17,25,26]. The expression levels, localizations, and affinities of each Gβ and Gγ subunit influences intracellular signaling cascades through the formation of specific Gβγ dimers and the specificity of each dimer for GPCRs[5,25,27,28].

Given the diversity seen for the expression and affinity of Gβ and Gγ subunits, as well as the affinity of Gβγ-effector interactions, it is likely that specific dimers could permit specialized roles in signal transduction pathways through association with particular GPCRs. Despite many attempts to understand G protein βγ specificity for particular GPCRs, much remains unclear due to a lack of specific antibodies or other methods of confidently assaying such preferences. Indeed, as yet only *in vitro* data exists which describes Gβγ specificity, and for only a few GPCRs[29–31]. For example, activated α_{2a}-adrenergic receptors (α_{2a}ARs) are found to interact with $G\alpha_{i1}$,

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$G\beta_1$, $G\beta_2$, $G\gamma_2$, $G\gamma_3$, $G\gamma_4$, and $G\gamma_7$ as shown by a fluorescence resonance energy transfer (FRET) assay[32,33] while M_4 muscarinic receptors interact with $G\alpha_o$, $G\beta_3$, and $G\gamma_4$[34]. Lack of tissue -specific determinants of specificity in heterologous expression systems created a gap between understanding *in vitro* and *in vivo* specificity of G protein βγ. As the interaction Gβγ dimers with particular GPCRs in the CNS may determine their role in regulating synaptic transmission, or their impact in neurological disease and GPCR targeted drug mechanism, further elucidation of G protein specificities *in vivo* is necessary.

α_{2a}ARs are $G_{i/o}$-coupled GPCRs[35,36] that are widely distributed in the peripheral and central nervous systems[37,38], are expressed in both adrenergic and non-adrenergic neurons, and are located in both pre- and post-synaptic[39] terminals. Presynaptic α_{2a}ARs in adrenergic neurons are called autoreceptors (auto-α_{2a}ARs) and act to inhibit exocytosis and prevent norepinephrine release. α_{2a}ARs in non-adrenergic neurons are called heteroreceptors (hetero-α_{2a}ARs)[37], and these also inhibit neurotransmitter release. Hetero-α_{2a}ARs activity is known to play a role in working memory, hypotension, bradycardia, sedation, analgesia, and hypnosis[37]. Using mRNA *in situ* hybridization and immunohistochemical analysis, auto- and hetero-α_{2a}ARs have been found in the locus coeruleus, cerebral cortex, hypothalamus, hippocampus, and amygdala[37,40–43]. Multiple polymorphisms within the *ADRA2A* gene have been identified, which variously increase α_{2a}ARs expression and alcohol dependence, reduce glucose-stimulated insulin release and antidepressant responsiveness, and alter memory and behavior[44–46]. In addition, the dysregulation of α_{2a}ARs, by increasing the amount of norepinephrine released, enhances fear memory and impairs spatial working memory[47,48]. Though the main mechanism of inhibition of exocytosis is via Gβγ subunits[49–51], it is unclear which G protein βγs are involved in these downstream signals of α_{2a}ARs.

With the development of transgenic mice including Hemagglutinin tagged (HA)-α_{2a}ARs knock-in (HA-α_{2a}ARs) and FLAG-α_{2a}ARs transgenic mice, the physiological implications of α_{2a}ARs can be further studied. HA-α_{2a}ARs mice were generated utilizing a homologous recombination gene targeting strategy to express HA-α_{2a}ARs in the endogenous mouse *ADRA2A* gene locus[52]. Expression and distribution of HA-α_{2a}ARs in these mice is identical to those of wildtype mice[52], as they are expressed in both adrenergic and non-adrenergic neurons which represent both auto- and hetero-α_{2a}ARs. Conversely, FLAG-α_{2a}ARs transgenic mice express FLAG-α_{2a}ARs only in adrenergic neurons, as the transgene is under the control of the dopamine-β-hydroxylase (Dbh) promoter[37]. These mice were then crossed with α_{2a}AR knockout (α_{2a}ARs KO) mice, such that only FLAG-α_{2a}ARs autoreceptors are present. The expression and function of this mice is identical to that of α_{2a}ARs autoreceptor[49]. By comparing with the wildtype, FLAG-α_{2a}ARs, and α_{2a}ARs knock-out mice, the different physiological functions of auto- and hetero-α_{2a}ARs were characterized. Auto-α_{2a}ARs play a role in bradycardia and hypotension while hetero-α_{2a}ARs are involved in anesthetic sparing, hypothermia, analgesia, bradycardia, and hypotension[37]. Given the physiological importance of α_{2a}ARs, and the different roles of auto-and hetero-α_{2a}ARs, the signaling mechanisms of α_{2a}ARs in both adrenergic and non-adrenergic neurons need to be further elucidated.

Together with our previous study quantifying the change in abundance and localization of each neuronal Gβ and Gγ subunit[28], the differences in physiological functions of auto- and hetero-α_{2a}ARs[37] suggests that the different α_{2a}ARs may utilize unique Gβγ dimers to regulate auto- vs. hetero-α_{2a}ARs specific downstream signaling pathways. Although $G\beta_1\gamma_2$ is the most abundant neuronal Gβγ dimer, other Gβγ combinations may be mediating auto- or hetero-α_{2a}AR signaling. For example, $G\beta_2\gamma$ and $G\beta_4\gamma$ dimers may specifically interact with adrenergic and opioid GPCRs[30]. In this paper, we test this hypothesis by using FLAG-α_{2a}ARs, HA-α_{2a}ARs, α_{2a}AR KO, and wildtype mice, together with various biochemical approaches such as a co-immunoprecipitation (co-IP) and a quantitative multiple reaction monitoring (MRM) method to identify and quantify Gβ and Gγ subunits. We measured and compared the interaction of overall (HA-α_{2a}ARs) or auto-α_{2a}ARs with neuronal Gβ and Gγ subunits for the first time, and depict the *in vivo* Gβγ specificity to auto- and hetero-α_{2a}ARs.

