The *in vivo* specificity of synaptic G\(\beta\) and G\(\gamma\) subunits to the \(\alpha_{2a}\) 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 \(\alpha\), \(\beta\), and \(\gamma\) subunits, with 16 \(\alpha\)_s, 5 \(\beta\)_s and 12 \(\gamma\)_s subunits. Though much is known about the specificity of \(G\alpha\) subunits, the specificity of \(G\beta\gamma\) is activated by a given GPCR and that activate each effector *in vivo* is not known. Here, we examined the *in vivo* G\(\beta\gamma\) specificity of presynaptic \(\alpha_{2a}\)-adrenergic receptors (\(\alpha_{2a}\)ARs) in both adrenergic (auto-\(\alpha_{2a}\)ARs) and non-adrenergic neurons (hetero-\(\alpha_{2a}\)ARs) for the first time. With a quantitative RMR proteomic analysis of neuronal G\(\beta\) and G\(\gamma\) subunits, and co-immunoprecipitation of tagged \(\alpha_{3}\)ARs from mouse models including transgenic FLAG-\(\alpha_{3}\)ARs and knock-in HA-\(\alpha_{3}\)ARs, we investigated the *in vivo* specificity of G\(\beta\) and G\(\gamma\) subunits to auto-\(\alpha_{2a}\)ARs and hetero-\(\alpha_{2a}\)ARs activated with epinephrine to understand the role of G\(\beta\gamma\) specificity in diverse physiological functions such as anesthetic sparing, and working memory enhancement. We detected G\(\beta_{3,2}\), G\(\gamma_{12}\), G\(\gamma_{19}\), and G\(\gamma_{14}\) with activated auto \(\alpha_{2a}\)ARs, whereas we found G\(\beta_{3,4}\), and G\(\gamma_{12}\) preferentially interacted with activated hetero-\(\alpha_{2a}\)ARs. Further understanding of *in vivo* G\(\beta\gamma\) specificity to various GPCRs offers new insights into the multiplicity of genes for G\(\beta\) and G\(\gamma\), and the mechanisms underlying GPCR signaling through G\(\beta\gamma\) 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-G\(\alpha\)s and G\(\beta\gamma\) subunits are released from the GPCR and interact with various effectors to initiate downstream signaling cascades. Theoretically, 60 different combinations of G\(\beta\gamma\) dimers are possible (5 G\(\beta\) \(\times\) 12 G\(\gamma\) subunits)\(^4-8\). However, not all theoretical G\(\beta\gamma\) dimers exist, are equally expressed, or interact with G\(\alpha\) subunits, receptors, effectors, and downstream signaling factors\(^5,9-17\). For example, G\(\beta_{3,1}\), and G\(\beta_{5}\), dimerize with all G\(\gamma\) subunits, while G\(\beta_{1}\), and G\(\beta_{2}\) are unable to dimerize with G\(\gamma_{1}\) and G\(\gamma_{11}\)\(^8\). In addition, G\(\beta_{3,1}\) has low-affinity interaction with G\(\gamma\) subunits\(^18,19\) and preferentially forms a stable dimer with the RGS R7 subfamily\(^20-24\). Similarly, G\(\beta_{3,1}\) shows a stronger association than G\(\beta_{3,2}\)\(^17,25,26\). The expression levels, localizations, and affinities of each G\(\beta\) and G\(\gamma\) subunit influences intracellular signaling cascades through the formation of specific G\(\beta\gamma\) dimers and the specificity of each dimer for GPCR\(^2,5,25,27,28\).