Results

The interaction of α_{2a} adrenergic receptors and Gβγ. To study the specificity of neuronal Gβγ subunits to synaptic α_{2a}ARs, we used brain synaptosomes from wildtype, α_{2a}AR KO, HA- and FLAG-α_{2a}AR mice. Because no GPCR antibodies are specific enough to co-IP α_{2a}ARs and Gβγ, we used HA- and FLAG-α_{2a}ARs expressing mice to overcome this limitation. Wildtype and α_{2a}-ARs KO mice were used as controls for HA- and FLAG-α_{2a}ARs mice. Synaptosomes from these mice were resuspended in a buffer with (stimulated) or without (unstimulated) epinephrine. DSP, a lipid-soluble thiol cleavable crosslinker, was added to ensure the receptor and Gβγ remained intact during co-IP experiments. The synaptosomes were then lysed and co-IPed for HA- or FLAG-α_{2a}ARs and Gβγ (Fig. 1A), which was validated by Western blot. Input represents total proteins present in lysate after the preclear while supernatant (Sup) represents what proteins are left in lysate after the co-IP with HA or FLAG specific antibodies (see Materials and Methods for more details). In wildtype and α_{2a}ARs KO mice, no α_{2a}AR and Gβγ interactions were detected following receptor stimulation (Fig. 1B,C). Here, we detected HA- and FLAG-α_{2a}ARs interacting with Gβγ only following α_{2a}AR stimulation (Fig. 1B,C).

Limit of $G\beta_1$ detection and quantification. To determine the number of co-IPs needed to detect Gβ and Gγ subunits in our MRM method, we used a serial dilution of purified $G\beta_1\gamma_1$ and monitored four non-heavy labeled proteolytic peptides of $G\beta_1$ to determine the limits of detection and quantitation (LOD/LOQ) (Supplementary Table 1)[53]. Because $G\beta_1\gamma_1$ is easily purified from the bovine retina, we chose it as our standard. It is used as a control to make sure that our method is running correctly and accurately. Previously, we have validated how each Gβ and Gγ are detected in our quantitative method[28]. Because $G\gamma_1$ is not present in the brain but only in photoreceptors, we only monitored $G\beta_1$ with mass spec. Below 10 pg of $G\beta_1\gamma_1$, we couldn't confidently identify the presence of $G\beta_1$ in samples. Between 10 pg to 250 pg, we were able to detect $G\beta_1$ but total area under the curve (AUC) didn't increase as the amount of purified $G\beta_1\gamma_1$ was increased (Supplementary Fig. 1). This suggests that we need more than 250 pg of $G\beta_1$ to detect and quantify proteins using our MRM method. We subsequently found using quantitative Western blots, that ~400–700 ng of Gβγ was pulled down with FLAG-α_{2a}ARs per half mouse brain

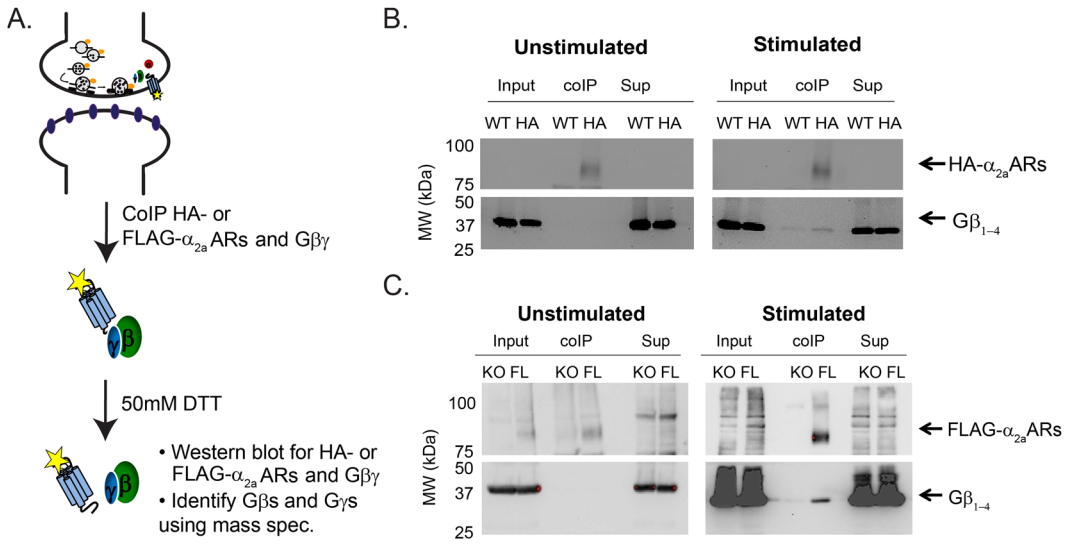

Figure 1. Co-immunoprecipitation of adrenergic α_{2a} receptors and Gβγ. Workflow of co-immunoprecipitation (coIP) experimental protocol (**A**), and representative Western blot of coIP of the HA-α_{2a}ARs (**B**) or FLAG-α_{2a}ARs (**C**) and Gβs following the resuspension of synaptosomes with unstimulated or stimulated buffers (stimulated, 100 μM epinephrine). Gels are cut out at 50 kDa to separate receptor (HA- or FLAG-α_{2a}ARs) and Gβ blots. The exposure times of receptor (HA- or FLAG-α_{2a}ARs) blots are 300 secs and 120 secs, respectively. The exposure times of Gβ blots are 300 secs for HA-α_{2a}ARs and 100 secs for FLAG-α_{2a}ARs coIP. The co-IP lane represents proteins immunoprecipitated with HA or FLAG specific antibodies. HA-α_{2a}ARs and FLAG-α_{2a}ARs are ~75 kDa while Gβs are ~33 kDa. HA-α_{2a}ARs and FLAG-α_{2a}ARs interact with Gβγ upon the activation of the receptors (stimulated). Sup: depleted supernatant.

used (10 co-IPs/half mouse brain) (data not shown). However, the previous limit of quantification experiment suggests that we need more than 4 ng of Gβγ for quantification[28]. Thus, using a half brain per condition, we can detect and quantify neuronal Gβ and Gγ despite our previously described technical challenges[28].

$G\beta_2$, $G\beta_4$, $G\gamma_2$, $G\gamma_3$, $G\gamma_4$, and $G\gamma_{12}$ specifically interact with neuronal α_{2a} adrenergic receptors. We examined the Gβ and Gγ subunits interacting with α_{2a}ARs to distinguish which Gβ and Gγ subunits interact with auto- vs. hetero-α_{2a}ARs. In Figs 2 and 3, we applied the quantitative MRM method[28] to co-IP samples of wildtype (WT) and HA-α_{2a}ARs mouse synaptosomes. Using SDS-PAGE gel, we excised Gβ and Gγ bands and added the heavy labeled proteolytic peptides to quantify each neuronal Gβ and Gγ subunit[28] (see Materials and Methods). Because Gβγ can be sticky, we built in a number of negative controls. To identify nonspecific interactions of Gβ and Gγ subunits, we used both unstimulated WT (WT no epi) and HA-α_{2a}AR (HA-α_{2a}AR no epi) samples as controls. In addition, we used stimulated WT (WT + epi) samples to detect nonspecific interactions with other receptors (non-HA-α_{2a}AR-mediated interactions). Thus the first three conditions in each graph in Figs 2 and 3 were to detect non-specific interactions of Gβγ, while the last detected interaction of Gβγ isoforms with epi-stimulated HA-α_{2a}AR.

$G\beta_2$ and $G\beta_4$ were significantly enriched with HA-α_{2a}ARs stimulated with epi (Fig. 2B,C). More $G\beta_4$ was detected than $G\beta_2$ In contrast, $G\beta_5$ did not interact with HA-α_{2a}ARs. Next, we examined the specificity of Gγ subunits to α_{2a}ARs to determine possible Gβγ dimer interactions with α_{2a}ARs. From the 6 detectable and quantifiable neuronal Gγ subunits[28], $G\gamma_2$, $G\gamma_3$, $G\gamma_4$, and $G\gamma_{12}$ were significantly enriched with HA-α_{2a}ARs upon epinephrine stimulation (Fig. 3A–C and E). We detected $G\gamma_2 > G\gamma_3 \approx G\gamma_4 > G\gamma_{12}$. $G\gamma_7$ and $G\gamma_{13}$ in stimulated HA-α_{2a}ARs + epi samples were equal to, or less, than corresponding control samples, suggesting these Gγs are present nonspecifically (Fig. 3D,F). From the subunits we have detected, we postulate that there may be as many as 8 different combinations of Gβγ dimers *in vivo* ($G\beta_2\gamma_2$, $G\beta_2\gamma_3$, $G\beta_2\gamma_4$, $G\beta_2\gamma_{12}$, $G\beta_4\gamma_2$, $G\beta_4\gamma_3$, $G\beta_4\gamma_4$, and $G\beta_4G\gamma_{12}$) which may interact with α_{2a}ARs in adrenergic and non-adrenergic neurons. Based on their detection levels, $G\beta_2\gamma_2$, $G\beta_2\gamma_3$, and $G\beta_2\gamma_4$ may be more likely to interact with α_{2a}ARs than other Gβγ dimers. $G\beta_2\gamma_{12}$, $G\beta_4\gamma_2$, $G\beta_4\gamma_3$, $G\beta_4\gamma_4$, and $G\beta_4G\gamma_{12}$ are less abundant Gβγ dimers interacting with α_{2a}ARs. Further biochemical analysis will be needed to validate the presence of these Gβγ dimers and their specificities with α_{2a}ARs in both adrenergic and non-adrenergic neurons.

$G\beta_2$, $G\gamma_2$, $G\gamma_3$, and $G\gamma_4$ specifically interact with auto-adrenergic α_{2a} receptors. After identifying the specificities of Gβ and Gγ for α_{2a}ARs in both adrenergic and non-adrenergic neurons, we decided to examine the specificity to auto-α_{2a}ARs which are only present in adrenergic neurons. In previous studies, auto-α_{2a}ARs and hetero-α_{2a}ARs were shown to have very different physiological functions[37]. We wondered if these different physiological functions may be mediated by unique Gβ and Gγ specificities for the different receptor types or through specific effector interactions. We again applied a quantitative MRM method to TCA-precipitated and trypsin-digested co-IP samples of α_{2a}ARs KO and FLAG-α_{2a}ARs mouse synaptosomes.

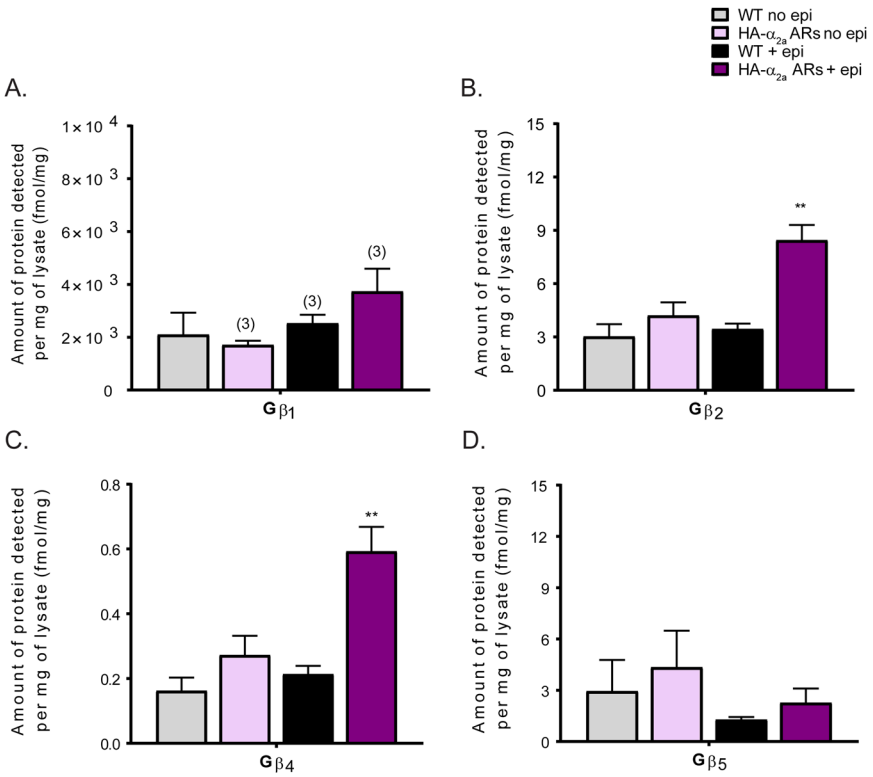

Figure 2. Gβ subunit specificity to α_{2a} adrenergic receptors**.** Quantification of Gβ subunits interacting with α_{2a}ARs in both adrenergic and non-adrenergic neurons (N = 4 unless otherwise noted on the graph with parentheses). Gβ subunits detected (fmol) from quantitative measurements were normalized by the amount of protein (mg), calculated using the volume and the protein concentration of precleared lysate used in co-IPs. We included several controls: unstimulated WT (WT no epi), HA-α_{2a}AR (HA-α_{2a}AR no epi), and stimulated WT (WT + epi) samples are all controls for the key sample, the Gβ and γ isoforms interacting with HA-α_{2a}AR. $G\beta_2$ and $G\beta_4$ specifically interact with activated α_{2a}ARs present in all synaptic terminals. Data were presented as mean ± SEM and compared by a one-way ANOVA, **P < 0.01. *Post hoc* analysis was performed with Tukey's multiple comparison test.