Given the diversity seen for the expression and affinity of G\(\beta\) and G\(\gamma\) subunits, as well as the affinity of G\(\beta\gamma\)-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 G\(\beta\gamma\) 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 G\(\beta\gamma\) specificity, and for only a few GPCRs\(^29-31\). For example, activated \(\alpha_{2a}\)-adrenergic receptors (\(\alpha_{2a}\)ARs) are found to interact with G\(\alpha_{1}\),
α measured and compared the interaction of overall (HA-post-synaptic terminals. Presynaptic different
ferent more than 250 pg of Gα while hetero-
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α action to inhibit exocytosis and prevent norepinephrine release. α expressing mice to overcome this limitation. Wildtype and quantitative Western blots, that ~400–700 ng of Gα specificities transmission, or their impact in neurological disease and GPCR targeted drug mechanism, further elucidation of G protein specificities in vivo is necessary.
a2ARs are Gαi-coupled GPCRs that are widely distributed in the peripheral and central nervous systems, are expressed in both adrenergic and non-adrenergic neurons, and are located in both pre- and post-synaptic terminals. Presynaptic α2ARs in adrenergic neurons are called autoreceptors (auto-α2ARs) and act to inhibit exocytosis and prevent norepinephrine release. α2ARs in non-adrenergic neurons are called heteroreceptors (hetero-α2ARs), and these also inhibit neurotransmitter release. Hetero-α2ARs activity is known to play a role in working memory, hypotension, bradyarrhythmia, sedation, analgesia, and hypnosis. Using mRNA in situ hybridization and immunohistochemical analysis, auto- and hetero-α2ARs have been found in the locus coeruleus, cerebral cortex, hypothalamus, hippocampus, and amygdala. Multiple polymorphisms within the ADR2A gene have been identified, which variously increase α2AR expression and alcohol dependence, reduce glucose-stimulated insulin release and antidepressant responsiveness, and alter behavior. In addition, the dysregulation of α2ARs, by increasing the amount of norepinephrine released, enhances fear memory and impairs spatial working memory. Though the main mechanism of inhibition of exocytosis is via Gβγ subunits, it is unclear which G protein βγ subunits are involved in these downstream signals of α2ARs.

With the development of transgenic mice including Hemagglutinin tagged (HA)-α2ARs knock-in (HA-α2ARs) and FLAG-α2ARs transgenic mice, the physiological implications of α2ARs can be further studied. HA-α2ARs mice were generated utilizing a homing strategy to express HA-α2ARs in the endogenous mouse ADR2A gene locus. Expression and distribution of HA-α2ARs in these mice is identical to those of wildtype mice, as they are expressed in both adrenergic and non-adrenergic neurons which represent both auto- and hetero-α2ARs. Conversely, FLAG-α2ARs transgenic mice express FLAG-α2ARs only in adrenergic neurons, as the transgene is under the control of the dopamine-β-hydroxylase (Dbh) promoter. These mice were then crossed with α2AR knockout (α2AR KO) mice, such that only FLAG-α2ARs autoreceptors are present. The expression and function of this mice is identical to that of α2AR autoreceptors. By comparing with the wildtype, FLAG-α2ARs, and α2ARs knock-out mice, the different physiological functions of auto- and hetero-α2ARs were characterized. Auto-α2ARs play a role in bradyarrhythmia and hypotension while hetero-α2ARs are involved in anesthetic sparing, hypothermia, analgesia, bradyarrhythmia, and hypotension. Given the physiological importance of α2ARs, and the different roles of auto- and hetero-α2ARs, the signaling mechanisms of α2ARs 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 subunits, the differences in physiological functions of auto- and hetero-α2ARs suggest that the different α2ARs may utilize unique Gβγ dimers to regulate auto- vs. hetero-α2ARs specific downstream signaling pathways. Although Gαi2 is the most abundant neuronal Gβγ dimer, other Gβγ subunit combinations may be mediating auto- or hetero-α2AR signaling. For example, Gβ2 and Gγ1 dimers may specifically interact with adrenergic and opioid GPCRs. In this paper, we test this hypothesis by using FLAG-α2ARs, HA-α2ARs, α2AR KO, and wildtype mice, together with various biochemical approaches such as 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-α2ARs) or auto-α2ARs with neuronal Gβ and Gγ subunits for the first time, and depict the in vivo specificity to auto- and hetero-α2ARs.

Results
The interaction of α2AR adrenergic receptors and Gβγ. To study the specificity of neuronal Gβγ subunits to synaptic α2ARs, we used brain synaptosomes from wildtype, α2AR KO, HA- and FLAG-α2AR mice. Because no GPCR antibodies are specific enough to co-IP α2ARs and Gβγ, we used HA- and FLAG-α2ARs expressing mice to overcome this limitation. Wildtype and α2ARs KO mice were used as controls for HA- and FLAG-α2ARs mice. Synaptosomes from these mice were resuspended in a buffer with (stabilized) 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-α2ARs and α2ARs in the same sample (Fig. 1A), which was validated by Western blot. Input represents total proteins present in lysate after the pre-clear 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 α2ARs KO mice, no α2AR and Gγ interactions were detected following receptor stimulation (Fig. 1B,C). Here, we detected HA- and FLAG-α2ARs interacting with Gγ only following α2AR stimulation (Fig. 1B,C).

Limit of Gβ3 detection and quantification. To determine the number of co-IPs needed to detect Gβ3 and Gγ subunits in our MRM method, we used a serial dilution of purified Gβ3 and monitored four non-heavy labeled proteolytic peptides of Gβ3 to determine the limits of detection and quantitation (LOD/LOQ) (Supplementary Table 1). Because Gβ3 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 Gβ3 and Gγ are detected in our quantitative method. Because Gγ is not present in the brain but only in photoreceptors, we only monitored Gβ3 with mass spec. Below 10 pg of Gβ3, we couldn’t confidently identify the presence of Gβ3 in samples. Between 10 pg to 250 pg, we were able to detect Gβ3, but total area under the curve (AUC) didn’t increase as the amount of purified Gβ3 was increased (Supplementary Fig. 1). This suggests that we need more than 250 pg of Gβ3 to detect and quantify proteins using our MRM method. We subsequently found using quantitative Western blots that ~400–700 ng of Gβ3 was pulled down with FLAG-α2ARs per half mouse brain.
samples as controls. In addition, we used stimulated WT (WT α) with other receptors (non-HA-G)

dimers are ~33 kDa. HA-Gα are ~75 kDa, while Gβ and Gγ are ~33 kDa. HA-Gα and non-adrenergic neurons.

physiological functions may be mediated by unique Gβγ dimers and their specificities with different receptor types or isoforms. Further biochemical analysis will be needed to validate the presence of these Gβγ dimers and their specificities with α2ARs in both adrenergic and non-adrenergic neurons.

Figure 1. Co-immunoprecipitation of adrenergic α2ARs and Gβγ. Workflow of co-immunoprecipitation (coIP) experimental protocol (A), and representative Western blot of coIP of the HA-α2ARs (B) or FLAG-α2ARs (C) and G3s 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-α2ARs) and G3s. The exposure times of receptor (HA- or FLAG-α2ARs) blots are 300 secs and 120 secs, respectively. The exposure times of G3 blots are 300 secs for HA-α2ARs and 100 secs for FLAG-α2ARs coIP. The co-IP lane represents proteins immunoprecipitated with HA or FLAG specific antibodies. HA-α2ARs and FLAG-α2ARs are ~75 kDa while G3s are ~33 kDa. HA-α2ARs and FLAG-α2ARs 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 Gβ for quantification. Thus, using a half brain per condition, we can detect and quantify neuronal Gβ and Gγ despite our previously described technical challenges.

Gβ3, Gβ4, Gγ2, Gγ3, Gγ4, and Gγ12 specifically interact with neuronal α2a adrenergic receptors. We examined the Gβ and Gγ subunits interacting with α2ARs to distinguish which Gβ and Gγ subunits interact with auto- vs. hetero-α2ARs. In Figs 2 and 3, we applied the quantitative MRM method to co-IP samples of WT and HA-α2ARs 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 (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-α2AR (HA-α2AR no epi) samples as controls. In addition, we used stimulated WT (WT epi) samples to detect nonspecific interactions with other receptors (non-HA-α2AR-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 isosforms with epi-stimulated HA-α2AR.