FLAG-α_{2a}ARs only express auto-α_{2a}ARs at the sympathetic presynaptic terminal, allowing us to study Gβ and Gγ subunit specificities to autoreceptors uniquely in sympathetic neurons. Similar to the previous experiment, α_{2a}ARs KO no epi and FLAG-α_{2a}ARs no epi samples were used as controls to identify nonspecific interactions, and α_{2a}ARs KO + epi samples were used to detect non-α_{2a}ARs associations. Here, $G\beta_2$ but not $G\beta_4$, showed a significant enrichment with auto-α_{2a}ARs (FLAG-α_{2a}ARs) (Fig. 4B). Again, $G\beta_1$ and $G\beta_5$ did not specifically interact with auto-α_{2a}ARs upon stimulation (Fig. 4A,D).

In contrast to the 4 Gγ subunits enriched with HA-α_{2a}ARs, we were able to detect $G\gamma_2$, $G\gamma_3$, and $G\gamma_4$ enriched with FLAG-α_{2a}ARs (Fig. 5A–C). Interestingly, we no longer saw enrichment of $G\gamma_{12}$ with FLAG-α_{2a}ARs (Fig. 5E) suggesting that $G\gamma_{12}$ may be a hetero-α_{2a}AR-specific Gγ subunit. As expected from the HA-α_{2a}AR study, $G\gamma_7$ and $G\gamma_{13}$ did not interact with FLAG-α_{2a}ARs (Fig. 5D,F). Although further validation is necessary, we speculate that $G\beta_2\gamma_2$, $G\beta_2\gamma_3$, and $G\beta_2\gamma_4$ may be the possible Gβγ dimers interacting with auto-α_{2a}ARs in sympathetic adrenergic neurons.

$G\beta_4$ and $G\gamma_{12}$ may specifically interact with heteroreceptors. Only a subset of Gβ and Gγ subunits from the HA-α_{2a}ARs study exhibited specificity to auto-α_{2a}ARs, suggesting that hetero-α_{2a}ARs may utilize those Gβ and Gγ subunits not associated with auto-α_{2a}ARs to regulate unique downstream signaling pathways. Without a transgenic tagged hetero-α_{2a}ARs mouse; however, we cannot directly measure the Gβ and Gγ subunits specific to hetero-α_{2a}ARs. However, in this study, we can infer the Gβ and Gγ specific to hetero-α_{2a}ARs by comparing and subtracting the results of our HA- and FLAG-α_{2a}ARs studies. By comparing the Gβ and Gγ subunits detected each set of experiments (which represent overall synaptic α_{2a}ARs and presynaptic α_{2a}ARs at the sympathetic terminal, respectively), we determined that $G\beta_4$ (Figs 2 and 4C) and $G\gamma_{12}$ (Figs 3 and 5E) may be heteroreceptor specific. As a result, it is possible that $G\beta_2\gamma_{12}$, $G\beta_4\gamma_2$, $G\beta_4\gamma_3$, $G\beta_4\gamma_4$, and $G\beta_4\gamma_{12}$ dimers may be left to interact with hetero-α_{2a}ARs.

Discussion

It is well defined that Gβγ dimers are released upon the activation of $G_{i/o}$-coupled GPCRs, such as the α_{2a}AR, and act as important signaling units to various downstream signaling cascades to ultimately mediate various physiological functions[54–61]. It is not known whether all 32 possible neuronal Gβγs (combined from the known expression of 4 neuronal Gβs and 8 neuronal Gγs[28]), are functional *in vivo*, however, how such sorting may take place to