Gβ3 and Gβ4 were significantly enriched with HA-α2ARs stimulated with epi (Fig. 2B,C). More Gβ4 was detected than Gβ3. In contrast, Gβ3 did not interact with HA-α2ARs. Next, we examined the specificity of Gγ subunits to α2ARs to determine possible Gβγ dimer interactions with α2ARs. From the 6 detectable and quantifiable neuronal Gγ subunits28, Gγ2, Gγ3, Gγ4, and Gγ12 were significantly enriched with HA-α2ARs upon epinephrine stimulation (Fig. 3A–C and E). We detected Gγ12 > Gγ3 ≈ Gγ1 > Gγ12, Gγ2, and Gγ14 in stimulated HA-α2ARs + 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 (Gβ3γ2, Gβ3γ3, Gβ3γ4, Gβ4γ2, Gβ4γ3, Gβ4γ4, Gγ2γ2, Gγ3γ2, Gγ3γ3, Gγ4γ2, and Gγ4γ4) which may interact with α2ARs in adrenergic and non-adrenergic neurons. Based on their detection levels, Gβ3γ2, Gβ4γ3, and Gγ4γ4 may be more likely to interact with α2ARs than other Gβγ dimers. Gβ3γ2, Gβ4γ3, Gγ2γ2, Gγ3γ3, and Gγ4γ4 are less abundant Gβγ dimers interacting with α2ARs. Further biochemical analysis will be needed to validate the presence of these Gβγ dimers and their specificities with α2ARs in both adrenergic and non-adrenergic neurons.

Gβ3, Gγ2, Gγ3, and Gγ4 specifically interact with auto-adrenergic α2a receptors. After identifying the specificities of Gβ and Gγ for α2ARs in both adrenergic and non-adrenergic neurons, we decided to examine the specificity to auto-α2ARs which are only present in adrenergic neurons. In previous studies, auto-α2ARs and hetero-α2ARs were shown to have very different physiological functions. 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 α2ARs KO and FLAG-α2ARs mouse synaptosomes.
FLAG-α2aARs only express α2aARs 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, α2aARs KO no epi and FLAG-α2aARs no epi samples were used as controls to identify nonspecific interactions, and α2aARs KO + epi samples were used to detect non-α2aARs associations. Here, Gβ2 but not Gβ4, showed a significant enrichment with auto-α2aARs (FLAG-α2aARs) (Fig. 4B). Again, Gβ1 and Gβ5 did not specifically interact with auto-α2aARs upon stimulation (Fig. 4A,D).

In contrast to the 4 Gγ subunits enriched with HA-α2aARs, we were able to detect Gγ2, Gγ3, and Gγ4 enriched with FLAG-α2aARs (Fig. 5A–C). Interestingly, we no longer saw enrichment of Gγ12 with FLAG-α2aARs (Fig. 5E) suggesting that Gγ12 may be a hetero-α2aAR-specific Gγ subunit. As expected from the HA-α2aAR study, Gγ7 and Gγ13 did not interact with FLAG-α2aARs (Fig. 5D,F). Although further validation is necessary, we speculate that Gβ2γ2, Gβ2γ3, and Gβ2γ4 may be the possible Gβγ dimers interacting with auto-α2aARs in sympathetic adrenergic neurons.

**Figure 2.** Gβ3 subunit specificity to α2a adrenergic receptors. Quantification of Gβ3 subunits interacting with α2aARs in both adrenergic and non-adrenergic neurons (N = 4 unless otherwise noted on the graph with parentheses). Gβ3 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-α2aAR (HA-α2aAR no epi), and stimulated WT (WT + epi) samples are all controls for the key sample, the Gβ and Gγ isoforms interacting with HA-α2aAR. Gβ3; and Gβ3 specifically interact with activated α2aARs 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.