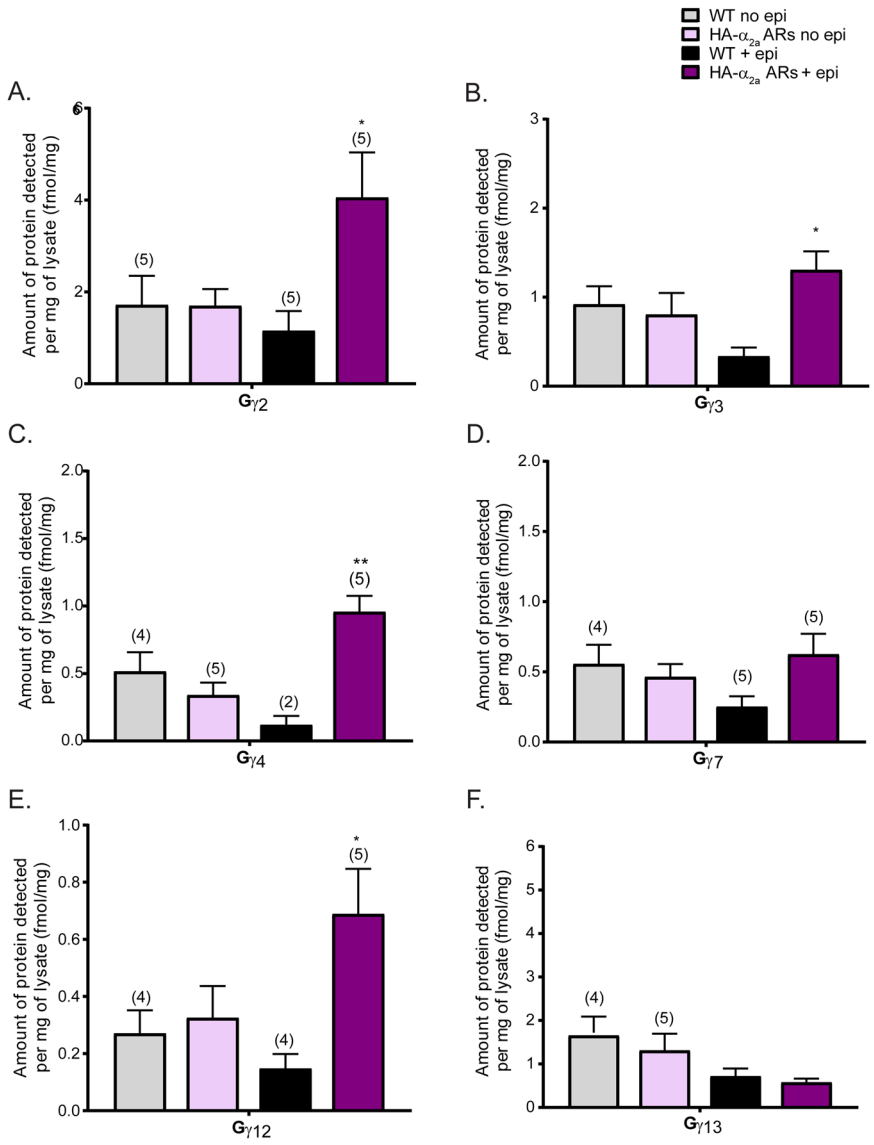

Figure 3. Gγ subunit specificity to α_{2a} adrenergic receptors. Quantification of Gγ subunit interactions with α_{2a}ARs in both adrenergic and non-adrenergic neurons (N = 4 unless otherwise noted on the graph). Gγ subunits detected (fmol) from quantitative measurements were normalized by the amount of protein (mg), calculated using the volume of precleared lysate used and the protein concentration of precleared lysate from BCA assay, used in co-IPs. Several controls were run: unstimulated WT (WT no epi), HA-α_{2a}AR (HA-α_{2a}AR no epi), and stimulated WT (WT + epi) samples. These are all controls for the key sample, the Gβ and γ isoforms interacting with HA-α_{2a}AR. $G\gamma_{|2}$, $G\gamma_{|3}$, $G\gamma_{|4}$, and $G\gamma_{|12}$ specifically interact with HA-α_{2a}ARs present in all synaptic terminals. Data were presented as mean ± SEM and compared by one-way ANOVA, *$P < 0.05$ and **$P < 0.01$. *Post hoc* analysis was performed with Tukey's multiple comparison test.

determine the formation of particular Gβγ dimers is not known, and very little is known of how the specificity of particular Gβγs plays a role in defining the specificity of signaling pathways[5,25,27–34].

***In vivo* specificity of α_{2a}ARs for Gβγ.** In this study, we have addressed the *in vivo* specificity of Gβ and γ interaction with the α_{2a}AR using MRM proteomics. We demonstrate that α_{2a}ARs preferentially interact with a subset of Gβ and Gγ subunits at synaptic terminals *in vivo*. Neuronal α_{2a}ARs (both auto- and hetero-α_{2a}ARs) interacted with $G\beta_2$, $G\beta_4$, $G\gamma_{|2}$, $G\gamma_{|3}$, $G\gamma_{|4}$, and $G\gamma_{|12}$ while auto-α_{2a}ARs interacted with $G\beta_2$, $G\gamma_{|2}$, $G\gamma_{|3}$, and $G\gamma_{|4}$ only. These findings suggest that Gβγs may shape signaling pathway specificity and that receptor and Gβγ interactions may be important in determining specific effector interactions.

In our previous study, we found $G\beta_1$ as the most abundant Gβ subunit in whole synaptosomes as well as at both pre- and post-synaptic fractions[28]. Interestingly, however, in this study we did not find a statistically significant interaction between $G\beta_1$ and HA-α_{2a}ARs upon receptor activation (Fig. 2A). Interestingly, we found $G\beta_2$ and $G\beta_4$ with activated α_{2a}AR instead, though there was more than 1,000-fold more $G\beta_1$ present at synapses. Despite the low abundance of $G\beta_4$ at the membrane[28], $G\beta_4$ binding to α_{2a}ARs, as well as the exclusion of the

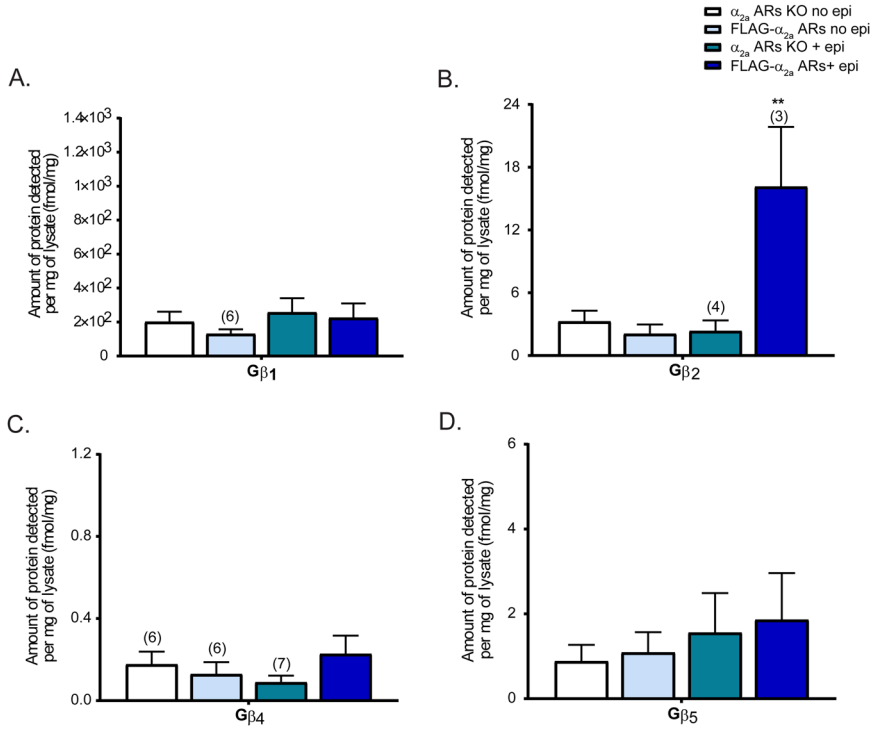

Figure 4. Gβ subunit specificity to auto-α_{2a} adrenergic receptors**.** Quantification of Gβ subunits interacting with auto-α_{2a}ARs (FLAG-α_{2a}-ARs) in adrenergic neurons (N = 5 unless otherwise noted on the graph). The data were analyzed identical to the study of α_{2a}ARs in both adrenergic and non-adrenergic neurons. Unstimulated α_{2a}ARs KO (KO no epi), FLAG-α_{2a}AR (FLAG-α_{2a}AR no epi), and stimulated KO (KO + epi) samples are controls. The difference between these epi-stimulated α_{2a}ARs KO and FLAG-α_{2a}AR represents the interaction of Gβ isoforms upon auto-α_{2a}ARs activation. $G\beta_2$ specifically interacts with auto-α_{2a}ARs. Data were presented as mean ± SEM and compared by one-way ANOVA, **P < 0.01. *Post hoc* analysis was performed with Tukey's multiple comparison test.

highly abundant $G\beta_1$, suggests a high specificity of this interaction. The numbers of receptors and effectors that specifically bind to unique Gβ and Gγ subunits may influence the abundance of certain Gβ and Gγ subunits at the membrane. For example, $G\beta_1$ may be specific to other receptors that are more abundant than α_{2a}ARs at synaptic terminals. Further studies are needed to determine these specificities, but these findings suggest that each receptor may utilize a unique set of Gβγ dimers to finely regulate receptor-specific downstream signaling.