Gβ3 and Gγ12 may specifically interact with heteroreceptors. Only a subset of Gβ3 and Gγ12 subunits from the HA-α2aARs study exhibited specificity to auto-α2aARs, suggesting that hetero-α2aARs may utilize those Gβ3 and Gγ12 subunits not associated with auto-α2aARs to regulate unique downstream signaling pathways. Without a transgenic tagged hetero-α2aARs mouse; however, we cannot directly measure the Gβ3 and Gγ12 subunits specific to hetero-α2aARs. However, in this study, we can infer the Gβ3 and Gγ12 specific to hetero-α2aARs by comparing and subtracting the results of our HA- and FLAG-α2aARs studies. By comparing the Gβ3 and Gγ12 subunits detected each set of experiments (which represent overall synaptic α2aARs and presynaptic α2aARs at the sympathetic terminal, respectively), we determined that Gβ3 (Figs 2 and 4C) and Gγ12 (Figs 3 and 5E) may be heteroreceptor specific. As a result, it is possible that Gβ3γ12, Gβ3γ12, Gβ3γ12, Gβ3γ14, and Gβ3γ12 dimers may be left to interact with hetero-α2aARs.

**Discussion**

It is well defined that Gβγ dimers are released upon the activation of Gαi- and Gαo-coupled GPCRs, such as the α2aAR, and act as important signaling units to various downstream signaling cascades to ultimately mediate various physiological functions. It is not known whether all 32 possible neuronal Gβγ dimers (combined from the known expression of 4 neuronal Gβs and 8 neuronal Gγs), are functional in vivo, however, how such sorting may take place to
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.

In vivo specificity of α2aARs for Gβγ. In this study, we have addressed the in vivo specificity of Gβ and γ interaction with the α2aAR using MRM proteomics. We demonstrate that α2aARs preferentially interact with a subset of Gβ and Gγ subunits at synaptic terminals in vivo. Neuronal α2aARs (both auto- and hetero-α2aARs) interacted with Gβ2, Gγ2, Gγ3, Gγ4, and Gγ12 while auto-α2aARs interacted with Gβ3, Gγ2, Gγ10, and Gγ12 only. These findings suggest that Gβγ’s 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 Gβ3 as the most abundant Gβγ subunit in whole synaptosomes as well as at both pre- and post-synaptic fractions. Interestingly, however, in this study we did not find a statistically significant interaction between Gβ3 and HA-α2aARs upon receptor activation (Fig. 2A). Interestingly, we found Gβ2 and Gβ3 with activated α2aAR instead, though there was more than 1,000-fold more Gβ3 present at synapses. Despite the low abundance of Gβ3 at the membrane, Gβ4 binding to α2aARs, as well as the exclusion of the

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**Figure 3.** Gγ subunit specificity to α2a adrenergic receptors. Quantification of Gγ subunit interactions with α2aARs 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 pre-precipitated lysate from BCA assay, used in co-IPs. Several controls were run: unstimulated WT (WT no epi), HA-α2aAR (HA-α2aAR no epi), and stimulated WT (WT + epi) samples. These are all controls for the key sample, the Gγ and γ isoforms interacting with HA-α2aAR. Gγ2, Gγ3, Gγ4, and Gγ12 specifically interact with HA-α2aARs 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.
highly abundant Gβ1, 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, Gβ1 may be specific to other receptors that are more abundant than α2aARs 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 Gγ12 and HA-α2aARs but not with auto-α2aARs (Figs 3 and 5E). Although Gγ12 was one of most abundant Gγ subunits at the membrane fraction in our previous study, it was not specifically associated with auto-α2aARs, providing evidence for high specificity of the Gγ12 subunit at the hetero-α2aARs. This suggests a Gβ4γ12 dimer at hetero-α2aARs. In addition, Gβ5 showed no specific interaction with α2aARs (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 Gαi-mediated signal transduction pathways.