Moreover, we detected a minor interaction between $G\gamma_{12}$ and HA-α_{2a}ARs but not with auto-α_{2a}ARs (Figs 3 and 5E). Although $G\gamma_{12}$ was one of most abundant Gγ subunits at the membrane fraction in our previous study[28], it was not specifically associated with auto-α_{2a}ARs, providing evidence for high specificity of the $G\gamma_{12}$ subunit at the hetero-α_{2a}ARs. This suggests a $G\beta_4\gamma_{12}$ dimer at hetero-α_{2a}ARs. In addition, $G\beta_5$ showed no specific interaction with α_{2a}ARs (Figs 2 and 3D), which supports previous studies that demonstrate it preferentially forms a stable dimer with the RGS R7 subfamily *in vivo* to modulate postsynaptic $G\alpha_i$–mediated signal transduction pathways[20–24].

As previously addressed[28], we experienced some technical challenges in detecting and quantifying Gγ subunits with this method. The amount of detected Gγ subunits was not similar to the amount of detected Gβ subunits. This difference may be due to the differences in peptide yield, which could stem from post–translational modifications, sample preparation artifacts, and differences in peptide re-solubilization efficiencies, all of which can lead to systematic errors in quantification[62]. Because of these, we are unable to calculate absolute protein quantities, but we can accurately determine the expression pattern of neuronal Gβ and Gγ subunits and compare within Gβ and Gγ subunits.

No evidence for pre-coupling of α_{2a}AR GPCRs *in vivo*. The association of receptor and G protein prior to receptor activation ("pre-coupling") has been suggested in some studies, but still remains unclear[1,63–68]. For example, in *in vitro* FRET assay, activated α_{2a}ARs were found to interact with $G\beta_1$[32,33]. However, in our study using synaptosomes from brain tissue, we do not see significant basal association between α_{2a}ARs and Gβ and Gγ. And we see only non-specific interaction between $G\beta_1$ and α_{2a}AR, even though it is highly abundant pre-synaptically. By contrast, we saw significant interactions of $G\beta_2$ and $G\beta_4$ with α_{2a}ARs, but only after epinephrine activation of α_{2a}ARs.

α_{2a}AR autoreceptors vs. heteroreceptors. Our findings suggest that unique Gβγ combination may play specific roles in mediating interactions with receptors. We found different Gβ and Gγ subunits in

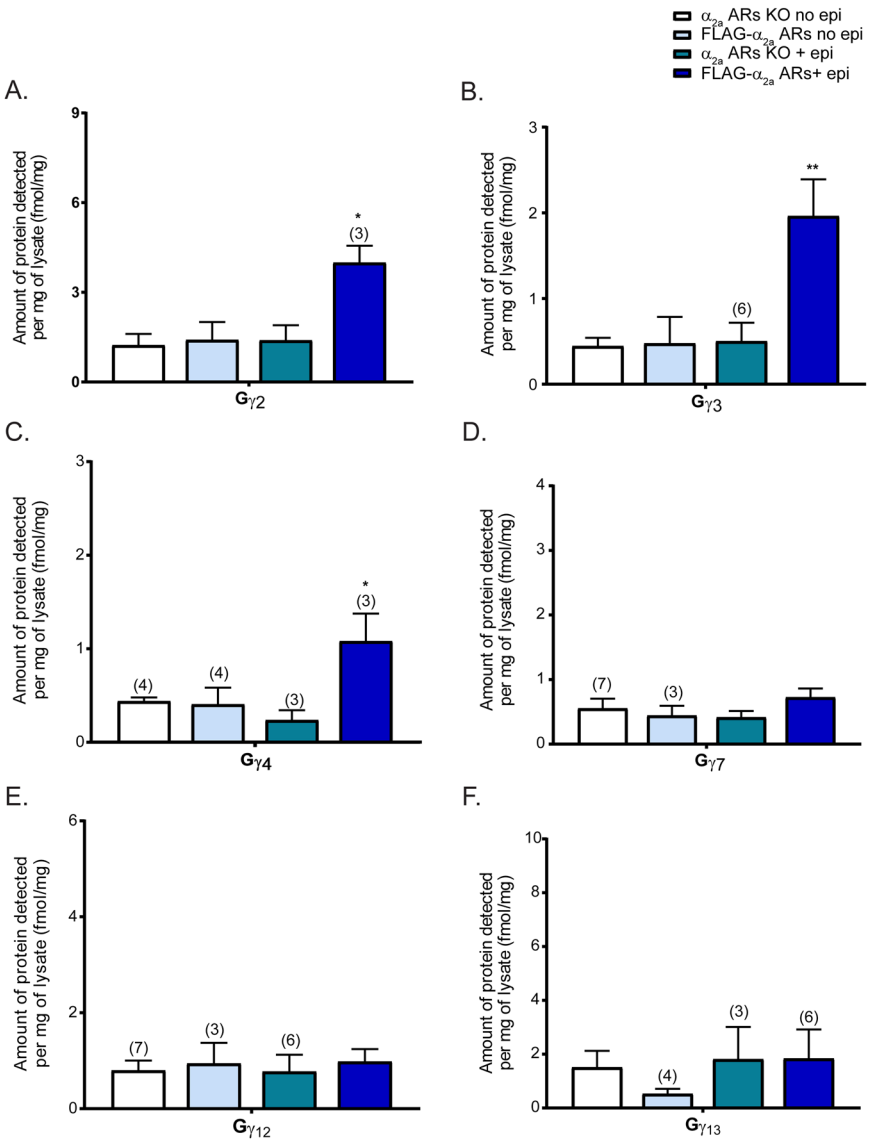

Figure 5. Gγ subunit specificity to auto-α_{2a} adrenergic receptors. Quantification of Gγ subunits interacting with auto-α_{2a}ARs on adrenergic neurons (N = 5 unless otherwise noted on the graph). The data were analyzed identical to the study of α_{2a}ARs in both adrenergic and non-adrenergic neurons. Unstimulated α_{2a}ARs KO (KO no epi), FLAG-α_{2a}AR (FLAG-α_{2a}AR no epi), and stimulated KO (KO + epi) samples are controls. The difference between these epi-stimulated α_{2a}ARs KO and FLAG-α_{2a}AR represents the interaction of Gγ isoforms upon auto-α_{2a}ARs activation. G$\gamma_{|2}$, G$\gamma_{|3}$, and G$\gamma_{|4}$ specifically interact with auto-α_{2a}ARs. Data were presented as mean ± SEM and compared by one-way ANOVA, *P < 0.05 and **P < 0.01. *Post hoc* analysis was performed with Tukey's multiple comparison test.

FLAG-tagged autoreceptors as compared to total HA-tagged α_{2a}ARs. This suggests that Gβγ specificities to receptors may change based on the cell type and localization of receptors. We estimate Gβ and Gγ subunit interactions with hetero-α_{2a}ARs by subtraction of presynaptic autoreceptor-associated Gβs and Gγs from total HA-α_{2a}AR-associated Gβs and Gγs, yielding the finding that $G\beta_2$ may be auto-α_{2a}AR specific, while $G\beta_4$ may be hetero-α_{2a}ARs specific. For Gγ subunits, G$\gamma_{|2}$, G$\gamma_{|3}$ and G$\gamma_{|4}$ were determined to be auto-α_{2a}ARs specific, while G$\gamma_{|12}$ was hetero-α_{2a}ARs specific. (Table 1). Overall, hetero-α_{2a}ARs may associate with G protein heterotrimers paired with $G\beta_4\gamma_{|12}$ to mediate hetero-α_{2a}AR-specific phenotypes such as sedation and anesthetic sparing[37]. One difference between these two mice is that heteroreceptors may be found either pre- or post-synaptically, whereas autoreceptors are only pre-synaptic.

We were not able to separate these two populations of heteroreceptors to determine whether this localization makes a difference. We were able to compare the results of these two studies side-by-side as similar levels of proteins were detected for most Gβ and Gγ subunits, however, one limitation of our studies is that we were unable to determine the differences in co-IP efficiency of HA- and FLAG- antibodies and the number of receptors in digested samples to calculate the relative Gβ and Gγ enrichment with hetero-α_{2a}ARs. Again, future studies with refined methodologies are needed to determine the functional consequences of identified specificities.

G proteins	α_{2a} ARs	Auto-α_{2a} ARs	Hetero-α_{2a} ARs (estimated)
$G\beta_2$	++	++	−
$G\beta_4$	+	−	+
$G\gamma_{2}$	+++	+++	−
$G\gamma_{3}$	++	++	−
$G\gamma_{4}$	+	+	−
$G\gamma_{12}$	+	−	+

Table 1. Gβ and Gγ specificities to hetero-α_{2a}ARs. The number of + denotes abundance. +: interaction with receptor detected; −: no interaction was detected.

Because HA-α_{2a}ARs represent both auto- and heteroreceptors and are found throughout the brain, we did not specify the neuronal type nor the location of receptors in the synaptosomes. $G\beta_2$ and $G\beta_4$ were previously identified to interact with α_{2a}ARs[30], and in this study these Gβ subunits are identified to interact with $G\gamma_{2}$, $G\gamma_{3}$, $G\gamma_{4}$, $G\gamma_{12}$ subunits. The rank order of Gγ specificity to overall neuronal α_{2a}ARs is similar to the Gγs found in whole and fractionated synaptosomes in the previous study[28]. It still remains unclear which Gγ subunits associate with each Gβ subunit. Though the rules for specificity determination are unknown, we assume that multiple factors affect the specificity: the preference of these Gβ subunits for Gγ subunits, the localization of receptors, and effector availability. The protein abundance and location of Gγ subunits will affect the Gβγ dimerization and their specificity to α_{2a}ARs.

Gβ and Gγ subunit specificity to α_{2a}ARs studied *in vitro*. Numerous *in vitro* studies have attempted to determine the specificity of Gβγ dimerization and their selectivity in interacting with various GPCRs and effectors[11,69,70]. Similar to our observations, $G\beta_2$, $G\beta_4$, $G\gamma_{2}$, $G\gamma_{3}$, and $G\gamma_{4}$ were previously shown to be strongly associated with α_{2a}ARs[32,71]. Using FRET, Gibson and Gilman demonstrated that endogenous α_{2a}ARs preferentially stimulated $G\alpha_{i1}$ heterotrimers paired with $G\beta_1$ or $G\beta_4$, and $G\alpha_{i3}$ heterotrimers paired with $G\beta_2$[32]. They also found that $G\beta_2$ association permitted 2-fold higher receptor activation, which was lost when $G\beta_2$ was replaced with $G\beta_1$. This result and our studies suggest that α_{2a}ARs with $G\alpha_{i3}\beta_2\gamma$ heterotrimers may be most likely to be present at the *in vivo* synaptic terminals. Moreover, $G\beta_2\gamma$ and $G\beta_4\gamma$ dimers were determined to interact with adrenergic and opioid GPCRs, while $G\beta_1\gamma$ and $G\beta_3\gamma$ dimers, particularly $G\beta_1\gamma_3$ and $G\beta_3\gamma_4$, may preferentially couple with somatostatin and muscarinic M4 GPCRs[29–31]. However, no specificity was identified based on the localization of receptors. In addition to the identify of Gα and Gγ subunits, the localization of receptor may play a role in α_{2a}AR selectivity of $G\beta_2$ and $G\beta_4$ over $G\beta_1$. Depending on the localization of receptor, α_{2a}ARs may also preferentially interact with specific effectors. Based on our results and previous biochemical studies, $G\beta_2\gamma_2$, $G\beta_2\gamma_3$, and $G\beta_2\gamma_4$ may be auto-α_{2a}ARs specific, while $G\beta_4\gamma_{12}$ may be hetero-α_{2a}ARs specific.

Other *in vitro* G protein specificity studies[71–74] depict a different Gβ and Gγ specificity than seen in our study. The gap between *in vitro* and *in vivo* detection of G protein specificity may be explained by tissue-specific determinants of specificity that are not present in heterologous expression systems, or difference in expression and availability of Gβ and Gγ subunits for *in vitro* studies. It is clear that Gβγ subunits are sticky, and this is why we provided multiple controls for non-specific effects. Future studies will be needed to address these differences.