As previously addressed, 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. 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 α2a 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. However, in our study using synaptosomes from brain tissue, we do not see significant basal association between α2a ARs and Gβ3 and Gγ. And we see only non-specific interaction between Gβ3 and α2a AR, even though it is highly abundant pre-synaptically. By contrast, we saw significant interactions of Gβ2 and Gβ3 with α2a ARs, but only after epinephrine activation of α2a ARs.

α2a AR autoreceptors vs. heteroreceptors. Our findings suggest that unique Gβγ combination may play specific roles in mediating interactions with receptors. We found different Gβ3 and Gγ subunits in...
FLAG-tagged autoreceptors as compared to total HA-tagged α2aARs. 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-α2aARs by subtraction of presynaptic autoreceptor-associated Gβs and Gγs from total HA-α2aAR- and FLAG-α2aAR-associated Gβs and Gγs, yielding the finding that Gγ2 may be auto-α2aAR specific, while Gγ3 may be hetero-α2aAR specific. For Gγ subunits, Gγ2, Gγ3, and Gγ4 were determined to be auto-α2aAR specific, while Gγ12 was hetero-α2aAR specific. (Table 1). Overall, hetero-α2aARs may associate with G protein heterotrimers paired with Gβ12, Gγ3, and Gγ4 to mediate hetero-α2aAR-specific phenotypes such as sedation and anesthetic sparing37. 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-α2aARs. Again, future studies with refined methodologies are needed to determine the functional consequences of identified specificities.

Figure 5. Gγ subunit specificity to auto-α2a adrenergic receptors. Quantification of Gγ subunits interacting with auto-α2aARs on adrenergic neurons (N = 5 unless otherwise noted on the graph). The data were analyzed identical to the study of α2aARs in both adrenergic and non-adrenergic neurons. Unstimulated α2aARs KO (KO no epi), FLAG-α2aAR (FLAG-α2aAR no epi), and stimulated KO (KO + epi) samples are controls. The difference between these epi-stimulated α2aARs KO and FLAG-α2aAR represents the interaction of Gγ isoforms upon auto-α2aARs activation. Gγ2, Gγ3, and Gγ4 specifically interact with auto-α2aARs. 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.
Because HA-α3-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. Gβ3 and Gβ4 were previously identified to interact with α3-ARs30, and in this study these Gβ subunits were identified to interact with Gγ2, Gγ3, Gγ4, and Gγ12 subunits. The rank order of Gγ specificity to overall neuronal α3-ARs is similar to the G-γ found in whole and fractionated synaptosomes in the previous study30. 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 Gβ/γ dimerization and their specificity to α3-ARs.

### Gβ and Gγ subunit specificity to α3-ARs studied in vitro

Numerous in vitro studies have attempted to determine the specificity of Gβ/γ dimerization and their selectivity in interacting with various GPCRs and effectors11,49,70. Similar to our observations, Gβ2, Gβ3, and Gγ2, Gγ3, and Gγ4 were previously shown to be strongly associated with α3-ARs32,71. Using FRET, Gibson and Gilman demonstrated that endogenous α3-ARs preferentially stimulated Goα3 heterotrimers paired with Gβ3, Gβ4, or Gγ1 and Goα3 heterotrimers paired with Gβ2. They also found that Gβ2 association permitted 2-fold higher receptor activation, which was lost when Gβ2 was replaced with Gβ3. This result and our studies suggest that α3-ARs with Goβ3/γ2–γ4 heterotrimers may be most likely to be present at the in vivo synaptic terminals. Moreover, Gβ2 and Gβ3 dimers were determined to interact with adrenergic and opioid GPCRs, while Gβ1 and Gβ3 dimers, particularly Gβ1γ3 and Gγ3, preferentially couple with somatostatin and muscarinic M4 GPCRs29,30. However, no specificity was identified based on the localization of receptors. In addition to the identify of Go and Gγ subunits, the localization of receptor may play a role in α3-AR selectivity of Gβ and Gγ over Gα. Depending on the localization of receptor, α3-ARs may also preferentially interact with specific effectors. Based on our results and other multisubunit biochemical studies5,52,54,55,78, it seems that Goβ3/γ2 can be auto-α3-AR specific, while Gβ2/γ4 may be hetero-α3-AR specific.