Role of Gα subunits in determining Gβγ specificity to α_{2a}AR receptors. In addition to Gβγ, Gα may also define the selectivity of $G_{i/o}$–coupled GPCRs such as α_{2a}ARs. Unlike $G\alpha_s$, much less is known about how GPCRs selectively activate inhibitor $G\alpha_{i1-3}$ and $G\alpha_o$ subunits. Recent cryo-electron microscopy (cryoEM) studies reporting the structures of $G_{i/o}$ bound GPCRs, such as μ-opioid[75], adenosine A_1[76], $5HT_{1B}$[77], and light receptor rhodopsin[78], determine the interaction of these receptors with G_i or G_o and suggest the conformational re-arrangements on the GPCR cytoplasmic site may affect the binding of specific G proteins. Interestingly, they found different interactions of $G_{i/o}$ bound GPCRs and Gβ subunits[79]. However, the role of Gβγ in GPCRs-G protein specificity is unclear in these studies due to the modification of the proteins and the resolution of cryoEM structures. Moreover, the studies of $GABA_B$ heteromeric receptors with $GABA_{B1}$ and $GABA_{B2}$ have suggested hetero-dimerization of GPCRs may also affect the binding interactions of Gβγ with the receptor[80,81]. Further studies are needed to determine how Gα subunits affect the specificity of Gβγ.

As a $G_{i/o}$–coupled GPCR, α_{2a}ARs couple to $G\alpha_{i1-3}$ and $G\alpha_{o1-2}$. In a previous study by Richardson and Robishaw, $G\alpha_i$-containing heterotrimers were highly coupled to α_{2a}ARs[71]. Further, $G\alpha_i$ subunits were demonstrated to mediate sedative anesthetic-sparing effects, but not inhibition of evoked release[82], and $G\alpha i_1$ were found to preferentially associate with $G\beta_1\gamma_3$ over $G\beta_1\gamma_1$ or $G\beta_1\gamma_{10}$[71]. This suggests that Gα−mediated selectivity additionally contributes to the specificity of α_{2a}AR signaling through G proteins and their physiological functions. Further studies will be needed to understand the specific associations of Gα subunits with the Gβ and Gγ subunits observed here and their roles in known α_{2a}AR-mediated physiological effects.

Conclusions

With the quantitative MRM method[28], we now can further elucidate the *in vivo* Gβ and Gγ specificities to other GPCRs as well as Gβγ effectors, and validate previous *in vitro* studies of the Gβγ dimerization and their selectivity in interacting with various GPCRs and effectors[11,69,70]. In the CNS, numerous Gβ and Gγ subunits exhibit interesting subcellular localizations[28,83]. We do not yet fully understand the importance of these localizations and their

physiological role, however. This study begins to piece together the puzzle why multiple different isoforms of Gβ and Gγ subunits exist. Further efforts and development of tools, such as knockout or tissue-specific knockout animals, will be needed to determine the specificity and roles of each unique Gβγ dimer in regulating various GPCR signaling cascades, and their impacts on neurological diseases and GPCR targeted drug mechanisms. Eventually this will allow us to determine how cells precisely regulate multiple downstream mechanisms to modulate signal intensity and specificity.

GPCR specificity to G proteins is defined by the Gα subunit preferred by a given GPCR. Whether GPCRs also have preference for Gβ and Gγ subunits is not well investigated. Here, we measured the *in vivo* specificity of presynaptic α_{2a}ARs to a subset of neuronal Gβ and Gγ subunits using a previously published proteomic approach. We found that Gβγ dimers, other than the most abundant $G\beta_1\gamma_2$, are also involved in α_{2a}ARs-mediated signaling cascades *in vivo*. In addition, auto- and hetero-α_{2a}ARs exhibit specificity to different Gβ and Gγ subunits. The variety of potential Gβγ dimers identified implies that the specificity of Gβγs to signaling pathways could be in part mediated through the receptors and their locations on particular types of neurons.

Materials and Methods

See supplementary for more details.

Animals. Adult, male HA- and FLAG-alpha2a adrenergic receptors (α_{2a}ARs), α_{2a}ARs knockout (KO), and wildtype mice[37,52] were used. 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. Mouse anti-HA-agarose (Sigma, A2095), mouse anti-FLAG (Sigma, F3165) mouse anti-HA (Covance, 901514, 1:750), rabbit anti-FLAG (Sigma, F7425, 1:100), and rabbit anti-Gβ (Santa Cruz, sc-378, 1:10,000 and 1:5000) were used.

Synaptosome. Crude synaptosomes were isolated from mouse brain tissue, as described previously[53,84,85] and stimulated with 100 μM epinephrine (epi). This mimics the local synaptic concentration of epinephrine and it is a commonly used concentration in alpha2a adrenergic receptor studies[86–88]. They were frozen in liquid nitrogen and stored at −80 °C.

Co-immunoprecipitation (Co-IP). Crude synaptosomes were gently resuspended in 4 mL of RIPA buffer using a 25-gauge needle to lyse membranes and diluted to 1 mg/ml. Homogenates were centrifuged to separate the triton-soluble and insoluble fractions. Triton-soluble fractions were used for co-IP by incubating with either an anti-HA or FLAG antibody and Protein G agarose beads overnight. For elution, 100 μL of 1X sample buffer with DTT and 5% βME were used for HA-α_{2a}ARs and wildtype samples while 15.09 μg FLAG peptide was used for FLAG-α_{2a}ARs and α_{2a}ARs KO samples. Elutants were TCA precipitated and resuspended in 100 μL of 1x sample buffer with DTT and 5% βME. All samples were stored at −80 °C freezer for Western blot or MRM analysis.

Immunoblot analysis. To examine the results of IP, Western blot analysis was performed on equal volumes of input, co-IP, and supernatant samples using 10% SDS-PAGE gels. Using Western Lightning™ Chemiluminescence Reagent Plus (Perkin-Elmer) and Bio-rad Western blot imager, Western blots were developed.

Heavy labeled peptide cocktail. A heavy labeled peptide cocktail was made as described previously[28].

Quantitative MRM of Gβ and Gγ subunits. Co-IP samples containing Gβ and Gγ subunits were separated, digested, and analyzed by a TSQ Vantage triple quadrupole mass spectrometer (Thermo Scientific)[28]. To allow comparisons between G proteins co-IPed from multiple mice, quantitative Gβ and Gγ subunits detected (fmol) were normalized by the amount of protein (mg) used in co-IPs. The amount of protein used in co-IPs was calculated using the volume of precleared lysate used and the protein concentration of precleared lysate from BCA assay.

Statistical analysis. One-way analysis of variance (ANOVA) with a Tukey post hoc test was used to account for differences in protein expression 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).

Data Availability

All data generated or analyzed during this study are included in this published article (and its Supplementary Information files).

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Y.Y., K.B., W.H.M. and H.E.H. participated in research design. Y.Y., K.B., and W.H.M. conducted experiments. K.H., R.G., L.H., Y.C. and Q.W. contributed in mouse breeding and sampling. Y.Y. performed data analysis. Y.Y., W.H.M., K.B. and H.H. wrote or contributed to the writing of manuscript. All authors reviewed the results and approved the final version of the manuscript.

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