Other in vitro G protein studies7,11,49,70,71 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 Gβ/γ 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 Gαi subunits in determining Gβγ specificity to α3-AR receptors

In addition to Gβ, Gαi can also mediate the selectivity of Gαi-coupled GPCRs such as α3-ARs. Unlike Gαi, much less is known about how GPCRs selectively activate inhibitor Gαi1–3 and Gαi2 subunits. Recent cryo-electron microscopy (cryoEM) studies reporting the structures of Gαi bound GPCRs, such as μ-opioid34, adenosine A1, SHT1B, and light receptor rhodopsin34, determine the interaction of these receptors with Gαi or Gαi 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 Gαi bound GPCRs and Gβ3 subunits34. However, the role of Gβγ in GPCRs-G protein specificity is unclear in these studies due to the modifications of the proteins and the resolution of cryoEM structures. Moreover, the studies of GABAγ heteromeric receptors with GABAγ and GABAγ have suggested hetero-dimerization of GPCRs may also affect the binding interactions of Gβγ with the receptor29,34. Further studies are needed to determine how Go subunits affect the specificity of Gβγ.

As a Gαi–coupled GPCR, α3-ARs couple to Gαi1–3 and Gαi2. In a previous study by Richardson and Robishaw, Gαi-containing heterotrimers were highly coupled to α3-ARs71. Further, Gαi subunits were demonstrated to mediate sedative anesthetic sparing effects, but not inhibition of evoked release34,59, and Gαi were found to preferentially associate with Gβ3γ3 over Gβ2γ3 or Gβ1γ4. This suggests that Gαi-mediated selectivity additively contributes to the specificity of α3-AR signaling through G proteins and their physiological functions. Further studies will be needed to understand the specific associations of Go subunits with the Gβ and Gγ subunits observed here and their roles in known α3-AR-mediated physiological effects.

### Conclusions

With the quantitative MRM method28, we can now further elucidate the in vivo Gβ and Gγ specificities to other GPCRs as well as Gγ effectors, and validate previous in vitro studies of the Gβγ dimerization and their selectivity in interacting with various GPCRs and effectors11,69,70. In the CNS, numerous Gβ3 and Gγ subunits exhibit interesting subcellular localizations8,83. We do not yet fully understand the importance of these localizations and their

| G proteins | α3-ARs | Auto-α3-ARs | Hetero-α3-ARs (estimated) |
|------------|--------|-------------|--------------------------|
| Gβ2        | ++     | ++          | −                        |
| Gβ3        | +      | −           | +                        |
| Gγ2        | +++    | +++         | −                        |
| Gγ3        | ++     | +           | −                        |
| Gγ12       | +      | −           | +                        |

Table 1. Gβ and Gγ specificities to hetero-α3-ARs. The number of + denotes abundance. +: interaction with receptor detected; −: no interaction was detected.
physiological role, however. This study begins to piece together the puzzle why multiple different isoforms of Gβγ 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 α2a-ARs to a subset of neuronal Gα and Gγ subunits using a previously published proteomic approach. We found that Gβγ dimers, other than the most abundant Gαβγ, are also involved in α2a-ARs-mediated signaling cascades in vivo. In addition, auto- and hetero-α2a-ARs exhibit specificity to different Gα and Gγ subunits. The variety of potential Gαβγ dimers identified implies that the specificity of Gαβγ 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 (α2a-ARs), α2a-ARs knockout (KO), and wildtype mice 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 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. 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 μg 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.

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). 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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Author Contributions
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.E.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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